Quantitative Polymerase Chain Reaction

EMD Team Fact Sheet—November 2011

This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. Please review the Introduction to EMDs Fact Sheet along with this one. A glossary is included at the end of this fact sheet.

Why is quantitative polymerase chain reaction relevant?

Quantitative polymerase chain reaction (qPCR) and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) are used to quantify the abundance and activity of specific microorganisms or expressed genes in pathways capable of biodegradation of contaminants present at a contaminated site. When microorganisms capable of biodegradation of the contaminants are relatively abundant and active under existing subsurface conditions, monitored natural attenuation (MNA) can be an effective site management approach; qPCR provides a means to establish such situations. On the other hand, qPCR results can reveal when contaminant-using microorganisms are present but not thriving, thus providing evidence that enhanced bioremediation options may need to be explored to stimulate the microbial community. Enhanced bioremediation, or biostimulation, involves addition an amendment such as an electron donor (e.g., lactate, emulsified vegetable oil) or acceptor (e.g., oxygen, sulfate) for the specific purpose of stimulating growth and activity of microorganisms capable of biodegradation of the contaminants. Thus, project managers can use qPCR in the characterization phase, remedy selection, monitoring, and site closure to assess microbial growth and activity. Within the environmental restoration industry, qPCR analyses have been offered on a commercial basis since 2002. In total, qPCR has been used to evaluate contaminant biodegradation in all but four states in the United States and countries representing six different continents.

What does qPCR do?

qPCR is an analytical method used to determine the number of copies of specific genes or DNA targets present in a sample. Depending on which gene is investigated, the qPCR analysis can quantify functional genes (e.g., trichloroethene [TCE] reductase [*tceA*]), specific microorganisms (e.g., *Dehalococcoides*), or groups of related microorganisms (e.g., sulfate-reducing bacteria) in soil, sediment, or groundwater samples. Like polymerase chain reaction (PCR, see the PCR Fact Sheet), qPCR requires specific primers to enable it to selectively amplify (and detect) individual low-abundance genes in samples containing many millions of other genes. As the name suggests, qPCR adds quantitation to the underlying PCR technology.

Since development in the early 1990s, qPCR has been used to study a wide variety of environmental processes, including biodegradation of chlorinated solvents such as perchloroethene (PCE), TCE, etc. (Davis et al. 2008, Hendrickson et al. 2002, Lee et al. 2008); petroleum hydrocarbons such as benzene, toluene, ethylbenzene, and xylenes (Baldwin et al. 2010); polycyclic aromatic hydrocarbons (DeBruyn, Chewning, and Sayler 2007); fuel oxygenates such as methyl *tert*-butyl ether (Hristova et al. 2003); and radionuclides (Amos et al. 2007). qPCR analyses have potential applicability in other environmental monitoring efforts, such as tracking of fecal microorganisms, so-called “microbial source tracking,” and identifying and tracking microorganisms potentially related to chemical compounds originating from industrial or energy-related activities.

How are the data used?

Data generated from qPCR analyses provide information about the identity and abundance of specific microorganisms that may be present and capable of biodegrading identified contaminants. During biodegradation processes, microorganisms break down contaminants using enzymes. The genes a microorganism possesses not only enable identification of the microorganism but also determine which enzymes that microorganism can produce and therefore which contaminants it can biodegrade. As mentioned, qPCR and RT-qPCR are used to measure the abundance of specific genes in a sample of

soil or groundwater. Depending on which genes are quantified, this information is indicative of the abundance of genes that encode for a particular enzyme (e.g., *tceA*) or the abundance of microorganisms that are known to biodegrade specific contaminants (e.g., *Dehalococcoides*). Quantification of specific genes or microorganisms capable of biodegradation of a contaminant provides a direct line of evidence that helps answer questions about site remediation.

Example Environmental Remediation Questions qPCR and RT-qPCR Can Help Answer

- **Site Characterization**
  - Assess current conditions and potential for biodegradation
    - Are microorganisms capable of biodegradation of the contaminant present?
    - If so, how many are present?
    - Are key contaminant-degrading microorganisms (qPCR) and biodegradation pathways (RT-qPCR) active?
  - Preliminary identification of remediation alternatives
- **Remediation**
  - Is MNA feasible?
    - Are microorganisms capable of biodegradation of the contaminant present?
    - Are contaminant-degrading microorganisms present in sufficient abundance under existing site conditions?
    - Are contaminant-degrading microorganisms and biodegradation pathways active under existing site conditions (RT-qPCR)?
  - Is biostimulation necessary? Should an amendment be added?
    - Will adding an amendment such as an electron donor (e.g., emulsified vegetable oil) or an electron acceptor (e.g., oxygen) stimulate growth of contaminant using microorganisms?
    - Will amendment addition promote activity of contaminant-degrading microorganisms?
  - Is bioaugmentation necessary?
- **Monitoring**
  - Monitored natural attenuation
    - Are contaminant-degrading populations maintained over time under existing site conditions?
  - Biostimulation
    - Did amendment addition promote growth or activity of contaminant-using microorganisms?
    - Are contaminant-degrading populations maintained over time?
    - Should a second amendment addition be considered?
  - Bioaugmentation
    - Did microbial culture survive in situ?
    - Is the microbial culture maintained over time?
- **Closure**
  - Are contaminant-degrading populations abundant and stable, suggesting that contaminant degradation is likely to continue?

In terms of site characterization and remedy selection, MNA is unlikely to be a feasible site management strategy when qPCR results indicate that contaminant-using populations are not present under existing site conditions. Conversely, when target microbial populations are detected, MNA is at least possible. However, when target microbial populations or activity are low, MNA may not achieve site closure goals in an acceptable time frame, and enhanced bioremediation options may need to be considered. For example, at TCE-impacted sites, the detection of *Dehalococcoides* indicates the potential for complete reductive dechlorination. However, Lu, Wilson, and Kampbell (2006) suggest that a *Dehalococcoides* population of 10^7 cells per liter is required for “generally useful” rates of reductive dechlorination and effective MNA. With increased use in the industry, empirical relationships between biodegradation rates for different types of contaminants and other target microbial populations will be developed to aid in assessment of MNA. Additionally, RT-qPCR can identify whether or not specific genes are being expressed for contaminant biodegradation.

Enhanced bioremediation, or biostimulation, involves addition of an amendment such as an electron donor (e.g., lactate, emulsified vegetable oil) or acceptor (e.g., oxygen, sulfate) for the specific purpose of stimulating growth and activity of microorganisms capable of biodegradation of the contaminants. Thus, qPCR or RT-qPCR results should reveal an increase in the abundance or activity of contaminant-degrading microorganisms relative to the baseline in response to the amendment. When qPCR analyses, performed as a component of site characterization, indicate that contaminant-degrading microorganisms are not present or present in low abundance, bioaugmentation (i.e., addition of microorganisms) may be a viable remedy to promote bioremediation. Similar to the discussion for biostimulation, qPCR analyses are used to document the in situ maintenance of key members of the commercial culture (e.g., *Dehalococcoides*) or may reveal decreases that suggest that another injection of electron donor or microbial culture may be required to permit continued biodegradation.

How does it work?

qPCR—qPCR is a laboratory analytical method that selectively amplifies and quantifies genes of interest in a sample. Sample preparation involves harvesting and concentrating microbial cells from the soil or groundwater sample (e.g., by filtration) and breaking these cells open to release their deoxyribonucleic acid (DNA). As described in the PCR Fact Sheet, a typical reaction mixture contains template DNA (environmental DNA), short DNA primers specific to the target gene, DNA building blocks (deoxyribonucleotide triphosphate [dNTPs]), and a heat-stable DNA-synthesizing enzyme (DNA polymerase). This mixture is repeatedly cycled through a precise temperature sequence that leads to the exponential increase in the number of copies of the target gene (amplification). For qPCR, fluorescent (light-emitting) dyes or fluorescently labeled "probes" which adhere or attach to the DNA are also added to the reaction mixture. During the amplification process, fluorescence from the dye or released from the probe is measured. As the number of target gene copies increases, the amount of light emitted increases and eventually exceeds a threshold level. The number of cycles required to exceed this threshold level of light emission, the threshold cycle (C_t), is proportional to the initial amount of target gene in the sample. The amount of the target gene in an environmental sample is calculated using a calibration curve relating C_t values to known amounts of the target gene (standards).

RT-qPCR—In RT-qPCR, ribonucleic acid (RNA) rather than DNA is extracted from the sample and converted (reverse transcribed) into DNA known as "complementary" or "cDNA." The remainder of the procedure is the same as described for qPCR. RNA is a short-lived type of molecule central to production of proteins including enzymes, with RNA rather than DNA as the basis of the analysis. RNA involvement in enzyme production, including those responsible for contaminant biodegradation, is a two-step process. First, the gene (DNA) is transcribed into a short-lived, intermediate molecule called messenger RNA (mRNA). In the second step, mRNA is translated into the corresponding protein (enzyme). A typical cell contains literally thousands of individual genes, many encoding enzymes responsible for a broad spectrum of different functions. Naturally, a cell does not need all of those enzymes to be produced all of the time. In the interest of efficiency, the microorganism regulates enzyme production where transcription of some genes into mRNA is "turned off" and "turned on" only when needed. Therefore, RT-qPCR with RNA rather than DNA as the basis of the analysis quantifies the expression of target genes and activity of specific microorganisms.

How are the data reported?

qPCR results are often presented as gene copies per milliliter of groundwater or per gram of soil. In many cases, a cell contains only one copy of the target gene such that gene copies and cell numbers are equal. For instances when a cell contains multiple copies of the target gene, the reported number can be converted based on knowledge of the number of target gene copies per cell. RT-qPCR results are reported as gene copies per milliliter of groundwater or per gram of soil. An active microorganism transcribes many mRNA or rRNA copies even from a single target gene.

Advantages

- Cultivation-dependent techniques like plate counts are laborious, time-consuming, and most importantly under-representative, because the overwhelming majority (>99%) of microorganisms present in the environment cannot be grown in the laboratory (Amann, Ludwig, and Schleifer 1995 and references therein). Cultivation-independent methods like qPCR and RT-qPCR do not require growing the target microorganisms and provide more accurate quantification than traditional methods.
- A qPCR analysis is capable of detecting specific microorganisms or target genes in the complex mixture of other (nontarget) microorganisms present in environmental samples.
- Results are typically available within days.
- Typical method detection limits (100 target gene copies) are several orders of magnitude lower than the target population required to achieve a "reasonable" rate of contaminant biodegradation.
- Can be performed with DNA obtained from a variety of sample types (e.g., water, soil, sediment, passive microbial sampling devices).
- qPCR analyses have been developed to monitor key microorganisms and processes responsible for biodegradation of a broad spectrum of common environmental contaminants (see Table 1).
- RT-qPCR is used to assess target gene expression and estimate microbial activity.

Limitations

- The development of qPCR analyses is based on known biodegradation pathways and gene sequences. With ongoing research, additional qPCR analyses will be developed to expand the applicability of the technique to other contaminants and newly identified biodegradation pathways.
- Although not particularly common, qPCR results can be affected by the presence of some metals or humic acids in the environmental sample. Samples exhibiting PCR inhibition should be readily identified with basic quality assurance (QA)/quality control (QC) procedures. Furthermore, inhibition can often be eliminated with minor modifications to the DNA extraction procedure.
- RT-qPCR must be used to distinguish between dead cells containing the target gene and live cells.
- Though each laboratory employs its own methodology, prescribed standardized protocols for sample collection, storage, preservation, DNA extraction, and nucleic acid targets do not currently exist. Efforts to generate standard operating procedures (SOPs) are currently under way (Lebrón et al. 2008, Hatt et al. 2011).

Available qPCR Targets

Table 1 shows the gene targets for qPCR analyses that are currently (2011) commercially available for assessing biodegradation pathways for a broad spectrum of contaminants. Additional qPCR and RT-qPCR analyses may be available at academic and research laboratories; others will be developed as new biochemical pathways are discovered and corresponding genes are identified. Selecting an appropriate qPCR analysis depends on the contaminant and the most likely biodegradation pathway, given the current redox conditions.

Sampling Protocols

Sample matrices that can be analyzed by qPCR include soil, sediment, groundwater, and filters. Sampling for microbiological samples can be easily incorporated into routine environmental monitoring programs. The following items are typical requirements for microbiological sampling: (a) use of aseptic sample collection techniques and sterile containers, (b) shipment of the samples to the laboratory within 24 hours of sample collection, and (c) maintenance of the samples at 4°C during handling and transport to the laboratory. Sample collection techniques and containers may vary depending on the matrix sampled and the laboratory analyzing the samples. Users should work with the analytical laboratory to ensure sampling protocols for collecting, handling, and transporting the samples are in place and understood.

Table 1. Current qPCR gene targets for assessing biodegradation pathways

Contaminant Group	Contaminants	Target	Redox Conditions	Environmental Relevance / Data Interpretation
Chlorinated Solvents	PCE, TCE, DCE, VC	***Dehalococcoides***	Anaerobic	• Only known group of bacteria capable of complete dechlorination of tetrachloroethene (PCE) and/or trichloroethene (TCE) to ethene.
Chlorinated Solvents	TCE and DCE	**TCE Reductase**	Anaerobic	• *Dehalococcoides* functional genes encoding reductive dehalogenases for trichloroethene (TCE) and *cis* -dichloroethene (*cis* -DCE).
Chlorinated Solvents	VC	**Vinyl Chloride Reductase**	Anaerobic	• *Dehalococcoides* functional genes encoding reductive dehalogenases for vinyl chloride (VC).
Chlorinated Solvents	TCA, PCE, TCE	***Dehalobacter***	Anaerobic	• Capable of dechlorination of PCE and TCE to *cis* -DCE. • Some strains capble of converting TCA, a common co-contaminant at PCE/TCE-impacted sites, to chloroethane.
Chlorinated Solvents	PCE, TCE	***Desulfuromonas***	Anaerobic	• Capable of dechlorination of PCE and TCE to *cis* -DCE using acetate as an electron donor.
Chlorinated Solvents	PCE, TCE, DCA, DCP	***Desulfitobacterium***	Anaerobic	• Capable of dechlorination of PCE and TCE to *cis* -DCE. • Some strains capable of dechlorination of chlorophenols, 1,2-dichloroethane and 1,2-dichloropropane.
Chlorinated Solvents	Chlorobenzene	**Toluene Dioxygenase**	Aerobic	• Catalyzes biodegradation of chlorobenzene by incorporation of oxygen into the aromatic ring.
Petroleum Hydrocarbons	BTEX	**Ring-hydroxylating Toluene Monooxygenases**	Aerobic	• Catalyzes the initial (and sometimes second) hydroxylation of BTEX compounds.
Petroleum Hydrocarbons	BTEX	**Phenol Hydroxylase**	Aerobic	• Catalyzes further oxidation of BTEX compounds.
Petroleum Hydrocarbons	T, X	**Toluene/Xylene Monoxygenase**	Aerobic	• Attacks toluene and xylenes at the methyl group.
Petroleum Hydrocarbons	B, T, chlorobenzene	**Toluene Dioxygenase**	Aerobic	• Catalyzes biodegradation of benzene and toluene by incorporation of oxygen into the aromatic ring.
Petroleum Hydrocarbons	Naphthalene	**Naphthalene Dioxygenase**	Aerobic	• Catalyzes aerobic biodegradation of naphthalene and other PAHs by incorporation of oxygen into the aromatic ring.
Petroleum Hydrocarbons	Naphthalene and PAHs	**Naphthalene-Inducible Dioxygenase**	Aerobic	• Catalyzes aerobic biodegradation of naphthalene and other PAHs by incorporation of oxygen into the aromatic ring.
Petroleum Hydrocarbons	Alkanes	**Alkane Monooxygenase**	Aerobic	• Catalyzes biodegradation of straight chain petroleum hydrocarbons.
Petroleum Hydrocarbons	MTBE	**MTBE utilizing PM1**	Aerobic	• Targets *Methylibium petroleiphilum* PM1, one of the few bacteria isolated that is capable of growth on MTBE
Petroleum Hydrocarbons	T, X	**Benzylsuccinate Synthase**	Anaerobic	• Targets gene encoding enzyme in anaerobic biodegradation of toluene.
Cometabolism Chlorinated Solvents	TCE	**Methanotrophs**	Aerobic	• Targets two types of methane oxidizing bacteria (methanotrophs) • Indicates the potential for cometabolic oxidation of TCE
Cometabolism Chlorinated Solvents	TCE	**Soluble Methane Monooxygenase**	Aerobic	• Targets the soluble methane monooxygenase gene • Soluble methane monooxygenases are generally believed to support faster cometabolism of TCE
Perchlorate	Perchlorate, chlorate	**Perchlorate Reductase**	Anoxic	• Catalyzes reduction of perchlorate and in most isolates reduction of chlorate to chlorite • Presence indicates the potential for aerobic BTEX biodegradation.
Polychlorinated biphenyls	PCBs (lightly chlorinated)	**Biphenyl Dioxygenase**	Aerobic	• Catalyzes catalyzes initial oxidation of lightly chlorinated PCB congeners.

Quality Assurance/Quality Control

To date, most EMDs do not have standardized protocols accepted by the U.S. Environmental Protection Agency (EPA) or other government agencies. However, most laboratories work under SOPs and good laboratory practices, which can be provided to the user (e.g., consultant, state regulator) on request.

Data quality should be evaluated by detailing the laboratory requirements in a site-specific QA project plan (QAPP). This plan should include identification of the EMDs being used; the field sampling procedures, including preservation requirements; the SOPs of the laboratory performing the analysis; and any internal QA/QC information available (such as results for positive and negative controls). Data reports include a lower quantification limit, a practical quantification limit, and data quality "flags" such as estimated value (J) similar to those of more routine chemical analyses. In 2004 EPA published *Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples*, which contains sections relevant to qPCR.

Under the Strategic Environmental Research and Development Program (SERDP) Project ER-1561, standard protocols are being developed, with guidance for collecting, preserving, storing, transporting, and processing samples for analyses, as well as evaluating results under various conditions (e.g., potential inhibitors and the impacts, if any, caused by monitoring well structure). Additionally, standardized *Dehalococcoides* reference materials and internal microbial controls are being designed to facilitate comparison of qPCR results between laboratories and to monitor the efficiency of each step from sample collection to quantification for qPCR methods. An assessment of the biases affecting the accuracy, precision, and reproducibility of qPCR analyses has been reported (Lebrón et al. 2008, Hatt et al. 2011).

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Glossary

contaminant-degrading population—The group of organisms that are capable of degrading a particular contaminant.

Dehalococcoides—A specific group (genus) of bacteria. *Dehalococcoides* species are the only microorganisms that have been isolated to date which are capable of complete reductive dechlorination of PCE and TCE to ethene. Some *Dehalococcoides* species are also capable of reductive dechlorination of chlorinated benzenes, chlorinated phenols, and polychlorinated biphenyls.

DNA (deoxyribonucleic acid)—A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix (EPA 2004).

dNTPs (deoxyribonucleotide triphosphates)—dNTPs are incorporated into DNA during elongation (EPA 2004).

electron acceptor—A chemical compound that accepts electrons transferred to it from another compound (based on EPA 2011).

electron donor—A chemical compound that donates electrons to another compound (based on EPA 2011).

enzyme—Any of numerous proteins or conjugated proteins produced by living organisms and facilitating biochemical reactions (based on EPA 2004).

functional gene—A segment of DNA that encodes an enzyme or other protein that performs a known biochemical reaction. For example, the functional gene *tceA* encodes the reductive dehalogenase enzyme that initiates reductive dechlorination of TCE. Other genes can code for RNA entities which can regulate the activity of other DNA target sequences.

gene—A segment of DNA containing the code for a protein, transfer RNA, or ribosomal RNA molecule (based on Madigan et al. 2010).

microbial community—The microorganisms present in a particular sample.

nucleic acid—A complex biomolecule consisting of a long "backbone" of organophosphate sugars with nucleotide bases attached.

primers—Short strands of DNA that are complementary to the beginning and end of the target gene and thus determine which DNA fragment is amplified during PCR or qPCR.

probes—(1) short DNA strands (see *microarray probes*, Microarray Fact Sheet; *FISH probes*, FISH Fact Sheet; *qPCR probes*); (2) surrogate compounds (see *enzyme activity probes*, EAP Fact Sheet).

qPCR probes—Short, defined segments of DNA or RNA, that may or may not be labeled and that are designed to bind with the target gene if found in the environmental sample.

qPCR target (target gene)—The specific gene quantified by a particular qPCR analysis. For example, vinyl chloride reductase genes are the target genes in qPCR analyses performed to assess reductive dechlorination of vinyl chloride to ethene. Similarly, a qPCR analysis targeting the toluene dioxygenase gene is used to evaluate aerobic biodegradation of toluene and benzene.

RNA (ribonucleic acid)—Single-stranded nucleic acid that is transcribed from DNA and thus contains the complementary genetic information.

transcription—The first step in activation of a biochemical pathway where a complementary RNA copy is synthesized from a DNA sequence.

translation—The second step of gene expression where messenger RNA (mRNA) produced by transcription is decoded by the cell to produce an active protein.

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ABOUT ITRC

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