Environmental Biotechnology: A Bioremediation Perspective

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The term environmental biotechnology *has a certain air of modernity when in fact it has a long history of use, if one considers the underlying principles and not the appellation. However, as part of its complex meaning, there is a dynamic new definition and purpose in this discipline with regard to bioremediation. The ability to probe the environment at the molecular level with exquisite methods, to create a new awareness of fundamental biological processes therein, has created an important new paradigm in remediation engineering design and management. Further, biological lines of evidence made extremely robust through the merger of biotechnology and environmental science are poised to be incorporated into the very fabric of site evaluation and disposition at the regulatory level. At the operational level, the field of environmental biotechnology is driven by the "omics," the common suffix for disciplines like genomics, proteomics, and metabolomics. An introduction to these elements of the process is followed by a review of how they are being used right now in a commercial framework, with the understanding that the entire process is still in the formative stages of its vast potential. © 2005 Wiley Periodicals, Inc.*

INTRODUCTION

In a famous scene from the movie *The Graduate*, where the newly degreed Ben is advised as to his best career options, a family friend insists that it is all reducible to one word—*plastics*. Well, insufferability aside, we might gently offer that the future of the environmental industry may in fact be in *biotechnology*. That being the case, we can expect the terminology to follow, and so by the merger of key words we arrive at *environmental biotechnology*. But what does that really mean? Moving into the possibilities, we discover that environmental biotechnology has a very broad and expanding definition. While the full scope will be considered momentarily, preemptively, the focal point of this discussion is on the use of diagnostic tools that are grounded in molecular biology and specifically molecular genetics for a variety of environmental remediation objectives.

As we grow our definitions, the intent is to use molecular biological tools (MBTs) to resolve the nature of the microbial ecology at contaminated sites. This, in turn, can and should influence the design and management of bioremediation engineering and, to its furthest extent, open up new paradigms for resolving the ultimate issue—site closure. Alternatively, we can say that we are interested in what MBTs can do for the disposition of sites either by monitored natural attenuation (MNA) or by enhanced natural attenuation (ENA). As a secondary feature that needs to be referenced although we will not dwell on it, we acknowledge the role of bioaugmentation—in essence "microbial

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intervention."This may be a natural follow-through to what diagnostics teach us about a contaminated site—especially if it embraces the use of recombinant organisms, either in controlled environments or in open systems.

So let us return to the full context of environmental biotechnology, wherein we can circumscribe our efforts and intent with limited confusion given the varied dimension and evolution of the subject as it now stands. In 2001, a worthy textbook entitled *Environmental Biotechnology* appeared, authored by Bruce Rittmann and Perry McCarty, two prominent professors of civil and environmental engineering. In our view, they sought to make an immediate point in choosing that title. The message was that one should recognize that the term *environmental biotechnology* has a deep history as well as a promising future. Consequently, we should recognize that environmental biotechnology involves a continuum of subjects, from the traditional domains of sewage, wastewater, and drinking water treatment through to the more recently established bioremediation sciences, which now embrace new disciplines where strange terms like *qPCR*, *DNA microarrays*, and *MALDI-TOF* abound. These are, in effect, the tools of the trade for practicing some of the "omics" we will soon discuss and that serve as the portal to the characterization of site microbial ecology from which all of the applied engineering aspects flow.

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This particular track, where new kinds of biological evidence are gathered for environmental decision making, is not the only one to consider. What do we say about environmental sensors if molecular biology and specifically molecular genetic elements are harnessed in the service of rapid and highly sensitive detection? This is also a segment of environmental biotechnology and in fact can play a role in the diagnostic aspects of our core objectives (Mulchandani & Sadik, 2000). Then, to further complicate things, we are witnessing a new and valid definition of environmental biotechnology from the halls of the Industrial and Environmental Division of the Biotechnology Industrial Organization (BIO), which is a dominant force advocating the business of biotechnology across a wide spectrum of applications (http://www.bio.org).

Interestingly, and quite recently in fact, within the context of BIO, environmental biotechnology is about "pollution prevention" rather than "pollution therapeutics," if we equate bioremediation with a therapeutic process. By *prevention*, we refer to the rapidly developing field of industrial biotechnology that encompasses "green chemistry," as in the production of biodegradable plastics and the generation of biofuels and other value-added products from cellulosics. Industrial biotechnology uses genetically enhanced microorganisms and engineered enzymes to accomplish these goals. Since it is all viewed as environmentally beneficial relative to the nonsustainable alternatives and is rooted in biotechnology, it has also been proclaimed environmental biotechnology.

Lastly, honorable mention goes to yet another definition—the use of biotechnology to track the status of environmental degradation. In this version of environmental biotechnology, which is functionally related to bioremediation objectives, we can explore the true nature of genomic diversity in the environment and chart the changes over time as a function of impacts—anthropogenic or otherwise. This science capitalizes on the fact that the vast majority of the microbial world is unculturable in the laboratory but can be expressed as a total DNA profile. These patterns, the "metagenomics" of the system, can serve as a "canary in the mine" and function as a metric for planetary management at many different scales. This too may also be referred to as environmental biotechnology.

Points of Interrelationship

While the term *environmental biotechnology* may evoke a wide array of perceptions, one thing is clear: there is an interrelationship between the elements such that events in one area establish the possibilities and effect change in another. While there seems to be some sorting out required for the way the term *environmental biotechnology* is used, there is a critical cross-fertilization that is important to recognize. For example, the resources that are now flowing into industrial biotechnology at a rapidly increasing rate will impact environmental remediation through the development of techniques, infrastructure, and even politics as biotechnology is seen to be a larger and larger contributor to resolving certain challenges faced by our society. This latter aspect may serve to be critical because as biotechnology becomes more "user-friendly" and develops a positive image through medicine, defense, energy independence, and pollution prevention, it will facilitate the entry of the applications into environmental decision making.

Going back to a previous step, before the advent of industrial biotechnology, the real drivers in this process that are at the true core of the revolution are medicine and defense, because they have drawn and will continue to draw in billions of dollars a year in investment. With the force of this condition, the spinoffs into the environmental arena are inevitable. So, the thesis is that in the field of environmental science we have inherited a legacy. The fact that environmental biotechnology may have this meaning or that meaning is only a reflection of a greater reality. In essence, a scientific revolution occurred, and we are experiencing a trickle-down effect. The new science will touch and transform the old science. The integration of biotechnology into the many facets of our existence where biology in some form resides says that it will inevitably be part of our site remediation attitudes, strategies, and operations. The question is therefore not about *if* it will all happen, but rather *when* it will all take place and how it will impact the remediation industry.

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Can "natural law" itself provide any answers or any clues to this process? Perhaps it can. The eminent evolutionary biologist Stephen Jay Gould presented in his book *The Panda's Thumb* the theory of punctuated equilibrium, which offers that evolution is characterized by long periods of calm that are disturbed by dramatic and sudden transformations leading to radical change (Gould, 1980). We submit that we are now on the verge of such a transition regarding the applications of biotechnology to environmental science. In this instance, we can point to two things that will bring on the punctuated equilibrium: the financial drivers just cited and the fact that the environmental contamination problems we face are so pervasive and intractable that a new paradigm is overdue. Regarding the latter, we ask the following question: How can environmental biotechnology, in the context we have framed it with respect to the remediation sciences, lead the remediation industry to creative and sensible ways of dealing with the crush of problems that in some cases remain unsolvable even given unlimited resources and all the good intentions?

Carpe Diem—Time for a Change in the Face of the Intractable

Are real solutions to the vast array of environmental impacts truly out of reach? If this is the case, then do we gain traction in the arguments that we need new tools and technologies to "triage" the problems so that limited resources can be optimally ap-

plied? Let's look at a few examples of what is in front of us just in the sphere of groundwater remediation.

To begin with, the National Research Council (NRC) of the National Academies (including Sciences, Engineering, and Medicine) just issued a study entitled *Contaminants in the Subsurface: Source Zone Assessment and Remediation* (NRC, 2005). In this extensive and comprehensive report, it is clearly stated that "[t]he technical difficulties involved in characterizing and remediating source zones and the potential costs are so significant that there have been no reported cases of large DNAPL (dense nonaqueous phase liquid) sites where remediation has restored the site to drinking water standards."

Given the fact that the cost of site closure is often an enormous burden on site owners, and by extension to the overall economy, every reasonable tool needs to be marshaled to assist in new strategies for site closure.

From another direction, we have the appearance and potential gradual incorporation of the Technical Impracticability (TI) Waiver into environmental decision making. The TI Waiver is a grand way of saying—truthfully—that nothing feasible can be done to mitigate the contamination at a site. While this is still a developing concept, only applies to groundwater, and is reversible with new technology, there are still many examples of "No Further Action" or "Conditional Closure" status in the annals of contaminated-site decision making. These realities bracket one extreme regarding environmental cleanup and what can really be done under a variety of economic and technical constraints.

Faced with these facts, among others, we need to turn to the assessments that are now made possible through the use of biotechnology and integrate them into the decision-making process. We posit that the system of environmental management is in gridlock with a variety of jurisdictional standards and unmanageable problems. The task at hand is to seize the opportunity and bring the best elements of a maturing biotechnology to bear on the recalcitrant elements of environmental remediation.

The application of MBTs has momentum in transforming an empiric approach into a precise, fine-tuned, and science-based technology for accelerated remediation and disposition of contaminated sites. Given the fact that the cost of site closure is often an enormous burden on site owners, and by extension to the overall economy, every reasonable tool needs to be marshaled to assist in new strategies for site closure. This will generate further economic benefit by allowing the property in question to become saleable and/or positioned for development.

As a case in point, the bioremediation of a contaminant can be considered to follow a first-order decay function, so that the time required to reduce levels from approximately 10 ppm to 0.1 ppm would involve about six contaminant half-lives and typically take about two years. Is it then reasonable to invest another five half-lives of time (and money) to bring the site into compliance levels below 5 ppb? Rather we suggest that molecular diagnostics be used to monitor the process of interest and to prove that sites are moving toward closure. To the latter, we suggest that the right suite of MBTs can provide the basis for declaring a site to be in a "sound microbiological condition," such that if a level of 0.1 ppm is achieved, site closure is reasonable under the assumption that the rest of the asymptotic degradation process is to be expected. This triage of our contaminated-site inventory is a necessity so that limited available financial resources can be applied to environmental restoration in the most efficient manner. Hence, future efforts should focus on the expansion of the molecular tool kit to provide a comprehensive suite of prognostic and diagnostic tools for site prioritization, and deciding on the most promising remedial strategy to achieve site closure (Ritalahti et al., 2005).

A DIAGNOSTICS-DRIVEN PARADIGM FOR GROUNDWATER REMEDIATION

The Remediation Side

This article examines only one of many potential elements in the array of applied environmental biotechnology—the potential of diagnostics. Also, the discussion focuses our examples on one element of the problem set—groundwater bioremediation. This will help illustrate an important forward movement in bioremedial engineering and the concomitant impacts on the disposition of our inventory of contaminated sites. First, let us refresh our awareness of the scope and potential of bioremediation.

At the turn of the millennium, various polls abounded as to who were the greatest minds of the past thousand years. In one such enterprise, William Shakespeare showed up near the very top. The reason was interesting in that, arguably, across all of his plays the basic elements of any plot in all of playwriting to follow were already expressed. Similarly, we have shown that the seemingly novel terminology of environmental biotechnology has simpler beginnings, and so, not surprisingly, we see the same thing in the concept and practice of bioremediation.

Bioremediation technology uses microorganisms to reduce, eliminate, contain, or transform to benign products contaminants present in soils, sediments, water, or air. Bioremediation is not a new technology. Both composting of agricultural material and sewage treatment of household waste are based on the use of microorganisms to catalyze or conduct chemical transformation. Such environmental technologies have been practiced since the beginning of recorded history. Evidence of kitchen middens and compost piles dates back to 6000 B.C., and the more "modern" use of bioremediation began over 100 years ago with the opening of the first biological sewage treatment plant in Sussex, England, in 1891. However, the word *bioremediation* is fairly new. Its first appearance in peer-reviewed scientific literature was in 1987 (Hazen, 1997).

The last 15 years have seen an increase in the types of contaminants to which bioremediation is being applied, including solvents, explosives, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) (Natural and Accelerated Bioremediation Research [NABIR], 2004). Now, microbial processes are beginning to be used in the cleanup of radioactive and metallic contaminants, though these contaminants present special problems since they cannot be destroyed, only transformed or contained.

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There are a number of *ex situ* and *in situ* bioremediation methods currently available (Exhibit 1). *Ex situ* methods have been around longer and are better understood, and they are easier to contain, monitor, and control. However, *in situ* bioremediation has several advantages over *ex situ* techniques. *In situ* treatment is useful for contaminants that are widely dispersed in the environment, present in dilute concentrations, or otherwise inaccessible (e.g., due to the presence of buildings or structures). This approach can be less costly and less disruptive than *ex situ* treatments because no pumping or excavation is required. Moreover, exposure of site workers to hazardous contaminants during *in situ* treatment is minimal.

Broadly, bioremediation strategies can be further divided into two extremes—natural attenuation (NA) or enhanced natural attenuation. Natural attenuation relies on the intrinsic bioremediation capabilities of the impacted environment. Sites that are high in organic carbon and energy sources, with low contaminant concentrations and without

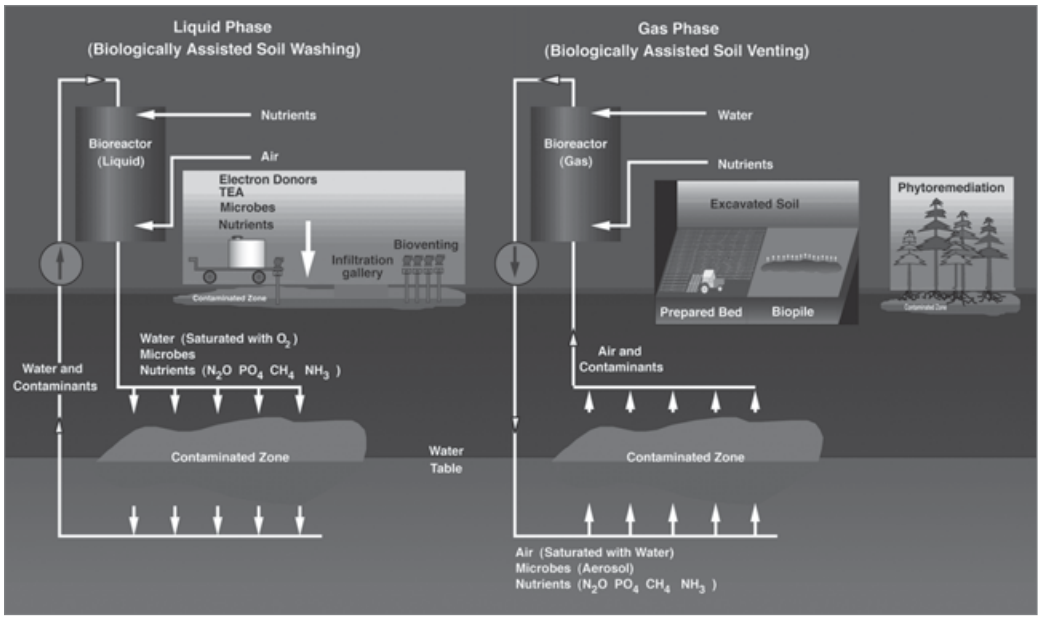

Exhibit 1. Bioremediation technologies

significant nutrient deficiencies, may be amenable to the degradation or transformation of the contaminants of concern without any intervention. The term *MNA* for monitored natural attenuation has already been referenced, and, as the name implies, this refers to NA with a layer of intervention at the monitoring level only. Enhanced natural attenuation is typically characterized by biostimulation and bioaugmentation strategies. Biostimulation can be aggressive or passive, in that electron donors, electron acceptors, and trace nutrients can be injected into the environment to stimulate indigenous organisms to increase biomass or activity to affect the contaminant. Passive biostimulation techniques include simple infiltration galleries or simply spreading fertilizer on the ground surface without any pumping or mixing. Bioaugmentation is the most aggressive aspect, since organisms are added to the contaminated environment.

Ideally, the most cost-effective and efficient approach to treat most large contaminant plumes is to use more aggressive approaches such as excavation and removal at the source, grading into MNA or ENA at the leading edge, or over time as the contaminant concentration declines. Rarely is a single remediation approach completely effective or cost-efficient. Indeed, combining aggressive physical and chemical treatment techniques like chemical oxidation or thermal desorption with bioremediation and bioaugmentation can provide advantages to managing some types of contaminants and allows the latter steps to be an effective polishing or sentinel strategy for the cleanup. MBTs can play a special role in a "treatment train" scenario, and this will be given special attention later.

The Diagnostics Side

Overview

Why diagnostics? The reason is simple—*nam et ipsa scientia potestas est*—or, in English, knowledge is power. In applied terms, with specific reference to groundwater remedia-

tion, the more we know about an aquifer and what ails it, the better we can design treatment, follow the healing process, and petition for site closure. We can stop "treating the whole cube," find an elusive contaminant source with greater accuracy, and focus efforts where they will do the most good. If properly coupled with ever-evolving aquifer probing technologies, the suite of new and future detection options that are grounded in molecular biology presents an exciting vision for the future.

It is like doing a CAT scan of the aquifer rather than an X-ray whereby more sophisticated projections of the condition of the subsurface are revealed. We will be able to routinely map the general chemistry of the aquifer, along with the compounds of concern and the relevant microbial populations, using miniaturized and multiplexed systems that employ novel capture chemistries. These will be wedded to revolutionary detection platforms that will rely on nanotechnology to separate and analyze in a small space. A conventional push probe that sips and deposits a continuous thin stream of water into real-time devices can generate all the data necessary to build a three-dimensional image of the subsurface. The era of a lab-on-a-chip or even using microbes themselves as the ultimate biosensors is upon us. In an example of the latter, using standard biochemical protocols, we can ask the microbes what the redox potential in the aquifer really is (normally hard to capture accurately) by measuring the microbial ubiquinone ratios that are redox-sensitive. And it is a better measure because it lets us know what redox potential the microbe is experiencing versus a more general "outside" measurement.

From a practical perspective, the imagery is critical to communicate the complexity of the information. We envision a new mechanism for conveying MBT-derived information, whereby the kinds of three-dimensional representations of an aquifer that are available now are applied to microbial ecological projections and become part of site design, management, and closure objectives. If one can see a detailed visual representation of what is going on at the level of microbial ecology (as opposed to standard graphical representations), then many possibilities unfold. We can make casual reference to these projections as "bug maps." Based on this information, deficiencies can be properly assessed, remedial strategies chosen, and when asymptotes are confronted, the argument can be made for a *minimal* level of MNA to conserve resources. In many ways, this is a more sophisticated extension of the contaminant distribution projections that were and still are ubiquitous from the average remedial action plan to the courtroom.

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Some of the available graphical modeling tools seem to take after George Lucas, as one can now "fly" through a subterranean site matrix. It cannot be overemphasized that presentation is critical. In the last decade, the scientific community has been visited by a refreshing new perspective from Edward Tufte (2001) on the visual presentation of scientific information, and we believe that these principles need to be studied and applied by the "environmental biotechnician." This way, the true value of the diagnostic information can be realized and communicated effectively to others downstream in the process—most notably the regulatory authorities.

The "Omics"

What then are the exact elements of the science of biotechnology that will support MBT-derived representations and allow us to fulfill the environmental mission to better resolve contaminated sites either physically or legally? The discussion centers on the "omics" as previously referenced, this being the shorthand for the common de-

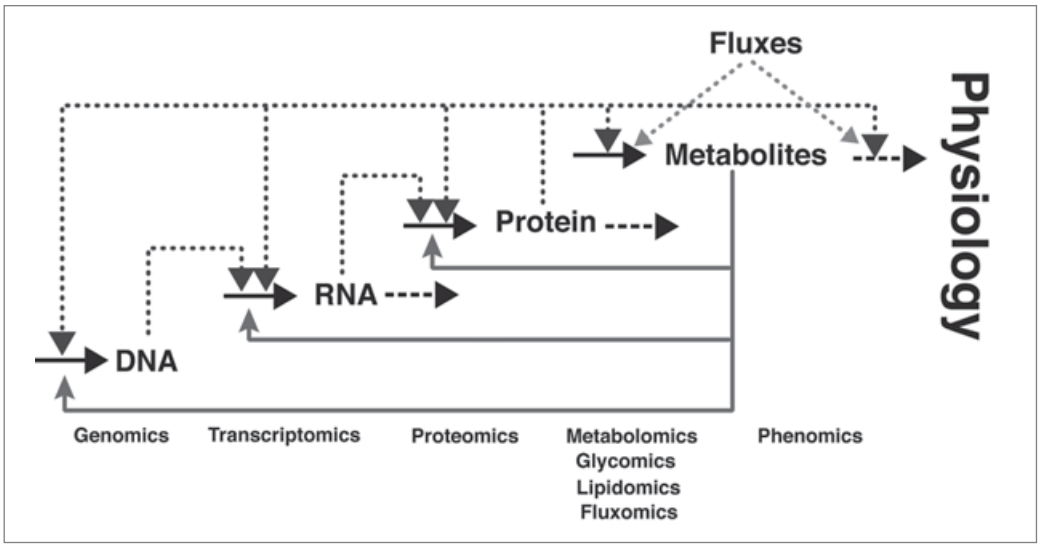

Exhibit 2. The hierarchy from the genome to final expression

nominator in terms such as *genomics*, *proteomics*, and *metabolomics*. Once again, as a corollary, we must emphasize that once we understand the potential shortcomings of the microbial ecology in a contaminated setting, the possibilities for biostimulation and bioaugmentation are present. Alternatively, we can seek to manipulate the aquifer geochemistry to favor natural or augmented microbiology to accelerate cleanup as a natural follow-through.

At the core of all MBT efforts is the elucidation of an organism's genetic code or genome. This is called sequencing. The human genome sequencing project was started in 1990 and was expected to take decades; however, it was completed in April 2003. Sequencing throughput has increased exponentially over the last ten years. Indeed, facilities like the Joint Genome Institute can now sequence the average bacterial genome before the first coffee break in the morning. More than 400 microbes have now been sequenced, but this total is expected to double in the next year. Whole communities can now be sequenced without ever culturing a single organism such that the biogeochemical relationships and syntrophy of entire communities can be determined. It is almost a magical thing, such that if these visions were articulated a decade ago, it would have had the weight of a career-ending move. Additional discussion regarding this issue is provided in the paragraphs that follow.

DNA codes for RNA, which codes for proteins, which produce metabolites, which lead to the physiology of the cell, the consortia, the community, and the ecosystem (Exhibit 2). Thus, with recent analytical advances and increasing understanding of cell structure and metabolism, the industry is able to increasingly examine other components in the cell to determine environmental relationships and biogeochemistry. As the sequence for different microbes has been annotated, it has enabled us to study the up and down regulation of genes being expressed—i.e., *transcriptomics*. Using technologies that detect mRNA, we can determine what genes are being turned on or off to provide code for protein production (Sayler et al., 2001). We can also use real-time polymerase chain reaction (qPCR) techniques to amplify sequences that are being expressed so we can see changes in expression of specific genes. These techniques are now being used to

determine if TCE degraders are present and active in environments where bioremediation is being considered or under way (Löffler et al., 2000).

Tyson et al. (2004) showed that the *metagenomics* from Iron Mountain, California, with a pH of 0.7 and a temperature of 42°C harbored an intricate relationship between iron and sulfate reducers (e.g., *Ferroplasma* spp. and *Leptospirillum* spp.). This has enabled a whole new area of *ecogenomics*, the study of genomes in an environmental context. In addition to metagenome analyses of the DNA sequence, we can also use techniques to look at specific components of the genome in highly conserved regions like the 16s ribosomal DNA/RNA segment to get specific identifications of species and look at evolutionary relationships between species. A number of other techniques for examining DNA from the environment have also been used over the past several years, one of the most popular being Terminal Restriction Fragment Length Polymorphism analysis (T-RFLP), which cuts the DNA and then examines the pattern of the fragments as an index of community structure change. The point is we are getting to know our environment in intricate ways, and once again it reveals an ability to approach contaminated sites and deal with design, management, and closure issues at a whole new level.

Drilling into this broader view a bit further, we can now take the 16s ribosomal DNA/RNA genomic sequences from microbial populations and display them on an array composed of glass slides or other substrates. These "16s" genetic sequences are the molecular equivalent of a fingerprint and can identify organisms to the point where this has now become the basis for taxonomic identification. Single-digit nanoliter drops of solution containing the sequences are deposited on a surface and these form microarrays. Based on the complementarity rules that govern molecular genetics, the nucleic acid sequences laid down on a microarray will hybridize to the corresponding sequences in an environmental sample. Through the ingenious use of fluorescent chemistry, signals from this event can be viewed. The power of this technique is significant. For example, thousands of microbial taxonomic sequences can be placed on a slide, as can the elements of an individual bacterial genome; in fact, the entire human genome can be arrayed on a couple of slides (Wilson et al., 2002).

Based on the complementarity rules that govern molecular genetics, the nucleic acid sequences laid down on a microarray will hybridize to the corresponding sequences in an environmental sample.

As a case in point, illustrating the use of genomics in particular, we can begin with the Department of Energy (DOE), which, in the case we will discuss, produced nuclear materials at the Hanford site for more than 40 years. What is significant about this connection is that DOE is one of the major supporters of MBT research through some $50 million in investment, about one-half of the government's total commitment in these areas. For more information, several key Web sites can be consulted (DOE Genomics:GTL Program: http://doegenomestolife.org; DOE Natural and Accelerated Bioremediation Research Program: http://www.lbl.gov/NABIR; and the Virtual Institute for Microbial Stress and Survival: http://vimss.lbl.gov).

At Hanford, chromium (Cr) was used to prevent corrosion in the cooling towers at the site and as an oxidizer in the nuclear fuel production process. Consequently, the site has a large plume of low-concentration hexavalent chromium (Cr[VI]) that is impacting the Columbia River. The Hazen group at the Lawrence Berkeley National Laboratory demonstrated that simple organic carbon compounds, like lactate, could stimulate iron reducers in the soil to reduce enough ferric iron (Fe[III]) to ferrous iron ([Fe[II]) so that the ferrous iron would reduce the insoluble hexavalent chromium to insoluble trivalent species (Cr [III]) and precipitate. In August 2004, 40 lbs of ^{13}C-labeled polylactate ester (a special preparation of the active ingredient in Hydrogen Release Compound, or

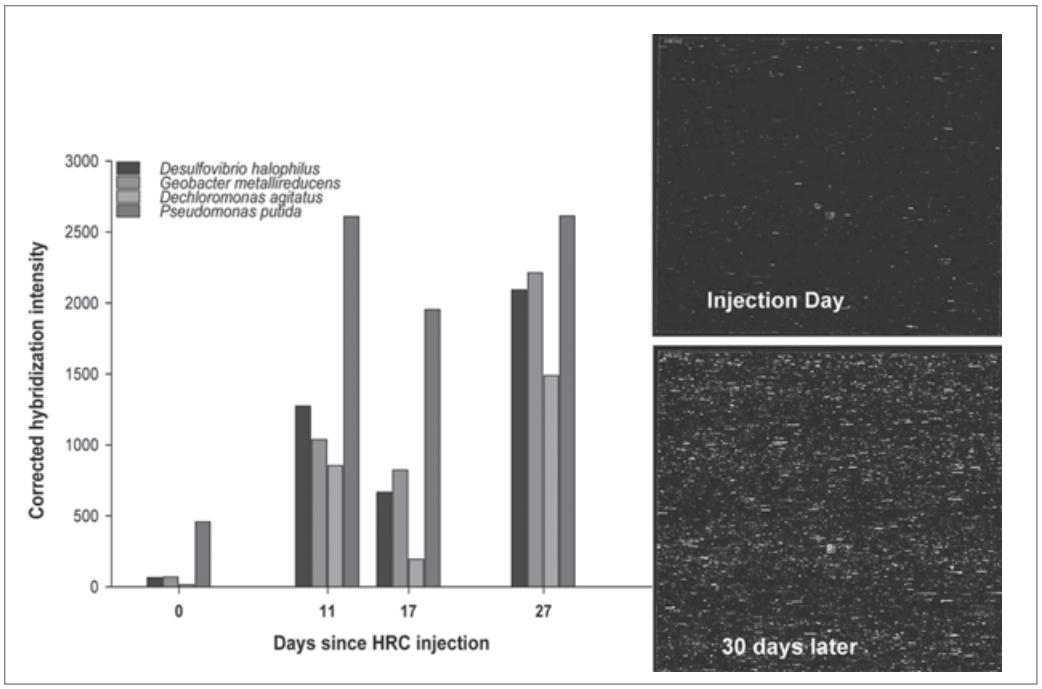

Exhibit 3. The use of microarrays in visualizing general and specific changes in the microbial ecology of an aquifer treated with an electron donor (HRC)

HRC®) was injected into a single well after doing pump tests, tracer tests, treatability studies, and baseline geophysics. The complete project design, methods, and results are given at http://esd.lbl.gov/ERT/hanford100h/.

In accordance with the HRC controlled release bioremediation mechanism (Koenigsberg & Sandefur, 1999), the polylactate ester hydrolyzes slowly to lactate in the aquifer, which is readily utilized as an electron donor by the indigenous bacteria. The HRC was labeled with ^{13}C so it could be determined if the microorganisms were utilizing the HRC. They were, and within two weeks, the total density of bacteria had increased more than two orders of magnitude, from $< 10^5$ cells/mL, to more than 10^7 cells/mL. Microbial community analyses with 16S ribosomal DNA/RNA microarrays for the entire known taxonomic database showed that the diversity increased dramatically (Exhibit 3). Analysis of the community structure after the injection showed an increase in denitrifiers, followed by increases in iron reducers and sulfate reducers. Even though nitrate was depleted and iron was reduced, sulfate depletion as a terminal electron acceptor was never complete, and subsequently methanogens were not observed in any of the samples. This stands as one of the first and most comprehensive correlative studies between a defined remedial action and the nature of the microbial ecology as illuminated through the use of MBTs. In this case, the focus was on genomics; however, there is more to the story of the "omics."

In the same way that genomes have been used in an ecological context to elucidate new understanding, we are now beginning to use proteins. The heavy lifting in this world is done with a technique called MALDI-TOF—an acronym for matrix-assisted laser desorption ionization–time-of-flight mass spectroscopy. Essentially, what one does is blast an environmental sample apart with a laser beam and collect and identify the

protein fragments. In more technical terms, a pulsed ultraviolet (UV) laser with nanosecond pulse width is focused on the sample ionizing the molecules. The ions are injected into a tube with the assistance of an electrical field and drift to a detector where the "time of flight" is proportional to their mass-to-charge ratio. In this way, each molecule yields a distinct signal and can be used to characterize a variety of biomolecules including proteins, with extreme sensitivity. This is the science of *proteomics*—one level up from the genomics revolution. In essence, it is functional genomics at the level of the protein products of the genome.

Recent studies by Ram et al. (2005) demonstrated that *metaproteome* analyses (i.e., determining all the proteins that are in an environment) could determine the relative abundance of a particular protein. Other seminal work in the field can be found in Halden et al. (2005). Thus, the use of proteomics could enable more specific determinations of the type of enzymatic reactions that the cells are currently capable of carrying out, not just which genes have been turned on or off to express the code for a particular protein. These studies are starting to show the effects that environmental stressors can have on bacteria and the pathways that bacteria use in stress response (Diaz, 2004).

That term, *stress response*, as it has just been used is a critical concept. Environmental contaminants are a source of stress for the life forms that reside in the impacted area. These sources of stress and the bacterial response can now be assessed for a variety of purposes. It can be the basis of an index for how much a site has been damaged, which can have implications in the triage concept and even in the legal definition of the impacts to a natural resource. On the other side, it can be the basis of the metrics of restoration. Tracking environmental changes with MBTs at least gives a handle on a previously impenetrable problem and allows environmental professionals to manage progress for a given effort made.

Lipids can also be used to determine particular responses to stress in the environment and to determine if particular groups or even some species are present, depending on the specificity of the lipids in their membranes and cell walls. Since membranes and cell walls are integral to a cell's interaction with the environment, the study of lipids/fatty acids, *lipidomics*, enables a different approach to the ecological context of a cell. Fatty acids have been widely used for preliminary identification using fatty acid methyl ester (FAME) analyses. Similarly, there are phospholipid fatty acid (PLFA) analyses that have been a mainstay in a leading commercial laboratory (Peacock et al., 2004). These tests have been demonstrated to indicate relative biomass in the environment of specific groups of bacteria and the physiological status of those bacteria (MacNaughton et al., 1999).

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The most recent area, which has been developed the least, is the study of metabolites, *metabolomics*. Using hydrophilic interaction chromatography techniques coupled to tandem mass spectrometry (MS/MS) detection and capillary electrophoresis mass spectrometry (CE-MS) methods, we can detect amino acids, nucleosides, nucleotides, organic acids, redox cofactors, and the metabolic intermediates of glycolysis, just to name a few. Probably more important than just the myriad of metabolites that can be studied is the concept of studying them in the context of their dynamic changes or fluxes in the cell, *fluxomics*. Metabolomics and fluxomics will allow us to assess gene function and relationships to phenotypes, understand metabolism and predict novel pathways, assess effects of genetic and metabolic engineering, and gauge the effect of environment stress changes that lead to changes in gene expression and metabolite levels (Burja et al., 2003).

The final expression of the physiology of the cell or its phenotype, *phenomics*, can also be studied in an environmental context. New high throughput phenotype microarrays (Exhibit 4) enable rapid characterizations of a cell's phenotype to more than 2,000 assays. These same methods can be used to rapidly determine the ability of environmental isolates to degrade or transform contaminants and/or minimum inhibitory concentrations for different stressors in the environment. Other phenomic type analyses include real-time analyses using synchrotron Fourier Transform Infrared Spectroscopy (FTIR) of bacteria on basalt exposed to toluene and chromium, demonstrating use of toluene by specific microcolonies and reduction of hexavalent chromium to trivalent chromium oxides (Holman et al., 1999).

Gathering the necessary evidence for sound site remediation design and management can itself be a costly undertaking, since operative mechanisms and rates of bioremediation are typically site-specific.

One of the most critical concepts for being able to develop and use all these "omics" in an environmental context is direct linkage to bioinformatics. Bioinformatics provides annotation of sequences, comparative genomics, pathway inference, pathway models, cell/environmental models, integration from biomolecules to ecosystems, and models for environmental biotechnology verification and prediction (http://vimss.lbl.gov) (Exhibit 5). All of these studies are dependent upon and enabled by the bioinformatics and will require immense and well-integrated databases and models if we are to take full advantage of the "omics" revolution in environmental biotechnology and bioremediation.

Clearly, after this discussion it should be apparent that we are now in an unprecedented time of biotechnology, and that leads to further realization that the impact to environmental science and site remediation is not a matter of "if" but rather of "when" and "how."

The First Levels of Integration—A More Pedestrian View

Earlier, we conveyed the sum and substance of the nature of bioremediation. A wide variety of groundwater contaminants are subject to biological transformations by different mechanisms and we can intervene with "food and bugs" or, to be more formal, substrates and microbes. We can even undertake more intense preliminary physical action such as chemical oxidation and "polish" a site with bioremediation.

Problems with the Status Quo—Indirect Lines of Evidence

Gathering the necessary evidence for sound site remediation design and management can itself be a costly undertaking, since operative mechanisms and rates of bioremediation are typically site-specific. A central component of site design, particularly with MNA, involves implementation of a groundwater-monitoring program to assess whether the desired bioprocesses are occurring, or are likely to occur with treatment. In addition to assessing contaminant concentrations and trends, these strategies typically involve measurements of concentrations and distributions of numerous geochemical parameters that are considered to be major indicators of actual and potential biological activity in groundwater. These parameters are generally termed *bioparameters* and can include a range of electron acceptors, by-products of various metabolic processes, and the oxidation/reduction potential (ORP). Data from the bioparameter measurements, in conjunction with contaminant trend data, are expected to help elucidate subsurface conditions. Using these data, prevalent bioprocesses can be deduced, as in aerobic versus anaerobic growth, and the effectiveness of a given biological treatment strategy thereby

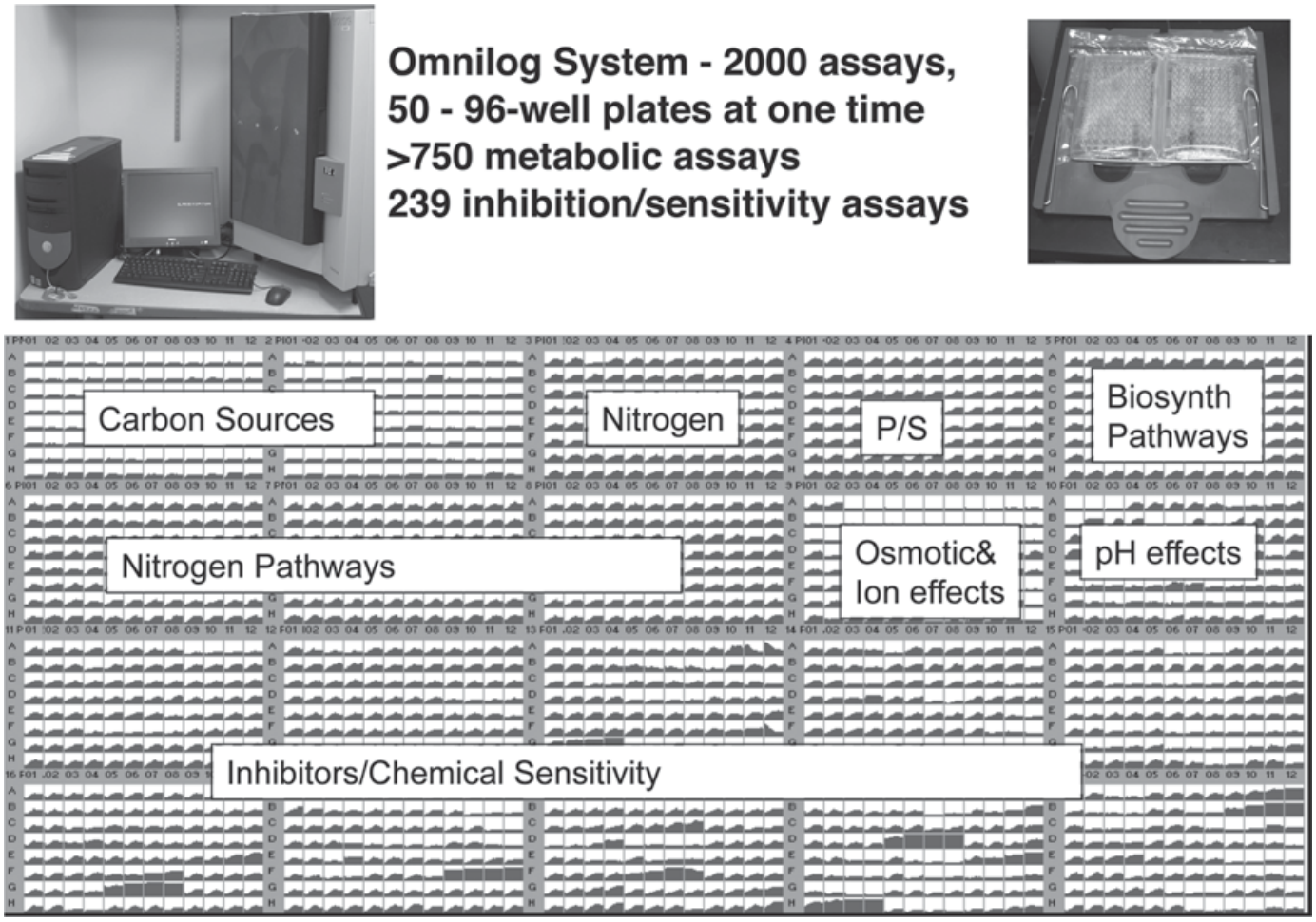

Exhibit 4. A phenotypic microarray

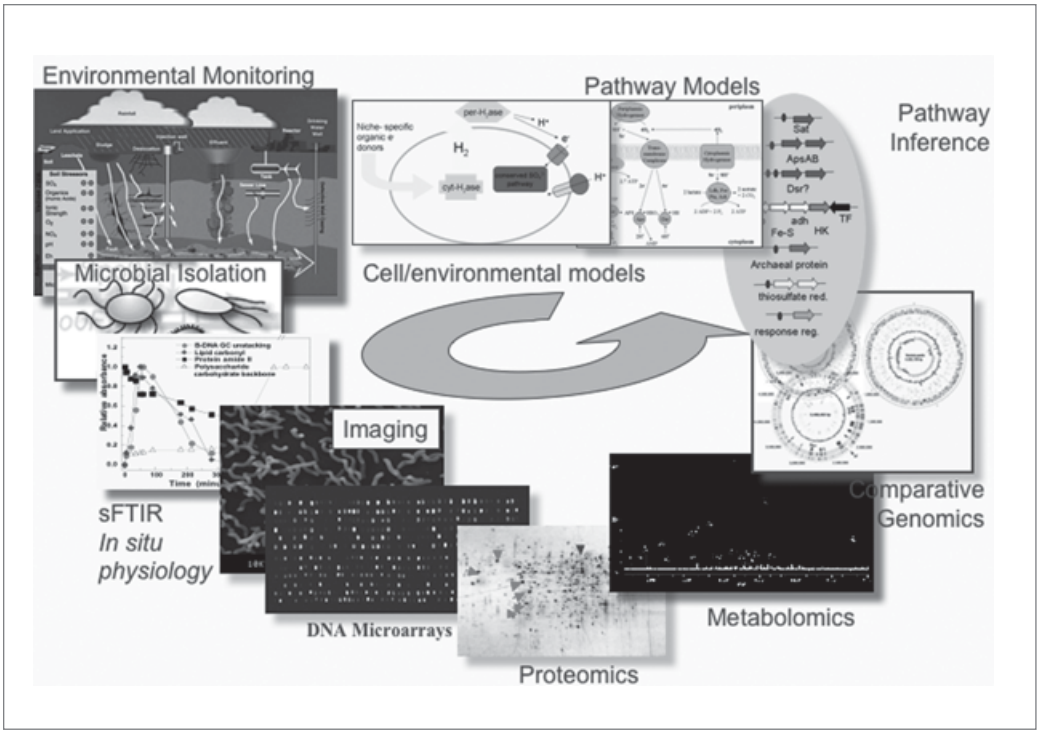

Exhibit 5. A relational view of the MBT landscape

assessed. In some instances, such evidence is necessary to avoid being forced to implement costly and often minimally effective techniques such as pump-and-treat systems, in an effort to achieve site closure.

The actual extent of site characterization required to support a biological management strategy varies from site to site depending on the regulatory environment. However, the goals remain the same: to deduce the prevalent bioprocesses in the subsurface and to determine whether biological treatment will prevent exposure of environmental receptors to the contamination. With respect to the prevalent bioprocesses, the key word here is "deduce"—that is, geochemical bioparameter data that amounts to circumstantial evidence of bioremediation.

Site managers have consistently found that in practice, measurement of numerous bioparameters is time-consuming and costly, and difficulties with data interpretation often occur, including major inconsistencies with contaminant plume data. Specifically, major costs are associated with preparation/mobilization (handling numerous sample containers, field instruments, field analytical kits, etc.), sampling, documentation, transport to the laboratory, validation (in excess of requirements for contaminants of concern), data management, and laboratory uncertainty and errors. Difficulties with data interpretation can include natural spatial variability in distribution and concentrations of naturally occurring anions and cations, dissolved oxygen (DO), ORP, sampling method–induced variability (particularly with DO and ORP), and field instrumentation errors (instrument and human error).

Experience has led to the conclusion that while measuring these bioparameters is theoretically a technically sound approach, the data obtained are often inconsistent and/or conflicting, and the insight gained from such monitoring is frequently minimal

and incommensurate with the level of effort and costs involved. Most important, with the current approach, it is often difficult or impossible to demonstrate convincingly that bioprocesses are occurring, when it is very likely that they are.

Introducing Biomarkers

An alternative method of assessing *in situ* bioprocesses that is more useful and cost-effective involves directly measuring various biochemical constituents of the bacteria themselves (i.e., "biomarkers"), which are indicative of their metabolic processes and therefore provide direct, relevant information regarding the environment in which they are growing. This technique provides a more accurate and more cost-effective assessment of *in situ* bioprocesses for sites undergoing bioremediation, as an expression of ENA or for MNA.

This technology is based on the principle that certain parameters of bacterial biochemistry are indicative of the conditions under which the bacteria live. That is, bacteria produce specialized, measurable, intercellular constituents that are involved in specific metabolic activities, which vary in response to the environment. The presence of, or relative quantities of, various constituents reflect specific metabolic activities and, hence, provide direct evidence of environmental conditions under which the organisms are growing *in situ*. This information can be used to show that certain bioprocesses are occurring and/or a particular set of conditions that favor degradation of a particular contaminant or class of contaminants is present (which is precisely the goal of geochemical bioparameter evaluation, as discussed earlier). However, since the microorganisms themselves are directly analyzed, and the biomarkers can be accurately measured, we can eliminate much of the ambiguity associated with analysis of bioparameters in groundwater.

Monitoring microorganisms in natural systems has in the past been complicated by two major factors. First, traditional techniques, which relied on isolation of bacteria from sediment and/or groundwater, were wholly inadequate. The results of these types of assays were not representative of the *in situ* microbial community. Normally, only a small fraction of the bacteria from a given site are culturable, and thus site investigators lose key information about potential remediative capabilities. The second complication involves groundwater-sampling techniques. Groundwater is inherently variable, and the heterogeneities associated with sampling groundwater for microbes, as well as geochemistry, often lead to confusing and conflicting results.

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The use of molecular and biochemical approaches like those described earlier provide a more effective and direct assessment of the microbial community than classical microbiological techniques. MBTs are useful to characterize natural attenuation or in site design to evaluate which remediation strategies are more effective. MBTs can provide crucial data to help manage a contaminated site through optimization of current remediation strategies, understand the microbial community response to a given bioremedial treatment, or answer questions remaining from the assessment of geochemical parameters. Finally, MBTs can provide additional lines of evidence to support site closure.

Commercially Available Biomarker Techniques

There are several different commercially available biomarker assays to help site managers in the design, management, and closure of contaminated sites (see www.microbe.com,

www.aerotechPK.com, www.regenesis.com, and www.sirem.com). Two of the most popular from among those that have been mentioned are the PLFA lipidomic analysis and the qPRC technique applied to taxonomic and/or functional genomic analysis. PLFA analysis is an effective tool for monitoring microbial responses to their environment; profiles simultaneously contain general information about the phylogenetic identity and physiological status of microbes. The microbial membrane from which the PLFA is derived reflects both the nature of the intracellular components and the extracellular environmental conditions. Thus, PLFA analysis tells us what types of microbes are present at a contaminated site and how they are reacting to environmental factors such as pollutants. The analysis is based on the extraction and separation of lipid classes, followed by quantitative analysis using gas chromatography/mass spectrometry (GC/MS). The individual fatty acids differ in chemical composition depending on the organism that produced them and the corresponding geochemical conditions.

The microbial membrane from which the PLFA is derived reflects both the nature of the intracellular components and the extracellular environmental conditions.

PLFA analysis provides quantitative insight into three important attributes of microbial communities: viable biomass (how many microbes are present), community composition (who is there), and metabolic activity (how they are feeling). PLFA analysis can answer specific questions in regard to contaminated sites such as if there is sufficient biomass to carry out a given bioremediation function and the effects of electron donor or acceptor addition on microbial community structure.

As stated before, advances in DNA-based microbial technology have revolutionized microbial monitoring by providing sensitive, rapid techniques to detect and quantify specific microorganisms. The "real-time" or "quantitative" (qPCR) technique is a kinetic approach whereby one measures the rate of the PCR reaction in the logarithmic stage and then back-calculates to the original amount of DNA present from the linear stage. In this way, a very good estimate of gene copies in the original sample can be calculated. Perhaps qPCR is best known for the ability to detect *Dehalococcoides sp*. (DHC), which are the only bacteria isolated that are known to reduce chlorinated ethenes all the way to ethene. Currently, there are two types of qPCR targets. The first is taxonomic, which targets specific microorganisms or groups with known metabolic (i.e., contaminant-degrading) capabilities. This type of assay is organism-specific. The second type of qPCR target is functional, and this assay targets genes that encode for enzymes involved in a specific microbial process such as reductive dechlorination. This assay is function-specific and not organism-specific and addresses the question "what do you do for a living?"

A Common Example of the Use of Biomarkers in Remediation Design

This case is chosen as typical for what the more responsive elements of the bioremediation industry are just beginning to experience, while more advanced elements of environmental biotechnology make their way to the fore. First as background, we discussed HRC earlier and noted that one of the key attributes is that it is a slow-release source of fermentable carbon. This viscous (20,000 centipoise) HRC substrate can then generate hydrogen and electrons that in turn drive the reductive dechlorination (dehalorespiration) of chlorinated solvents (such as chlorinated ethenes) and convert them to benign ethene. In this context, the controlled release features allow for the redox potential of the aquifer to be poised at more optimal levels for the key reductive dechlorination process. Excursions into the lower methanogenic regions are wasteful of substrate, such that methane is produced at the expense of ethene. Sometimes this balance is hard to con-

trol, and there is an engineering opportunity for designing and managing an HRC-treated site. At another extreme, sometimes sites are treated with fermentable substrates like a lactic acid solution with the viscosity of water and are too available for fermentation (i.e., too much of a good thing). Regulation of these systems is even more difficult and should be approached with caution, but in either case the MBTs are available.

In New York, the microbial response to an HRC application at a site contaminated with approximately 200 ppm of chlorinated ethenes was monitored using PLFA and two aspects of qPCR (taxonomic and functional). Samples were collected using Bio-Trap^{sm} samplers (passive samplers containing porous, colonizable beads used to collect bacteria over time) and analyzed for the abundance of total biomass referenced as eubacteria. DHC, and other bacterial groups known to compete for available hydrogen, methanogens, and sulfate-reducing bacteria, were also monitored.

Exhibits 6a and 6b depict the microbial response from a mid-plume area following about two years of quarterly monitoring. These results were able to show that DHC concentrations decreased initially, whereas methanogen concentrations increased three orders of magnitude. Initial observations suggest that methanogens were outcompeting the DHC for available hydrogen. However, after nine months, the system came into self-regulation from a single injection of slow-release HRC, which could be monitored with the specific MBTs as cited earlier. DHC concentrations had increased to a level of approximately 10^6 cells/bead and have continued to dominate the system at levels ranging from approximately 10^6 to 10^7 cells/bead.

This was the first use of a commercial assay for functional genes (tceA and bvcA) whereby the genes for key dechlorinating enzymes used by the microbes against the target contaminant degradation were measured. In this study, the background conditions registered nondetect or very low levels of these functional genes. Upon biostimulation with HRC, the functional gene concentration increased by orders of magnitude and precisely in the regions on the site where they were expected based on the HRC injection program.

The severity of certain primary phases of treatment such as chemical oxidation can be monitored such that an appropriate transition to bioremedial polishing steps can be better managed.

A Special Case

Earlier we discussed what is sometimes referred to as a "treatment train," whereby a more intensive treatment is used first and polished with a more passive operation. Such is the case with chemical oxidation followed by bioremediation (with reference to the subsets of both biostimulation and bioaugmentation). Given that chemical oxidation is having a strong renaissance in environmental remediation circles, a special use of MBTs in this application should be noted. The severity of certain primary phases of treatment such as chemical oxidation can be monitored such that an appropriate transition to bioremedial polishing steps can be better managed.

Chemical oxidation reactions involve a relatively immediate breaking of chemical bonds and the removal of electrons from the contaminant whereby the electrons are transferred to the oxidant. This mechanism (one of several) is often called "direct oxidation" and is a more instantaneous and energetic process, in contrast to bioremediation, which works more slowly and variably through the action of microorganisms. Sometimes, typically with chlorinated ethenes, one seeks to supplement the chemical oxidation remediation activity with the biologically mediated anaerobic reductive processes already described. If this is the plan, then the oxidiz-

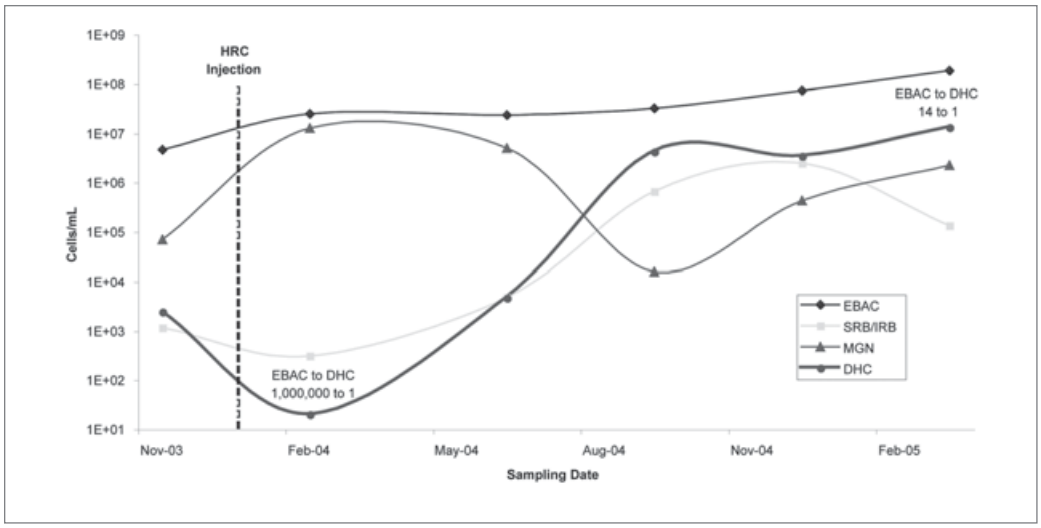

Exhibit 6a. Quantitative polymerase chain reaction (qPCR) result for samples collected from the mid-plume area between November 2003 and March 2005.

ing environment created by chemical oxidation can be inhibitory, albeit in a transient way (it is a misconception that aquifers are easily sterilized, particularly when considering that there are organisms that can survive autoclaving). In essence, the MBTs can be used to track the microbial ecology in such a way as to help optimize the transition from chemical oxidation to bioremediation. By dealing with the turning point from chemical to biological treatment, one can better assess the timing for adding substrates and organisms.

CLOSING THOUGHTS

A nonexhaustive set of "First Principles" are offered, largely derived from what has been presented—a "Top Ten" list of sorts.

1. Environmental biotechnology means different things to different people. Getting definitions in order at this early stage may help avoid confusion later on.
2. Molecular biological tools (MBTs) have a legacy of billions of dollars invested in them via medical science, homeland security, and now industrial initiatives, and this will continue and grow. It is not a matter of *if* this science will impact the remediation industry but rather *when* and *how* it will occur.
3. Applications for MBTs fall into three areas—site design, management, and closure. On the issue of site closure, MBTs are poised to redefine MNA and ENA dramatically if properly applied. It has been said that there is not enough money in America to clean it up by multiples. In a more scientifically grounded expression, the National Academy of Sciences has told us in very clear terms that, at least where NAPLs are concerned, they cannot document a single problem solved to the desired standards, regardless of the efforts made. More intelligent choices need to be made in the disposition of resources, and this is the stage on which MBTs will perform.

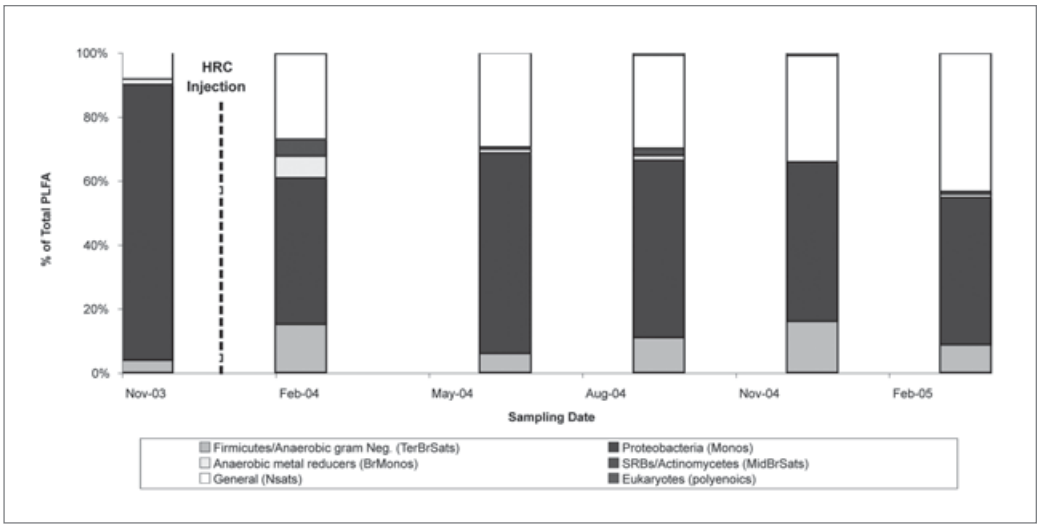

Exhibit 6b. Community structure profiles for PLFA analysis for samples collected from the mid-plume area between November 2003 and March 2005.

4. As with many things that follow the classical exponential scientific curve of progress, we may expect dramatic movements in the short term in the capabilities of biotechnology and, by extension, environmental biotechnology.
5. From a business perspective, integration is the key. Environmental biotechnology cannot exist in a vacuum. If coupled with intelligent and established remediation engineering, greater and sustained benefits will be realized from the new tools. Molecular diagnostics is a tool, not a solution.
6. Communication of complex scientific concepts is key. An appeal is made here that "Tuftian Dynamics" be considered and incorporated into the process.
7. It's not all about DNA. There are a host of other "omics" all able to play a vibrant role in the MBT strategic paradigm.
8. The arguments about problems with spatial variability in characterizing aquifers—especially at the microbiological level—will diminish with increased sample frequency. This, in turn, will be facilitated by current and emerging rapid field diagnostic capabilities.
9. In conjunction with the above, it is important to keep the science cheap and affordable—you might need lots of samples to make projections of microbial ecology for which we hereby coin the term *bug maps*. In line with Tufte (2001), keep the images simple and digestible. Remember the audience that has to deal with the output and complexity of the subject.
10. Don't overlook the potential for MBTs in the management of the transition of primary treatments like chemical oxidation to polishing steps like bioremediation. MBTs can be important in treatment train management.

REFERENCES

Burja, A. M., Dhamwichukorn, S., & Wright, P. C. (2003). Cyanobacterial postgenomic research and systems biology. Trends in Biotechnology, 21(11), 504–511.

Diaz, E. (2004). Bacterial degradation of aromatic pollutants: A paradigm of metabolic versatility. International Microbiology, 7(3), 173–180.

Gould, S. J. (1980). The panda's thumb: More reflections in natural history. New York: W.W. Norton and Co.

Halden, R. U., Colquhoun, D. R., & Wisniewski, E. S. (2005). Identification and phenotypic characterization of Sphingomonas wittichii strain RW1 by peptide mass fingerprinting using matrix-assisted laser desorption ionization-time of flight mass spectrometry. Applied and Environmental Microbiology, 71, 2442–2451.

Hazen, T. C. (1997). Bioremediation. Microbiology of the terrestrial subsurface. Boca Raton, FL: CRC Press.

Holman, H. Y. N., Perry, D. L., Martin, M. C., Lamble, G. M., McKinney, W. R., & Hunter-Cevera, J. C. (1999). Real-time characterization of biogeochemical reduction of Cr(VI) on basalt surfaces by SR-FTIR imaging. Geomicrobiology Journal, 16(4), 307–324.

Koenigsberg, S. S., & Sandefur, C. (1999). The use of hydrogen release compound for the accelerated bioremediation of anaerobically degradable contaminants: The advent of time-release electron donors. Remediation, 10(1), 31–53.

Löffler, F. E., Sun, Q., Li, J., & Tiedje, J. M. (2000). 16S rRNA gene-based detection of tetrachloroethene-dechlorinating Desulfuromonas and Dehalococcoides species. Applied and Environmental Microbiology, 66(4), 1369–1374.

MacNaughton, S. J., Stephen, J. R., Venosa, A. D., Davis, G. A., Chang, Y.-J., & White, D. C. (1999). Microbial population changes during bioremediation of an experimental oil spill. Applied and Environmental Microbiology, 65(8), 1766–1795.

Mulchandani, A., & Sadik, O. A. (2000). Chemical and biological sensors in environmental monitoring. Washington, DC: American Chemical Society.

Natural and Accelerated Bioremediation Research (NABIR). (2004). Bioremediation of metals and radionuclides: What it is and how it works (2nd ed.). Berkeley, CA: Lawrence Berkeley National Laboratory.

National Research Council. (2005). Contaminants in the subsurface: Source zone assessment and remediation. Washington, DC: National Academy of Sciences.

Peacock, A. D., Chang, Y.-J., Istok, J. D., Krumholz, L., Geyer, R., & White, D. C. (2004). Utilization of microbial biofilms as monitors of bioremediation. Microbial Ecology, 47, 284–292.

Ram, R. J., VerBerkmoes, N. C., Thelen, M. P., Tyson, G. W., Baker, B. J., Blake, R. C., et al. (2005). Community proteomics of a natural microbial biofilm. Science, 308(5730), 1915–1920.

Ritalahti, K. M., Löffler, F. E., Rasch, E. E., & Koenigsberg, S. S. (2005). Bioaugmentation for chlorinated ethene detoxification. Industrial Biotechnology, 1(2), 114–117.

Rittmann, B. E., & McCarty, P. L. (2001). Environmental biotechnology: Principles and applications. New York: McGraw-Hill.

Sayler, G. S., Fleming, J. T., & Nivens, D. E. (2001). Gene expression monitoring in soils by mRNA analysis and gene lux fusions. Current Opinion in Biotechnology, 12(5), 455–460.

Tufte, E. (2001). The visual display of quantitative information. Cheshire, CT. Graphics Press.

Tyson, G. W., Chapman, J., Hugenholtz, P., Allen, E. E., Ram, R. J., Richardson, P. M., et al. (2004). Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature, 428(6978), 37–43.

Wilson, K. H., Wilson, W. J., Radosevich, J. L., DeSantis, T. Z., Viswanatahan, V. S., Kuczmarski, T. A., et al. (2002). High-density microarray of small-subunit ribosomal DNA probes. Applied and Environmental Microbiology, 68(5), 2535–2541.

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