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Standard Guide for Application of Molecular Biological Tools to Assess Biological Processes at Contaminated Sites[1]

This standard is issued under the fixed designation E3354; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide provides a framework for the application of molecular biological tools (MBTs) to assess and characterize *in-situ* biological processes to improve contaminated soil and groundwater management. While the focus of this guide is on *in-situ* biological processes, some concepts of how to apply MBTs can also be applied to *ex-situ* bioremediation approaches (for example, biopiles, bioreactors) to support design, operation, and troubleshooting. The intent of this guide is to develop a consistent way in which MBTs are applied at contaminated sites, not to develop expertise. Technical experts need to be engaged whenscoping, planning, executing, and interpreting data for MBTs. Lastly, there is a brief description of isotopic techniques within section 5.2; however, the scope and focus of this guide is the use of nucleic acid-based MBTs to assess biological processes at contaminated sites.

1.2 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.3 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D6771 Practice for Low-Flow Purging and Sampling Used for Groundwater Monitoring

2.2 *U.S. EPA References:*[3]

U.S. EPA. 2018 Sampling, Laboratory and Data Considerations for Microbial Data Collected 32 the Field. EPA/600/R-164. 74 pg

U.S. EPA. 2008 A Guide for Assessing Biodegradation and Source Identification of Organic Ground Water Contaminants Using Compound Specific Isotope Analysis (CSIA). Ada, Oklahoma: Office of Research and Development, U.S. (EPA 600/R-08/148)

U.S. EPA. 1999 Use of monitored natural attenuation at superfund, RCRA corrective action, and underground storage tank sites. United States Environmental Protection Agency, Washington

U.S. EPA 1996 Ground Water Issue: Low-Flow (Minimal Drawdown) Ground-Water Sampling Procedures EPA/540/S-95/504

3. Terminology

3.1 This section includes definitions of key processes that are specific to molecular biological tools and use of abbreviations and acronyms. Definitions are adapted from other sources noted in the referenced documents. A full list of bioremediation-relevant microorganisms is beyond the scope of this guide.

3.2 *Definitions:*

3.2.1 *16S ribosomal ribonucleic acid (rRNA), n*—the RNA component of the 30S unit of the prokaryotic ribosome. The 16S rRNA is produced by 16S rRNA genes (sometimes referred to as 16S rDNA) which are gene sequences used to study bacterial phylogeny and taxonomy.

3.2.1.1 *Discussion*—The 16S rRNA gene has historically been the most common housekeeping genetic marker used by molecular biologists and microbiologists to identify and classify microorganisms (for example, genus and species).

3.2.2 *attenuation, n*—contaminant reduction over space and time due to physical (advection and dilution), chemical (volatilization, adsorption, abiotic transformation), and/or biological processes (biodegradation, biotransformation).

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from United States Environmental Protection Agency (EPA), William Jefferson Clinton Bldg., 1200 Pennsylvania Ave., NW, Washington, DC 20460, http://www.epa.gov.

3.2.3 *bioinformatics, n*—a subdiscipline of biology and computer science that focuses on the acquisition, storage, analysis and study of biological data, frequently applied to DNA, RNA, and protein sequences.

3.2.4 *biomarker, n*—a unique characteristic of a biomolecule, such as a specific DNA sequence, that can be measured and used as an indicator of a target microorganism or a specific biological process.

3.2.5 *bioaugmentation, n*—the introduction of microorganisms into the environment for the purpose of enhancing a beneficial biological activity.

3.2.6 *bioremediation, n*—cleanup of sites contaminated with hazardous, toxic, or radioactive substances or wastes, or any combination thereof using biological systems.

3.2.7 *biostimulation, n*—the introduction of electron donor, electron acceptor, and/or nutrients to the subsurface to promote biodegradation by an existing, native microbial community. Manipulation of pH or temperature may also be a form of biostimulation to enhance biodegradation.

3.2.8 *compound specific isotope analysis (CSIA), n*— analytical method that measures the ratio between the stable isotopes (for example, $^{13}C/^{12}C$, $^{2}H/^{1}H$, or $^{37}Cl/\ ^{35}Cl$) of a contaminant. As contaminants are degraded (for example, metabolism of aromatics, reductive dechlorination of chlorinated solvents), contaminants become enriched with the heavier isotopes (for example, ^{13}C) and the associated shift in isotopic ratios can be determined as evidence of bond breaking reactions.

3.2.9 *constituent of concern (COC), n*—an environmental constituent that is to be assessed, remediated, and or monitored in groundwater, soil, or soil gas at contaminated sites; sometimes referred to as a chemical of concern.

3.2.10 *conceptual site model (CSM), n*—a written and/or graphical representation of a site which includes potential sources and receptors of contaminants as well as the collection of physical, chemical, and biological processes governing contaminant fate and transport. These models are often iterative, revised as more information is learned, and used to aid decision-making throughout the site project lifecycle.

3.2.11 *deoxyribonucleic acid (DNA), n*—a biological macromolecule that carries genetic information. DNA consists of two long chains of nucleotides twisted into a double helix.

3.2.12 *electron acceptor, n*—a chemical compound that accepts electrons transferred to it from another compound. It is an oxidizing agent that is reduced through the process of accepting electrons.

3.2.13 *electron donor, n*—a chemical compound that donates electrons to another compound. It is a reducing agent that is oxidized through the process of losing electrons.

3.2.14 *enhanced attenuation (EA), n*—a remedial strategy that promotes biodegradation via addition of amendments (biostimulation) or cultured microorganisms (bioaugmentation) that stimulate biogeochemical reactions that result in the reduction of COCs *in-situ*.

3.2.15 *functional gene, n*—a segment of DNA that encodes an enzyme or other protein that performs a known biochemical reaction typically of use or interest beyond its role in the general metabolism of the cell (housekeeping gene).

3.2.15.1 *Discussion*—For example, the anaerobic Benzene Carboxylase (*abcA*) gene encodes an enzyme responsible for a known initial activation step in the anaerobic biodegradation pathway of benzene and is therefore a functional gene of interest to bioremediation practitioners.

3.2.16 *genomics, n*—a branch of molecular biology focusing on the structure, function, evolution, and mapping of genomes.

3.2.16.1 *Discussion*—While genetics may focus on the study of individual genes, genomics will consider the entire genetic information of an organism.

3.2.17 *genome, n*—the entire complement of genetic material for an organism.

3.2.18 *metagenomics, or meta-omics, n*—the study of genetic material derived directly from multiple host organisms or environmental samples containing multiple genomes, such as a mixed microbial community.

3.2.19 *molecular biological tools (MBTs), n*—a suite of molecular genetic analyses that can be used to characterize and evaluate microorganisms and their related activity.

3.2.19.1 *Discussion*—MBTs may also be referred to as or included in Environmental Molecular Diagnostics (EMDs).

3.2.20 *metabolism, n*—the myriad of chemical reactions in organisms that support life, including the conversion of the energy in substrates to energy available to perform cellular processes; the conversion of food to building blocks for proteins, lipids, nucleic acids, and some carbohydrates; and the elimination of metabolic wastes.

3.2.21 *metabolite, n*—a low molecular weight intracellular or extracellular molecule which is an intermediate or end product of biological processes required for organism growth, maintenance, and normal function.

3.2.22 *metabolic byproduct, n*—biochemical compounds that are generated by a microorganism during metabolic activity.

3.2.23 *metabolomics, n*—the analysis of intracellular and extracellular intermediates and end products generated during biological processes required for growth, maintenance, and normal function of an organism.

3.2.24 *microcosm study, n*—or "laboratory treatability studies" are a laboratory study in which samples of environmental media (for example, soil, sediment, and/or water) are tested in a contained environment, such as a bottle, to evaluate changes in concentration (or mass) of COCs over time.

3.2.24.1 *Discussion*— Microcosm studies are designed to mimic field conditions (to the extent possible) and may be used to evaluate remedial amendments, or determine natural degradation rates.

3.2.25 *monitored natural attenuation (MNA), n*—remedial approach that relies on demonstration through multiple lines of evidence, that naturally occurring physical (advection and dilution), chemical (volatilization, adsorption, abiotic transformation), and/or biological processes (biodegradation,

biotransformation) are able to reduce the mass and concentration of COCs to achieve site specific remedial objectives within a reasonable time frame.

3.2.26 *next generation sequencing (NGS), n*—colloquial term for massively parallel sequencing methods (for example, Illumina®) which allows the sequencing of millions of nucleic acid sequences from environmental samples simultaneously and can be used for target amplicon sequencing or metagenomic sequencing.

3.2.27 *nucleobase (base), n*—biological molecules that are cyclic organonitrogen compounds which are components of nucleotides that are part of fundamental structure of DNA and RNA.

3.2.27.1 *Discussion*—The five primary nucleobases are: adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U).

3.2.28 *nucleotide, n*—biological molecules that contain a nucleobase, a five-carbon sugar (for example, ribose (RNA) or deoxyribose (DNA)), and a phosphate group which make up DNA and RNA.

3.2.29 *primer, n*—a short, chemically synthesized, single-stranded DNA sequence that is used to prime the polymerase chain reaction to by complementary binding to a target gene sequence a thereby initiating the amplification of a targeted segment of DNA.

3.2.30 *prokaryote, n*—unicellular organism that lacks a membrane-bound nucleus. Prokaryotes are divided into two domains, Archaea and Bacteria.

3.2.31 *proteomics, n*—the analysis of the proteome, the entire set or subset of proteins that is produced or modified by an organism or system during a biological process, such as contaminant transformation.

3.2.32 *polymerase chain reaction (PCR), n*—a laboratory method used to make copies of a specific DNA sequence, allowing for amplification to a sufficient scale and resolution to study and identify specific organisms and functional genes

3.2.33 *quantitative polymerase chain reaction (qPCR), n*—a laboratory analytical technique for quantification of a target gene based on DNA polymerase chain reaction (PCR) technology, sometimes referred to as real-time PCR.

3.2.34 *reverse transcriptase qPCR (RT-qPCR), n*—a laboratory analytical technique for quantification of an expressed target gene (RNA) based on production of complementary DNA (cDNA) reverse transcribed from RNA.

3.2.35 *ribonucleic acid (RNA), n*—a single-stranded nucleic acid that is transcribed from DNA and contains the complementary genetic information.

3.2.35.1 *Discussion*—Messenger RNA (mRNA) is translated to amino acid sequences to synthesize proteins, including enzymes responsible for biodegradation. Ribosomal RNA (rRNA) forms structural components of ribosomes which are required for protein synthesis. Transfer RNA (tRNA) is used during production of proteins in the ribosome.

3.2.36 *stable isotope probing (SIP), n*—a technology that utilizes a synthesized form of the contaminant containing a stable isotope (for example, ^{13}C label) added to a sampler or study sample.

3.2.36.1 *Discussion*—If biodegradation is occurring, the isotope will be detected in the biomass (for example, phospholipids, DNA) and/or metabolic products (for example, volatile fatty acids, carbon dioxide or dissolved inorganic carbon or methane) over time.

3.2.37 *transcriptomics, n*—analysis of all RNA transcripts in a sample that are produced by the genome(s), under specific circumstances, or in a specific cell using high-throughput methods, such as microarray analysis.

3.2.38 *transcripts, n*—RNA synthesized from a DNA sequence (gene) that may then be translated in 295 proteins and ultimately the activation of a biochemical pathway (that is, gene expression).

4. Significance and Use

4.1 Contaminated sites subject to remediation are growing in complexity and associated remediation costs, presenting a challenge for managers of contaminated sites. The need to properly monitor, evaluate, and report remediation processes (including physical, chemical, and biological) characterizing site conditions and contaminant mass and attenuation is critical for the evaluation and selection of effective remediation strategies. Assessment and characterization of biological processes associated with contaminant attenuation is supported and improved by the accurate and consistent use of molecular biological tools (MBTs) including data acquisition, interpretation, and reporting.

4.2 The development of this guide through ASTM International is designed to meet the needs of managers of contaminated sites within the United States and elsewhere. The variety of available MBTs and the complexity with which they are currently being applied are not addressed in existing ASTM International Standards. The principal users of this guide should be industry project managers, regulators, consultants, analytical laboratories, and community stakeholders.

5. Overview of Molecular Biological Tools

5.1 This guide provides an overview and suggested application of a group of biological analyses collectively termed molecular biological tools (MBTs). MBTs are available to environmental practitioners for the purpose of directly assessing the contaminant-degrading capabilities and other activities of microorganisms present in the environment. MBTs can be applied to aqueous and solid media samples (for example, groundwater, wastewater, surface water, soils, sediments, etc.). There is a wide variety of MBTs, including but not limited to quantitative polymerase chain reaction (qPCR) and metagenomics, that can be applied depending on the selectivity desired (Fig. 1). For example, metagenomics allows for the sequencing on all DNA within a given sample, whereas target amplicon sequencing only sequences genes selected for with primers often across all microorganisms containing a gene (for example, all bacterial 16S rRNA genes within a sample) and qPCR often selects a gene unique to microbial group or

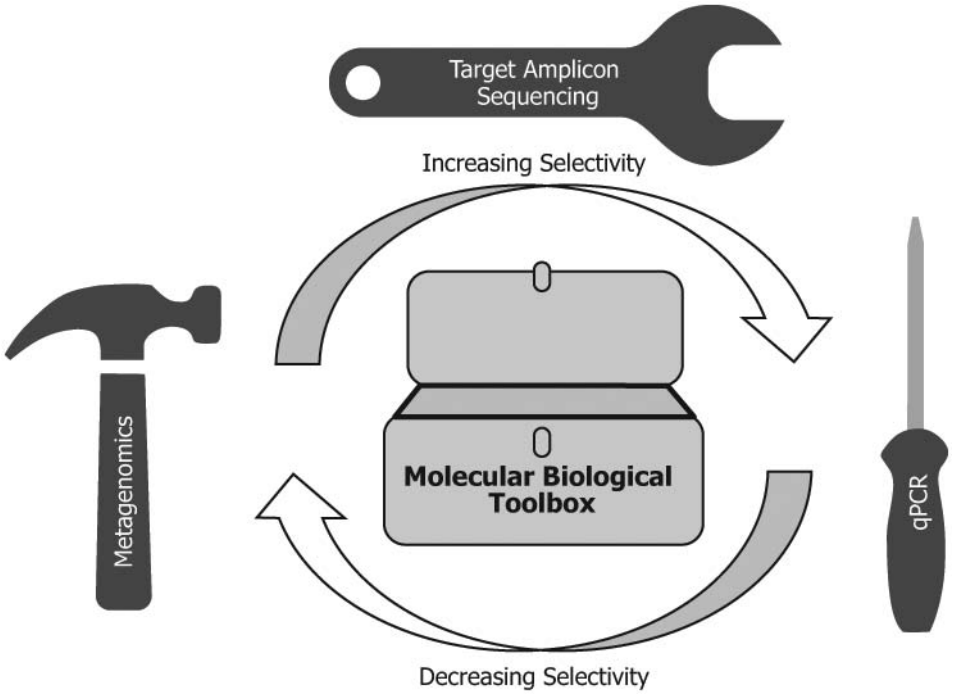
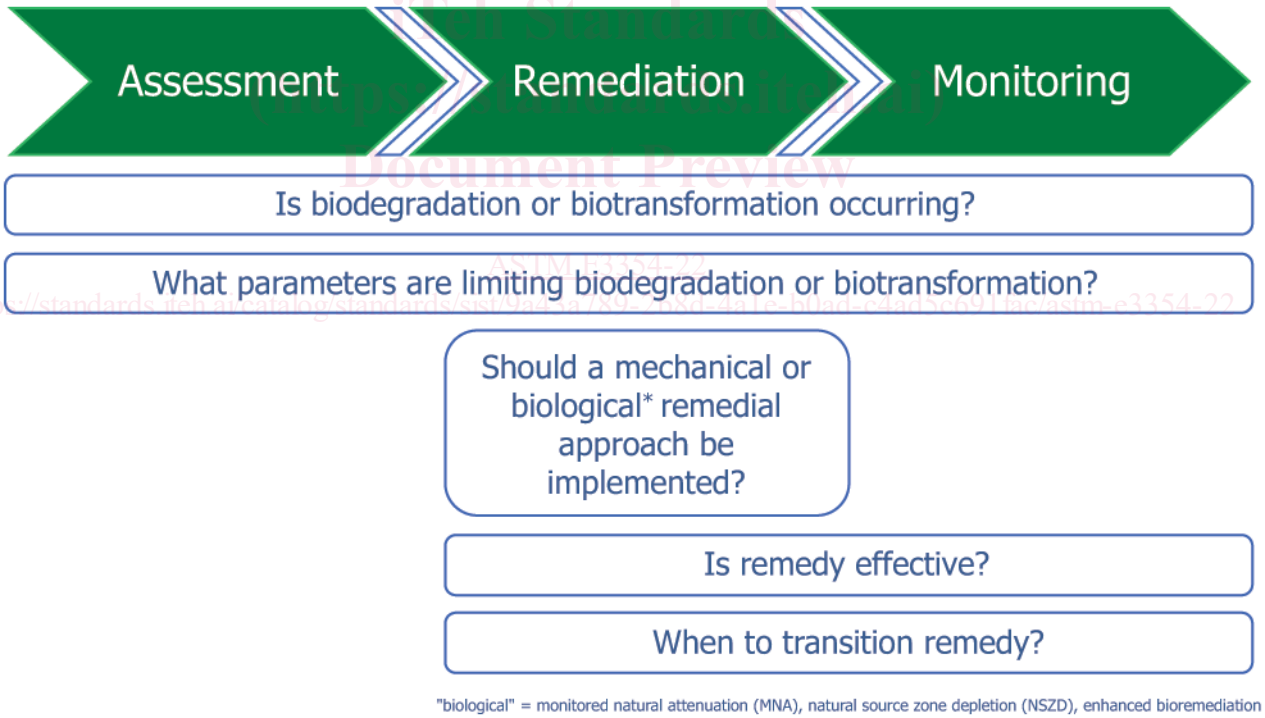


FIG. 1 Conceptualization of the molecular biological toolbox consisting of a range of tools with different levels of selectivity



NOTE 1—Consult Table 1 for additional associated questions that different types of MBTs can answer. Adapted by ITRC Figure ES-3 from Environmental Molecular Diagnostics Technical and Regulatory Guidance (2013).

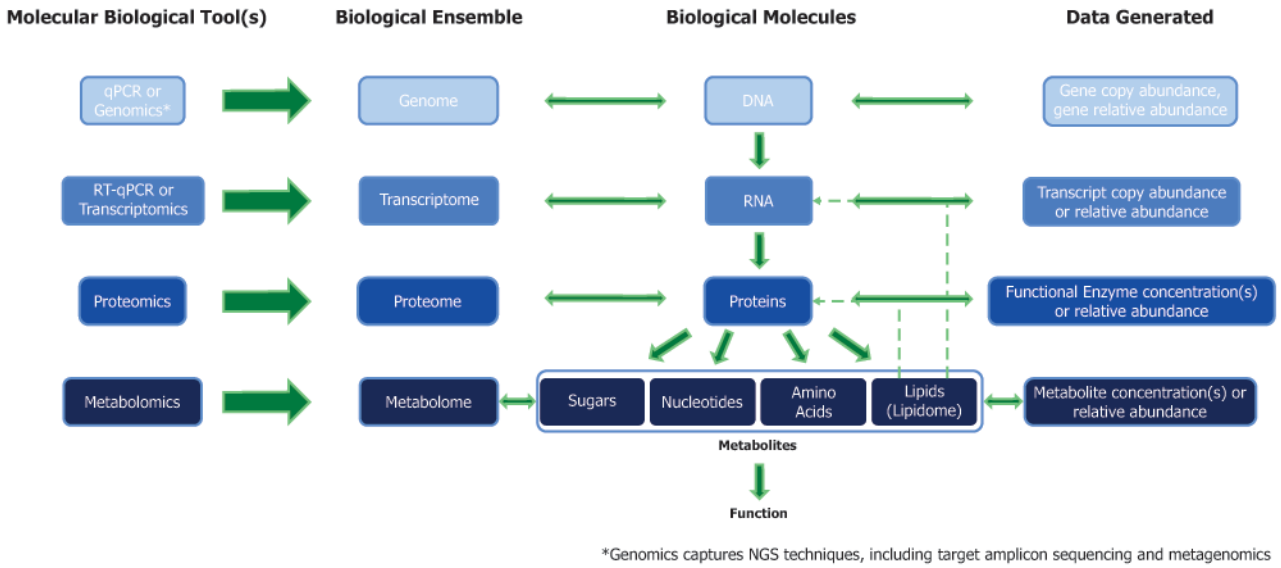
FIG. 2 Potential uses of MBT data to answer key site management questions through the project lifecycle

functional gene of interest (for example, 16S rRNA gene for *Dehalococcoides*). Further, within this document there is a brief description of isotopic techniques (for example, CSIA and SIP) within 5.2; however, the scope and focus of this guide is on the use of nucleic acid-based MBTs (for example, PCR and NGS) to assess the role of biological processes at contaminated sites. Additional information regarding the broader suite of available MBTs are available in Table 1 and Fig. 3.

5.2 *Available Molecular Biological Tools:*

TABLE 1 List of Molecular Biological Tools and Associated Questions, Type of Data Generated, Advantages and Disadvantages

Molecular Biological Tool	Abbreviation	Associated Questions	Type of Data Generated	Advantages	Disadvantages
Polymerase Chain Reaction (Targeted method)	PCR	Is a specific gene present?	•Presence / absence of gene	•Culture independent •Sensitive •Results are easy to understand and convey to stakeholders •Inexpensive Commercially available	•Positive results are dependent upon sequence similarity to a known gene. •Does not give information on activity •Does not differentiate between live and dead cell DNA
Quantitative Polymerase Chain Reaction (Targeted method)	qPCR	How many copies of a specific gene are present?	•Presence / absence of a gene •# of gene copies if present	•Culture independent •Sensitive •Quantitative •Results are easy to understand and convey to stakeholders •Inexpensive •Commercially available	•Positive results are dependent upon sequence similarity to a known gene. •Does not give information on activity •Does not differentiate between live and dead cell DNA
Reverse Transcription Polymerase Reaction (Targeted method)	RT-PCR	Is a specific gene actively being transcribed (used) and how much?	# of gene copies being transcribed	•Culture independent •Quantitative Identifies genes that are actively transcribed •Results are easy to understand and convey to stakeholders •Inexpensive •Commercially available	•Positive results are dependent upon sequence similarity to a known gene. •Activity is inferred from transcription •Only actively transcribed genes will be detected
16S rRNA Amplicon Sequencing (sometimes marketed as Next Generation Sequencing) (Targeted method)	16S sequencing (NGS)	What microorganisms are present? What microorganisms are numerically dominant?	Relative percentages of distinct 16SrDNA genes	•Culture independent •Provides information on what and how many organisms are present •Commercially available	•Sensitivity is inversely proportional to complexity of the community. •Does not give information on activity •Does not differentiate between live and dead cell DNA •Bioinformatic analysis can be a bottle neck to data usability and conveying results to stakeholders
Metagenomics (Non-targeted method)		What organisms and functional genes are present in the sampled microbial community?	Assemblage of the genetic material of the entire microbial community at a specific point in time	•Culture independent •Commercially available	•Does not give information on activity •Does not differentiate between live and dead cell DNA •Bioinformatic analysis can be a bottle neck to data usability and conveying results to stakeholders
Transcriptomics (Non-targeted method)		•What genes are being used by the microbial community? •Do they change under different conditions?	Assemblage of the genes being actively transcribed of the entire microbial community at a specific point in time	•Culture independent •Provides information on which physiological processes should be active	•Bioinformatic analysis can be a bottle neck to data usability and conveying results to stakeholders •Not commercially available
Proteomics (Non-targeted method)		What proteins/ enzymes are being produced by the microbial community?	Assemblage of the proteins being produced by of the entire microbial community at a specific point in time	Culture independent Can be used to reverse engineer a nucleic acid probe for a gene of interest Provides information on what processes organisms are actively carrying out in the environment.	Requires specialized expertise for separation and interpretation Not commercially available
Stable Isotope Probing (addition of stable isotope)	SIP	Is a specific target compound being biodegraded under in-situ conditions?	a) Shift of DNA to higher density indicating uptake b) Identification of degrading community through higher density DNA in 16SrDNA sequence fragments	•Can absolutely confirm biodegradation of target compound under *in-situ* conditions	•Limited to compounds that are used as sources of carbon by microbial community •When used with passive samplers the size of the microbial population may differ from the actual environment due to selective enrichment in the sampler.
Compound Specific Isotope Analysis (natural abundance of stable isotope (no addition)	CSIA	Is compound attenuation due to destructive reactions?	Change in ratio of heavy to light isotope(s) in the compound of interest relative to absolute concentration of the compound.	•Can confirm degradation of target compound under *in-situ* conditions (as opposed to non- degradative attenuation mechanisms). •Can provide information about the primary mechanism (biotic/ abiotic) of target compound destruction in a sample.	•Limited to degradation reactions that result in a strong isotope fractionation. •Detection limits for stable isotope are typically much higher than for mass measurements. •Significant method validation required for each compound of interest.
Metabolomics		What processes are active in the microbial community?	Assemblage of the metabolite profile of a cell or community at a specific point in time	Can provide information on the active physiological processes in the environment	•Requires specialized expertise for separation and interpretation •Not commercially available



NOTE 1—Adapted from schematic on "Metabolome" Wikipedia page (https://en.wikipedia.org/wiki/Metabolome).

FIG. 3 Conceptual schematic depicting overview and relationship between select MBTs, biological ensemble, biological molecules (DNA, RNA, proteins, and metabolites), and type data generated

5.2.1 *Polymerase Chain Reaction:*

5.2.1.1 The primary MBTs described in this guide are those based on the polymerase chain reaction (PCR). The goal of PCR analysis is the amplification of a target nucleic acid sequence (genomic DNA [gDNA] or complementary DNA [cDNA]) by several orders of magnitude. Once amplified, there should be sufficient copies of DNA for further analysis and interpretation. Through the amplification process, PCR technologies allow detection and analysis of DNA sequences even when they are initially present at low levels in the environment.

5.2.1.2 Many adaptations have been made to this technique, including reverse transcription PCR (RT-PCR), quantitative real-time PCR (qPCR), reverse transcription qPCR (RT-qPCR) and digital PCR (dPCR). In all cases, the PCR analysis relies on the use of a synthesized target sequence of DNA (that is, primers) to query total genomic DNA extracted from environmental samples for the presence of complementary sequences.

5.2.1.3 qPCR, allows for a targeted gene to not only be detected but also quantified. This technology is regarded as the "gold standard" nucleic acid analysis for contaminated site samples because of its high sensitivity, good reproducibility, quantification range, ease of use and relatively low costs. Quantification of a target DNA sequence (over time or space) using qPCR allows for evaluation of trends related to key organisms or functional genes involved in contaminant biodegradation.

5.2.2 *Next Generation Sequencing*—Next Generation Sequencing (NGS) is the simultaneous sequencing of multiple pieces of nucleic acid for the purpose of making observations about the full microbial community within a sample. This technology allows for assessment of the microbial community (that is, phylogenetics or who is there) and, in some cases where function is strongly linked to phylogeny, suggest or infer what the microbes do (for example, their function or role). NGS can be a useful tool to observe changes in microbial community structure both in microbial diversity and in function that may result from environmental contamination and remediation activities. NGS is not a fully quantitative tool, but rather provides insight on the proportions or abundances of microbes relative to others in a given community. Recent advances in sequencing technology have significantly reduced the cost of this analysis. Nevertheless, the organization and analysis of the sequenced DNA requires bioinformatic expertise which can be a bottleneck to producing and interpreting the results. NGS technology can be used for targeted amplicon sequencing, such as for the 16S rRNA gene, or for non-targeted sequencing of the entire metagenome (metagenomics) within a sample. When requesting NGS from a laboratory, it is important to specify targeted amplicon or metagenomic sequencing and understand laboratory NGS 382 capabilities.

5.2.3 *Omics*—Omics is a term that broadly refers to the study of biological processes at numerous levels, from the characterization of genes (genomics), mRNA (transcriptomics), proteins (proteomics) or metabolites (metabolomics) (see Fig. 3). While genomics is the study of an organism's entire genetic material, metagenomics is the study of genetic material of all of the organisms found in a specific environment. In the context of contaminated site management, these tools can provide yet another line of evidence to support the activity of specific physiological functions (for example, biodegradation of hydrocarbons or sulfate reduction), or document changes or differences in microbial populations.

5.2.4 *Isotopic Tools*—Isotopic tools rely on measurable differences between heavy and light stable isotopes of elements in a single compound.

5.2.4.1 *Compound specific isotope analysis*—Compound specific isotope analysis (CSIA) measures differences in relative abundance of stable isotopes within a COC, for example ^{13}C and ^{12}C, especially when chemical bonds are expected to be broken. For remediation strategies where COC mass is transformed or destroyed (through chemical transformation or biodegradation), the bonds in lighter isotopes (for example,

^{12}C) are preferentially broken resulting in an enrichment of the heavier isotopes (for example, ^{13}C) of the remaining parent substrate. These compound specific isotope changes can be linked to the extent of transformation or biodegradation of COC over time or distance (USEPA, 2008).

5.2.4.2 *Stable isotope probing*—Stable isotope probing (SIP) uses a microbial substrate (typically a site COC which is enriched in the heavier stable isotope and can be purchased from a specialized vendor) The heavy isotope contaminant is subjected to the site subsurface conditions and microbes that then may degrade the heavier contaminant. Biomass and biodegradation products are tracked over time to assess incorporation of the heavier isotope to demonstrate ongoing biodegradation processes. SIP can be performed in the lab and in the field using commercially available *in-situ* microcosm devices.

6. Application of Molecular Biological Tools

6.1 *Project Lifecycle*—The application of MBTs should be considered for use during the following stages of the contaminated site project lifecycle: assessment, remediation, and monitoring. Application of MBTs can be performed to answer different questions depending on the stage of the project lifecycle in which MBTs are applied (Fig. 2). MBTs can be complementary lines of evidence used to: (a) develop or refine biogeochemical processes within the conceptual site model (CSM) (b) reduce uncertainty of biological processes associated with remedy design, (c) monitor remedy performance and differentiate biological processes from chemical or physical processes, and (d) support communications with stakeholders. While nucleic acid-based MBTs cannot quantify field-scale biodegradation or biotransformation rates as of the time of writing this guide, there are past and ongoing efforts to assess the potential for field-scale rate quantification based on the abundance of biomarkers (Lu et al., 2006 (**1**),[4] Wilson et al., 2019 (**2**), Michalsen et al., 2021 (**3**), Adamson et al., 2022 (**4**).

6.1.1 *Assessment*—MBT data in conjunction with COC, or geochemical data, can be used to support site assessment by determining if key organisms or genes associated with biodegradation are present at the site. With the MBT and geochemical data set, a project manager can determine if the baseline site conditions are conducive to biodegradation processes. If COC attenuation has been observed, these data can help establish the mechanisms responsible for observed changes. If COC attenuation has not been observed, these data can help in the development of hypotheses as to the factors limiting biodegradation.

6.1.2 *Remediation*—Once the site has been assessed at the baseline phase, MBT data can support the selection and implementation of specific remedial strategies to initiate or enhance attenuation of COCs. For example, COC attenuation could be occurring slowly or have stalled due to a depletion of limiting electron donors, electron acceptors, or nutrients even if key microorganisms are present. In this case, the remedial strategy might be to alleviate the electron acceptor/donor or nutrient limitation through biostimulation with site-specific amendments (for example, emulsified vegetable oil (EVO), formate, sulfate, nitrate, oxygen, etc.). Alternatively, the key microorganisms may not be present in sufficient numbers because the site geochemistry is not supportive. For this latter case, the remedial strategy could be changing the overall geochemistry, for example pH, and possibly bioaugmentation, the addition of key microorganisms.

6.1.3 *Monitoring*—MBTs can be a powerful tool for monitoring the progression of an implemented remedy. MBTs can directly measure the presence of key microorganisms, functional genes that code for enzymes involved in biodegradation of COCs, and other relevant biogeochemical processes. Monitoring of microbial and functional genes, in conjunction with changes in COC concentrations and geochemical data, often provide compelling evidence to determine the effectiveness of remedial interventions. For example, MBT data may demonstrate an increased abundance of contaminant-degrading microorganisms or functional genes before measurable changes in contaminant concentration are observed. These data are especially relevant when there is significant contaminant mass present as sorbed phase or non-aqueous phase liquid (NAPL) in the area targeted by remediation. This observation may be considered a leading indicator of contaminant remediation. If required microorganisms are not detected at sufficient abundance, that finding may indicate the need for remedy adjustment or optimization.

6.2 *Value of Application:*

6.2.1 *Assessing Contaminant Biodegradation*—The commercial availability of PCR and qPCR technologies has resulted in their routine use for detecting presence or absence of specific microorganisms or functional genes known to biodegrade COCs and in the case of qPCR, quantification. For example, high abundance of Dehalococcoides mccartyi (an obligate organohalide respiring anaerobe) and vinyl chloride reductase genes serve as positive indicators of the potential for anaerobic biodegradation of chlorinated organics. As additional COC biodegradation pathways, and corresponding associated functional genes, are identified, qPCR assays are developed to target these novel genes. Currently, there are well documented qPCR assays to quantify microorganisms and functional genes that degrade a wide range of environmental contaminants. Table 2 lists a selection of some of the commercially available qPCR test targets as of October 2021. It is inevitable that as additional contaminant biodegradation and biotransformation pathways, microorganisms, and functional genes are identified and elucidated, new assays will continue to be developed and deployed.

6.2.2 *Supporting Remedy Transition*—Various remediation technologies are often combined to remove contaminant mass through physical processes (for example, volatilization or dissolution from NAPL) and then destroy it via chemical and/or biological processes (for example, chemical oxidation and biodegradation). Mechanical *in-situ* remediation approaches include technologies such as pump-and-treat, air sparging, soil vapor extraction, or multiphase extraction for contaminant removal from saturated and/or vadose zone. At sites being treated with mechanical processes, the remediation

[4] The boldface numbers in parentheses refer to a list of references at the end of this standard.

TABLE 2 Selection of Commercially Available Genetic Targets for Microorganisms and Functional Genes Associated with Contaminant Biological Processes

Contaminant Class	Redox	Acronym	Target	Most Common Relevance
Chlorinated Ethenes	Anaerobic	Dhc	*Dehalococcoides*	Reductively dechlorinates PCE, TCE, all DCE isomers, VC
		bvcA	BAV1 Vinyl chloride reductase (bvcA)	Dechlorination of cDCE and VC to ethene
		tceA	Trichloroethene reductase (tceA)	Dechlorination of PCE and TCE to cDCE and VC
		vcrA	Vinyl chloride reductase (*vcrA*)	Dechlorination of cDCE and VC to ethene
		Dhb	*Dehalobacter*	Partial dechlorination of PCE and TCE to cDCE
		Dsm	*Desulfuromonas*	Reductively dechlorinates PCE and TCE to cis-DCE using acetate as an electron donor
		Dsb	*Desulfitobacterium*	Reductively dechlorinates PCE and TCE to cis-DCE
		PCE-1	*Geobacter* Tetrachloroethylene Reductase (pceA)	Reductive dechlorination of PCE to cis-DCE
		PCE-2	*Sulfurospirillum* Tetrachloroethylene Reductase (pceA)	Reductive dechlorination of PCE to cis-DCE
		Dhg	Dehalogenimonas	Dechlorination of tDCE to VC and VC to ethene
		CER	Dehalogenimonas Vinyl Chloride Reductase	Dechlorination of VC to ethene
		TDR	Dehalogenimonas 1, 2-Trans Dichloroethene Reductive Dehalogenase (tdrA)	Reductive dechlorination of 1,2 trans DCE
		MBR	DHC MB Reductase	Potential biomarker for Production of trans DCE during dechlorination of PCE and TCE
		MGN	Methanogens via methyl coenzyme reductase mcrA/mrtA	Utilize hydrogen and can compete with halorespiring bacteria for available electron donor
		APS	Sulfate reducing bacteria via Adenosine 5' phosphosulfate reductase	Compete with halorespiring bacteria for available hydrogen
	Aerobic	sMMO	Soluble Methane Monooxygenase	Co-oxidation of TCE, cis-DCE, and vinyl chloride. Expressed at higher copper to biomass ratios
		pMMO	Particulate Methane Monooxygenase	pmoA is expressed at higher copper to biomass ratios and is capable of cometabolizing TCE at lower rates.
		RMO	Ring hydroxylating Toluene monooxygenase (toluene-3 and 4 monooxygenases)	Co-oxidation of TCE
		RDEG	Ring hydroxylating Toluene monooxygenase (toluene-2 monooxygenases)	Co-oxidation of TCE
		PHE	Phenol Hydroxylase	Co-oxidation of TCE
		TOD	Toluene and Benzene Dioxygenases	Co-oxidation of TCE
		EtnC	Ethene monooxygenase	Converts ethene and vinyl chloride to their respective epoxyalkanes
		EtnE	Epoxyalkane transferase	Mediates conjugation and breaking of the epoxide
		PPO	Propane monooxygenase	Cometabolism of TCE in the presence of propane
		BMO	Butane monooxygenase	Cometabolism of TCE in the presence of butane
Chlorinated Ethanes	Anaerobic	Dhb	*Dehalobacter*	Dechlorination of 1,1,1-TCA/1,1-DCA to chloroethane; 1,2-DCA /1,1,2-TCA to VC/ethene; 1,1,2,2-TeCA to tDCE
		Dhg	*Dehalogenimonas*	Dechlorination of 1,2- DCA to ethene; 1,1,2,2-TeCA to c/tDCE; 1,1,2-TCA to VC
		Dhc	*Dehalococcoides*	Dechlorination of 1,2-DCA to ethene; dechlorination of VC produced from 1,1,2-TCA dechlorination
		Dsb	*Desulfitobacterium*	Dechlorination of 1,1,2-TCA and 1,2-DCA
		DCA	1,1 Dichloroethane dehalogenase (dcrA)	Dechlorination of 1,1-DCA to chloroethane
		CFR	Chloroform Reductase (cfrA, ctrA, thmA)	dechlorination of 1,1,1-TCA to 1,1-DCA
		DCAR	1, 2 Dichloroethane dehalogenase (*Desulfitobacterium* and *Dehalobacter*)	Dechlorination of 1,2-DCA to ethene
		MGN	Methanogens via methyl coenzyme reductase mcrA/mrtA	Utilize hydrogen and can compete with halorespiring bacteria for available electron donor
		APS	Sulfate reducing bacteria via Adenosine 5' phosphosulfate reductase	Compete with halorespiring bacteria for available hydrogen
	Aerobic	sMMO	Soluble Methane Monooxygenase	Co-oxidation of 1,1,1-TCA and 1,2-DCA
		PPO	Propane monooxygenase	Cometabolism of 1,1,2-TCA in the presence of propane
		BTM	Butane monooxygenase	Cometabolism of 1,1,1-TCA in the presence of butane
Chlorinated Methanes	Anaerobic	Dhb	*Dehalobacter*	Reductive dechlorination of chloroform to DCM; fermentation of DCM to acetate
		DCM	*Dehalobacter* DCM	Degrade DCM through fermentation
		CFR	Chloroform Reductase (cfrA)	CFR converts chloroform to dichloromethane
	Aerobic	sMMO	Soluble Methane Monooxygenase	Co-metabolism of chloroform and dichloromethane
		DCMA	Dichloromethane dehalogenase	Dechlorination of DCM to formaldehyde under oxic conditions
Chlorinated Propanes	Anaerobic	Dhg	*Dehalogenimonas*	Converts TCP to allyl chloride; DCP to propene
		Dhc	*Dehalococcoides*	Converts DCP to propene
		Dhb	*Dehalobacter*	Converts DCP to propene

TABLE 2 *Continued*

Contaminant Class	Redox	Acronym	Target	Most Common Relevance
		Dsb	*Desulfitobacterium*	Dechlorination of TCP and DCP
		1,2 DCP	1,2 Dichlorpropane Dehalogenase	Dechlorination of 1,2 DCP to
	Aerobic	sMMO	Soluble Methane Monooxygenase	Capable of co-oxidation of 1,2-DCP 1,3-DCP and 1,2,3-TCP
		PPO	Propane monooxygenase	Capable of cometabolism of TCP
Chlorinated Benzenes	Anaerobic	Dhc	*Dehalococcoides*	Capable of Dechlorinating hexachlorobenzene, pentachlorobenzene, tetrachlorobenzene
		Dhb	*Dehalobacter*	Potential reductive dechlorination of DCBs and CB
		DECO	*Dehalobium chlorocoercia* DF-1	Capable of reductive dechlorination of HCB, PeCB and 1,2,3,5- TeCB.
		MGN	Methanogens via methyl coenzyme reductase mcrA/mrtA	Utilize hydrogen and can compete with halorespiring bacteria for available electron donor
		APS	Sulfate reducing bacteria via Adenosine 5' phosphosulfate reductase	Compete with halorespiring bacteria for available hydrogen
	Aerobic	PHE	Phenol Hydroxylase	Significant increases in numbers of bacteria containing PHE genes corresponded to increases in biodegradation of DCB isomers
		TOD	Toluene and Benzene Dioxygenases	Mediates the incorporation of both atoms of oxygen into the aromatic ring of benzene and substituted benzenes (toluene and chlorobenzene)
		RMO	Ring hydroxylating Toluene monooxygenase (toluene-3 and 4 monooxygenases)	Catalyze the initial and in some cases second oxidation of a variety of monoaromatic compounds including BTEX and CB
		TCBO	Trichlorobenzene and Biphenyl/ Isopropylbenzene Dioxygenases	Capable of aerobic biodegradation of a number of chlorinated benzenes including chlorobenzene, 1,2-dichlorobenzene, 1,2,4- trichlorobenzene, and 1,2,4,5-tetrachlorobenzene
Chlorinated Biphenyls	Anaerobic	Dhc	*Dehalococcoides*	Capable of reductive dechlorination of PCBs
		DECO	*Dehalobium chlorocoercia* DF-1	Shown to reductively dechlorinate doubly flanked chlorines in PCBs
		PCBR	*Dehalococcoides* PCB Reductase pcbA1, pcbA4, and pcbA5	May serve as biomarkers for the dechlorination of PCBs
	Aerobic	BPH	Biphenyl Dioxygenase	Initiates aerobic cometabolism of PCBs.
Chlorinated Phenols	Anaerobic	Dhc	*Dehalococcoides*	Capable of utilizing PCP and tetrachlorophenol (TeCP), trichlorophenol (TCP), and 2,3-dichlorophenol (2,3-DCP)
		Dsb	*Desulfitobacterium*	Some strains are capable of reductive dechlorination of PCP while others can utilize TCP and DCP
		MGN	Methanogens via methyl coenzyme reductase mcrA/mrtA	Utilize hydrogen and can compete with halorespiring bacteria for available electron donor
		APS	Sulfate reducing bacteria via Adenosine 5' phosphosulfate reductase	Compete with halorespiring bacteria for available hydrogen
	Aerobic	PCP	Pentachlorophenol Monooxygenase (pcpB, E, and R)	Initiates oxidation of PCP and aromatic ring cleavage
BTEX	Anaerobic	bssA	Benzylsuccinate Synthase	Mediates the first step in the anaerobic biodegradation of toluene and in some cases ethylbenzene and xylenes
		abcA	Anaerobic Benzene Carboxylase	Anaerobic biodegradation of benzene
		GMET	Geobacter metallireducens Functional Genes	Targets functional genes including a predicted oxidoreductase specifically required for anaerobic benzene metabolism
		BCR	Benzoyl Coenzyme A Reductase	Reduces the benzene ring structure of the central intermediate Benzyl-CoA
		ORM-2	*Deltaproteobacterium ORM-2*	ORM-2 anaerobic benzene degrader under sulfate reducing or methanogenic conditions
		SRB	Sulfate reducing bacteria via *dissimilatory sulfate reductase* (*dsrA*)	SRB are symbiotic partners to ORM-2 in anaerobic benzene degradation
		Pepto-ben	Benzene degrading Peptococcacae	Anaerobic benzene degradation under nitrate reducing conditions
	Aerobic	TOD	Toluene and Benzene Dioxygenases	Capable of degrading toluene, benzene, and chlorobenzene along with co-oxidation of a variety of compounds including ethylbenzene, p-xylene, and m-xylene
		RMO	Ring hydroxylating Toluene monooxygenase (toluene-3 and 4 monooxygenases)	Catalyze the initial oxidation and sometimes second oxidation steps in aerobic BTEX biodegradation
		RDEG	Ring hydroxylating Toluene monooxygenase (toluene-2 monooxygenases)	Catalyze the initial oxidation and sometimes second oxidation steps in aerobic BTEX biodegradation
		PHE	Phenol Hydroxylase	Catalyze the continued oxidation of phenols produced by RMOs
		TOL	Toluene /Xylene Monooxygenase	Biodegradation of toluene by attacking at the methyl group
		EDO	Ethylbenzene Dioxygenase	Responsible for aerobic biodegradation of alkylbenzenes including ethylbenzene and isopropylbenzene or cumene

TABLE 2 *Continued*

Contaminant Class	Redox	Acronym	Target	Most Common Relevance
		TCBO	Trichlorobenzene and Biphenyl/ Isopropylbenzene dioxygenases	Includes benzene and isopropylbenzene dioxygenases
MTBE and TBA	Aerobic	PM1	*Methylibium petroleiphilum* PM1	Capable of utilizing MTBE and TBA as growth supporting substrates
		TBA	Tert Butyl Alcohol Monooxygenase	Catalyzes the continued biodegradation of TBA, an intermediate produced during aerobic MTBE and ETBE biodegradation
		ETHB	P450 cytochrome monooxygenase	Initiates aerobic biodegradation of ETBE and is capable of co- oxidation of MTBE and TAME
Diesel, Naphthalene, and PAHs	Anaerobic	ANC	Anaerobic Naphthalene Carboxylase	Only known pathway for anaerobic biodegradation of naphthalene
		mnssA	Naphthyl-2-methyl-succinate Synthase	Initiates anaerobic biodegradation of 2-methylnaphthalene by catalyzing the addition of fumarate onto the methyl group
	Aerobic	NAH	Naphthalene Dioxygenase	Initiates aerobic metabolism of naphthalene by incorporating both atoms of molecular oxygen into the ring. Also capable of catalyzing oxidation of anthracene, phenanthrene, acenaphthylene, acenaphthene, and fluorine.
		NidA	Naphthalene-Inducible Dioxygenase	Capable of mineralizing naphthalene and degrading some higher molecular weight PAHs including pyrene and benzo[a]pyrene.
		PHN	Phenanthrene Dioxygenase	Phenanthrene/naphthalene dioxygenases capable of degrading phenanthrene and naphthalene but have broad specificity
		ARHA	Acenaphthylene Dioxygenase	Capable of catalyzing the degradation of acenaphthene, acenaphthylene, naphthalene, phenanthrene, anthracene and fluoranthene
		NAHM	Marine Naphthalene and PAH Dioxygenases (nahAc, phnA)	Quantifies naphthalene dioxygenase genes from marine organisms that are capable of catalyzing the degradation of naphthalene, 2-methylnaphthalene, phenanthrene, fluoranthene, and pyrene.
		PHE	Phenol Hydroxylase	Involved in aerobic biodegradation of BTEX and catalyze the continued oxidation of phenols produced by toluene monooxygenases and indicate the potential for aerobic BTEX biodegradation.
TPH -Alkanes and Crude Oil	Anaerobic	assA	Alkylsuccinate Synthase	Initiates anaerobic biodegradation of alkanes with chain lengths from C6 to at least C18
	Aerobic	ALKB	Alkane Monooxygenase	Initiates the aerobic biodegradation of n-alkanes with carbon lengths from C5 to C16
		ALMA	Alkane Monooxygenase	Catalyzes the aerobic biodegradation of C20-C32 alkanes by some Alcanivorax species that are dominant in marine systems
		CAR	Carbazole Dioxygenase	Catalyzes the oxidation of carbazole and other high molecular weight aromatics such as dibenzofuran
1,4 Dioxane	Aerobic	DXMO	Dioxane/Tetrahydrofuran Monooxygenase	Initiates aerobic metabolism of 1,4-dioxane by *P. dioxanivorans* CB1190. In other organisms however, DXMO/THFMO initiates metabolism of tetrahydrofuran and only co-oxidation of dioxane
		ALDH	Aldehyde Dehydrogenase	Co-expressed with DXMO/THFMO
	Aerobic Cometabo lism	PPO	Propane monooxygenase	Aerobic propane utilizing bacteria are capable of co-oxidation of dioxane when propane is added as a substrate
		RMO	Ring hydroxylating Toluene monooxygenase (toluene-3 and 4 monooxygenases)	Capable of co-oxidation of dioxane
		RDEG	Ring hydroxylating Toluene monooxygenase (toluene-2 monooxygenases)	Capable of co-oxidation of dioxane
		SCAM	Small Chain Alkane Monooxygenase	Induced by a wide variety of gaseous alkanes and are especially effective for 1,4-D cometabolism
Dioxin, dibenzofuran	Aerobic	dbfA	Dibenzofuran dioxygenase	Catalyzes the first step in the aerobic degradation of dibenzofuran. Can also degrade dibenzo-p-dioxin at lower levels.
Dibenzothiophene	Aerobic	dbtA	Dibenzothiophene dioxygenase	Catalyzes the degradation of the intermediate dibenzothiophene in *Burkholderia*
RDX	Aerobic	XplA	RDX degrading organisms via cytochrome P450 (xplA)	Targets the xplA gene for the aerobic degradation of RDX
Styrene	Aerobic	STY	Styrene Monooxygenase	Catalyzes the epoxidation of styrene to styrene oxide
Perchlorate	Anaerobic	pcrA	Perchlorate Reductase	Catalyzes the initial, rate-limiting step in the biodegradation of perchlorate. In some organisms, perchlorate reductase also catalyzes the reduction of chlorate to chlorite
		pcrAS	Perchlorate Reductase	Catalyzes the initial, rate-limiting step in the biodegradation of perchlorate as well as the reduction of chlorate to chlorite in *Sedimenticola* spp.

TABLE 2 *Continued*

Contaminant Class	Redox	Acronym	Target	Most Common Relevance
		DNF	Denitrifying Bacteria via dissimilatory nitrite reductase (nirS and nirK)	Many, but not all, perchlorate reducing bacteria will utilize nitrate as an electron acceptor potentially limiting perchlorate reduction.
Nitrogen, Nitrate, Nitrite, Ammonia	Anaerobic	NIF	Nitrogen Fixing Bacteria via nitrogenase (nifD)	Nitrogen fixation converts nitrogen gas into ammonia which can be assimilated by organisms
		DNF	Denitrifying Bacteria via dissimilatory nitrite reductase (nirS and nirK)	Responsible for converting nitrite to nitric oxide
		ADNF	Archaeal Denitrifying Bacteria via nitrite reductase (nirS and nirK)	Responsible for converting nitrite to nitric oxide in archaeal organisms
		Anammox	Anaerobic ammonia oxidation (*Brocadia, Kuenenia, Scalindua, Anammoxyglobus, Jettenia*)	Responsible for converting nitrite and ammonia directly into molecular nitrogen
		AMXNIRS	Anammox Nitrite Reductase (nirS)	Responsible for reducing nitrite to nitric oxide inside the anammoxosome
		AMXNIRK	Anammox Nitrite Reductase (nirK)	Responsible for reducing nitrite to nitric oxide inside the anammoxosome
	Aerobic	AMO	Ammonia oxidizing bacteria via ammonia monooxygenase (amoA)	Responsible for converting ammonia to hydroxyl amine which is then converted to nitrite by hydroxylamine oxidoreductase
		AOA	Ammonia oxidizing archaea via ammonia monooxygenase (amoA)	Responsible for converting ammonia to hydroxyl amine in archaeal organisms
		NOB	Nitrite oxidizing bacteria (Nitrospira)	Responsible for converting nitrite to nitrate
		NOR	Nitrite oxidizing bacteria via nitrite oxidoreductase (NOR/NXR)	Responsible for converting nitrite to nitrate
Prokaryotic Groups	Variable	AAB	Acetic Acid Bacteria via alcohol dehydrogenase (adhA) (Acetobacter, Gluconobacter, and Komagataeibacter)	Catalyzes the oxidation of ethanol to acetic acid which can be a potential cause of corrosion.
		AGN	Acetogens	Acetogenic bacteria are strict anaerobes that produce acetate from the conversion of H_2-CO_2, CO, or formate. The presence of acetic acid is known to exacerbate carbon dioxide corrosion of carbon steel
		AMGN	Acetoclastic Methanogens (*Methanosarcina*)	Acetoclastic methanogens dismutate acetate to form methane.
		APS	Sulfate reducing bacteria via Adenosine 5' phosphosulfate reductase	Anaerobic hydrocarbon oxidation/biogeochemical reduction/microbially induced corrosion
		ARG	*Archaeoglobus*	Targets a genus of sulfate reducing archaea
		ARC	Total Archaea	Quantifies total archaea
		BCE	*Burkholderia* Exopolysaccharide	Gene involved in the production of exopolysaccharide (EPS) and biofilm formation by some *Burkholderia* spp.
		CLAD	*Cladosporium*	*Cladosporium resinae* is such a common fuel contaminant that it has been described as the "kerosene fungus." *C. resinae* grows on hydrocarbons including alkanes to produce organic acids often linked to the corrosion of aluminum fuel tanks.
		DCS	*Deinococcus*	Genus of bacteria considered very efficient primary biofilm formers and therefore have been implicated in slime formation and biofouling.
		EBAC	Total Eubacteria	Quantifies total Eubacteria
		FEOB	Iron Oxidizing Bacteria (Gallionella, Leptothrix, Mariprofundus, and Sphaerotilus)	Iron oxidizing bacteria are a group of microorganisms commonly implicated in metal deposition and tubercle formation.
		FER	Fermenting Bacteria (Firmicutes)	Anaerobic bacteria that produce organic acids and hydrogen. Acid production can lead to localized drops in pH facilitating corrosion while hydrogen production can support growth of other MIC associated organisms including SRB.
		GEO	Geobacter	Iron reducing bacteria reduce insoluble ferric iron to soluble ferrous iron potentially facilitating the removal of protective corrosion products formed on exposed iron alloy surfaces. This assay targets a common iron reducing bacteria, *Geobacter*.
		GLK	Glycerol utilizing acetogens via glycerol kinase	Microbial degradation of glycerol, a byproduct of biodiesel production from fats, leads to the generation of volatile fatty acids (VFAs) (lactic and propionic acid) both of which have been observed at high concentrations in diesel tanks.
		IRB/SRB	Iron and Sulfate Reducing Bacteria	Quantifies iron and sulfate reducing Deltaproteobacteria
		IRA	Iron Reducing Archaea (Ferroglobus and Geoglobus)	Quantifies two genera of iron reducing archaea
		IRB	Iron Reducing Bacteria	Iron reducing bacteria reduce insoluble ferric iron to soluble ferrous iron potentially facilitating the removal of protective corrosion products formed on exposed iron alloy surfaces.