
Preparing for Electron Acceptor Addition & Enhanced Bioremediation

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1.0 Remedial Investigation and Pre-Injection Activities

While monitored natural attenuation (MNA) can be an effective remedy, low concentrations of electron acceptors such as dissolved oxygen, nitrate, and sulfate can limit petroleum hydrocarbon biodegradation under existing site conditions. When MNA will not meet site cleanup goals in an acceptable timeframe, air (bioventing/biosparging), oxygen (oxygen infusion), or an electron acceptor product (e.g. ORC®, PermeOx®, EAS®) is often added or injected to promote growth of petroleum hydrocarbon degraders and enhance bioremediation.

From a microbiology perspective, pre-injection design activities focus on determining the required amount of electron acceptor product and quantifying baseline (background) concentrations of BTEX and other petroleum hydrocarbon degrading bacteria to evaluate the effectiveness of electron acceptor injection. While many site-specific factors need to be considered, the data collected during site characterization and remedial investigation should answer the following questions:

What are current concentrations of contaminant degraders?

- Aerobic and anaerobic BTEX degraders?
- Aerobic MTBE and TBA degraders?
- Aerobic and anaerobic naphthalene and other PAH degraders?

Which electron acceptor should be added?

- Enhanced aerobic bioremediation with injection of an oxygen-releasing material?
- Enhanced anaerobic bioremediation with a sulfate-based product?

How much electron acceptor is needed?

- Sufficient to meet demand & support growth of petroleum hydrocarbon degraders?

1.1 What are the concentrations of BTEX and other petroleum hydrocarbon degrading bacteria under existing subsurface conditions?

Quantification of functional genes involved in both aerobic and anaerobic biodegradation during a remedial investigation provides insight into the potential for biodegradation under existing conditions and a benchmark to evaluate the impact of site activities. Simply put, increased concentrations of targeted functional genes demonstrate growth of contaminant degraders in response to treatment.



Submit groundwater samples from select monitoring wells for **CENSUS® qPCR** or **QuantArray®-Petro** analysis prior to electron acceptor injection. Quantification of baseline concentrations of BTEX and other petroleum hydrocarbon degrading bacteria will allow site managers to evaluate the microbial response to the planned electron acceptor injection and ultimately, the effectiveness of the remediation approach.

CENSUS® qPCR and QuantArray®-Petro are DNA-based molecular biological tools used to accurately quantify specific functional genes (e.g. benzene carboxylase, toluene monooxygenase) responsible for biodegradation of BTEX and other contaminants of concern as shown in the table below.

CENSUS® qPCR & QuantArray®-Petro Assays for Petroleum Hydrocarbon Sites

Aerobic BTEX

Toluene 3- and 4-Monooxygenases (RMO)	Xylene/Toluene Monooxygenase (TOL)
Toluene 2 Monooxygenase (RDEG)	Ethylbenzene/Isopropylbenzene Dioxygenase (EDO)
Phenol Hydroxylase (PHE)	Biphenyl/Isopropylbenzene Dioxygenase (BPH4)
Toluene/Benzene Dioxygenase (TOD)	

Aerobic MTBE

<i>Methylobium petroliphilum</i> PM1 (PM1)	TBA Monooxygenase (TBA)
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Anaerobic BTEX

Benzene Carboxylase (ABC)	Benzoyl Coenzyme A Reductase (BCR)
Benzylsuccinate synthase (BSS)	

Aerobic PAHs and Alkanes

Naphthalene Dioxygenase (NAH)	Alkane Monooxygenase (ALK)
Phenanthrene Dioxygenase (PHN)	

Anaerobic PAHs and Alkanes

Naphthylmethylsuccinate Synthase (NMS)	Alkylsuccinate Synthase (ASSA)
Naphthalene Carboxylase (ANC)	

Other

Total Bacteria (EBAC)	Sulfate Reducing Bacteria (APS)
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Quantitative polymerase chain reaction (qPCR) is a process whereby many copies of a specific target gene are generated. As each gene copy is made, a fluorescent marker is released, measured and used to quantify the number of target genes present in the sample. QuantArray® is a nano-fluidic platform for solution-phase qPCR which provides simultaneous quantification of a broad spectrum of genes of interest in a single analysis. For more information on CENSUS® qPCR and QuantArray®-Petro, please see the Microbial Insights website (www.microbe.com).

1.2 Aerobic or Anaerobic — Which electron acceptor product should be injected?

Enhanced aerobic bioremediation by biosparging, oxygen infusion, or injection of an oxygen-releasing material is very well established and the most common strategy for stimulating petroleum hydrocarbon biodegradation. However, petroleum hydrocarbons including BTEX are also susceptible to biodegradation under anaerobic conditions, and alternative electron acceptors such as sulfate can also support bioremediation in the field.



When selecting an electron acceptor,

- Review QuantArray®-Petro results for concentrations of key functional genes involved in aerobic and anaerobic biodegradation of BTEX and other petroleum hydrocarbons;
- Examine site geochemistry carefully and consult product vendors; and
- Consider an In Situ Microcosm (ISM) study to evaluate options (See [Section 2.0](#)).

1.3 How much electron acceptor is needed?

If the injected mass of the electron acceptor is too low, the electron acceptor (e.g. dissolved oxygen, sulfate) will not meet bioremediation demands and long-term performance will be limited. Conversely, overestimation of the required electron acceptor mass leads to increased costs with little improvement of overall treatment. Therefore, electron acceptor manufacturers/suppliers have developed design tools to estimate the required mass or volume of their specific products. The calculations are based on a wide variety of site-specific input parameters, including the size of the treatment area, hydraulic characteristics, contaminant concentrations, and geochemical conditions. Site managers should review these input parameters when considering enhanced bioremediation as a treatment approach to ensure that the necessary data are available or collected during the site assessment or remedial investigation.

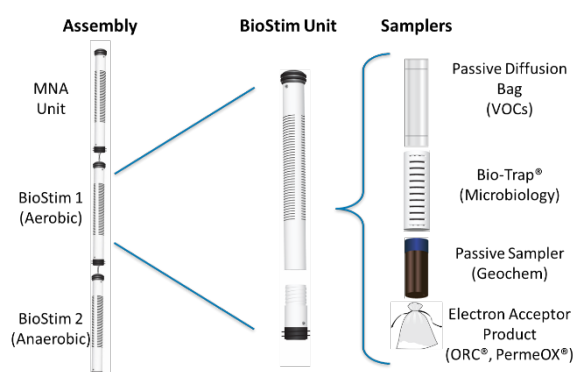


Consult your product manufacturer/vendor and use vendor design tools that calculate the recommended mass of electron acceptor product.

2.0 In Situ Microcosms (ISMs) vs Pilot Studies

At some sites, additional assessment is needed to screen remediation options before transitioning from site characterization to implementing a remedy at full scale. In such situations, site managers have frequently turned to laboratory microcosms or small pilot studies to evaluate bioremediation. However, duplication of *in situ* conditions in the laboratory is difficult, and the results often do not correlate to the field. Pilot studies are performed in the field, but are often prohibitively expensive as an investigative tool. *In Situ* Microcosms (ISMs) provide microbial, chemical, and geochemical evidence to cost-effectively evaluate biodegradation and screen remedial alternatives.

Figure 1: In Situ Microcosm (ISM)



ISMs can be tailored to investigate a wide variety of remediation approaches, but for petroleum hydrocarbon sites, ISMs typically consist of two or three units each corresponding to a common treatment option. In Figure 1 for example, the ISM assembly consists of a monitored natural attenuation (MNA) unit, an aerobic biostimulation unit (BioStim 1), and an anaerobic biostimulation unit (BioStim 2). The ISM assembly consisting of all units is suspended in an impacted monitoring well for 30 to 60 days.

Following in-well deployment, the ISM is recovered for analysis.

Each ISM unit contains a set of passive samplers: a passive diffusion bag sampler for quantification of contaminant concentrations, a permeable membrane sampler for geochemical conditions, and a Bio-Trap® sampler for microbial analyses (CENSUS® qPCR or QuantArray®-Petro). Comparison of the results between ISM units (MNA vs. Aerobic BioStim 1 vs. Anaerobic BioStim 2) allows the site manager to determine the impact of each remediation option on the concentrations of contaminants and contaminant-degrading bacteria, as well as on geochemical conditions.

Along with CENSUS® qPCR or QuantArray®-Petro, **stable isotope probing (SIP)** can be incorporated into an ISM study. SIP is an innovative tool that uses a ¹³C-labeled contaminant to conclusively evaluate biodegradation. Comparison of SIP results between ISM units provides a strong line of evidence when comparing the impact of different treatment approaches on contaminant biodegradation. For more information on ISMs and SIP, please see the Microbial Insights website (www.microbe.com).



Consider an *In Situ* Microcosm (ISM) study to provide the chemical, geochemical, and microbiological lines of evidence needed to cost-effectively screen remediation options.

3.0 Full-Scale Injection

3.1 Baseline Sampling and Analysis

Collection and analysis of baseline samples is absolutely critical – the results serve as the benchmark to evaluate the effectiveness of the selected treatment approach. If significant time has passed since the remedial investigation, conditions have changed, or site characterization studies were limited, an additional groundwater sampling event would be recommended to establish baseline conditions prior to full-scale injection.



Consider conducting a groundwater sampling event just prior to full-scale injection to establish baseline:

- Chemistry – Contaminant concentrations
- Geochemistry – Concentrations of electron acceptors and redox conditions
- Microbiology – Concentrations of functional genes responsible for biodegradation of BTEX and other petroleum hydrocarbons (CENSUS® qPCR or QuantArray®-Petro)

3.2 Performance Monitoring

Enhanced bioremediation is an established treatment strategy at sites impacted by petroleum hydrocarbons. Post-injection monitoring of contaminant concentrations and geochemical parameters provides a wealth of information. However, chemical and geochemical results alone do not demonstrate biodegradation. Decreases in contaminant concentrations can result from physical processes such as dilution. Consumption of electron acceptors, although an indirect indicator of microbial metabolic activity, does not necessarily indicate biodegradation of contaminants of concern. Moreover, contaminant desorption (rebound) can occur, so that clear decreasing trends in contaminant concentrations are not always readily evident. As shown in [Case Studies Section 4.0](#), incorporating CENSUS® qPCR or QuantArray®-Petro analysis into post-injection performance monitoring provides valuable, direct, and quantitative evidence to evaluate the effectiveness of electron acceptor addition in stimulating growth of desired BTEX and petroleum hydrocarbon degrading bacteria.



Include microbial analyses (CENSUS® qPCR or QuantArray®-Petro) along with traditional chemical and geochemical monitoring as an additional and direct line of evidence in evaluating the effectiveness of electron acceptor injection.

4.0 Case Studies

4.1 Baseline Samples – Bio-Trap® Samplers and QuantArray®-Petro

Groundwater at a former gasoline service station was impacted by leaking underground storage tanks and associated piping. While trend analysis suggested that BTEX and MTBE concentrations were stable to decreasing, enhanced bioremediation with injection of an oxygen-releasing material was selected as the corrective action plan to decrease time to closure.

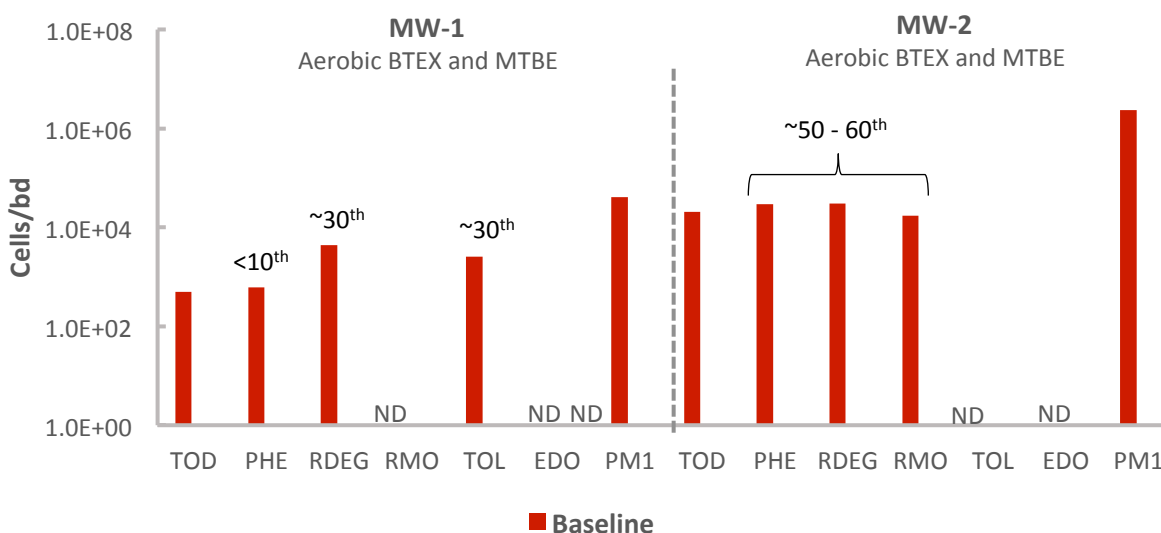
Due to the time period between the remedial investigation and the planned injection, a baseline sampling event was planned to answer several important questions including:

What are the current/baseline concentrations of aerobic BTEX and MTBE degraders?

- The baseline concentrations of aerobic BTEX and MTBE degraders will serve as the benchmark to evaluate the effectiveness of biostimulation as a treatment strategy.

QuantArray®-Petro analysis was performed on Bio-Trap® samplers deployed in select monitoring wells within the planned treatment area. A portion of the results for functional genes involved in aerobic BTEX and MTBE biodegradation are shown in Figure 2.

Figure 2: QuantArray-Petro results for functional genes responsible for aerobic BTEX and MTBE biodegradation



As shown in Figure 2, functional genes responsible for aerobic BTEX biodegradation (TOD, PHE, RDEG, RMO, and TOL) were detected at low (MW-1) to moderate (MW-2) concentrations during the baseline sampling event.

- The MI Microbial Database contains CENSUS® qPCR and QuantArray®-Petro results from environmental samples submitted from sites world-wide and is the largest compilation of field concentrations of contaminant-degrading microorganisms in the industry. Using the MI Database, site managers can compare their qPCR and QuantArray® results against others in the

MI Database allowing them to put their results in context and assess whether concentrations of contaminant degraders at their site are low, medium, or high. For more information on the MI Database, check the Microbial Insights website (www.microbe.com).

- During the baseline event, a variety of aromatic oxygenase genes were detected at MW-1, confirming the potential for aerobic BTEX biodegradation. However, concentrations were low, suggesting that aerobic biodegradation was limited under the prevailing subsurface conditions.
- For example, concentrations of toluene-2-monooxygenase (RDEG) and toluene/xylene monooxygenase (TOL) genes were on the order of $\sim 10^3$ cells/bead. Although demonstrating the presence of aerobic BTEX degraders, the concentrations of these functional genes ranked around the 30th percentile when compared to other sites and therefore would be considered below average. Likewise, phenol hydroxylase genes (PHE) were detected (10^2 cells/bd) but at low concentrations (<10th percentile), suggesting limited aerobic BTEX biodegradation potential under baseline redox conditions.
- At monitoring well MW-2, concentrations of ring hydroxylating toluene monooxygenase (RMO, RDEG) and phenol hydroxylase (PHE) genes were somewhat higher than observed at MW-1. However, concentrations of these aromatic oxygenase genes would be considered near average (50th – 60th percentiles) when compared against the MI Database. Thus, the results indicate a moderate potential for aerobic BTEX biodegradation under the baseline redox conditions.



Overall, QuantArray®-Petro results for the baseline sampling event indicated the presence of low to moderate concentrations of aerobic BTEX and MTBE utilizing bacteria under MNA conditions consistent with historical groundwater monitoring. The baseline results will serve as the benchmark to evaluate the effectiveness of the injection of an oxygen-releasing material at the site.

4.2 Post-Injection Performance Monitoring

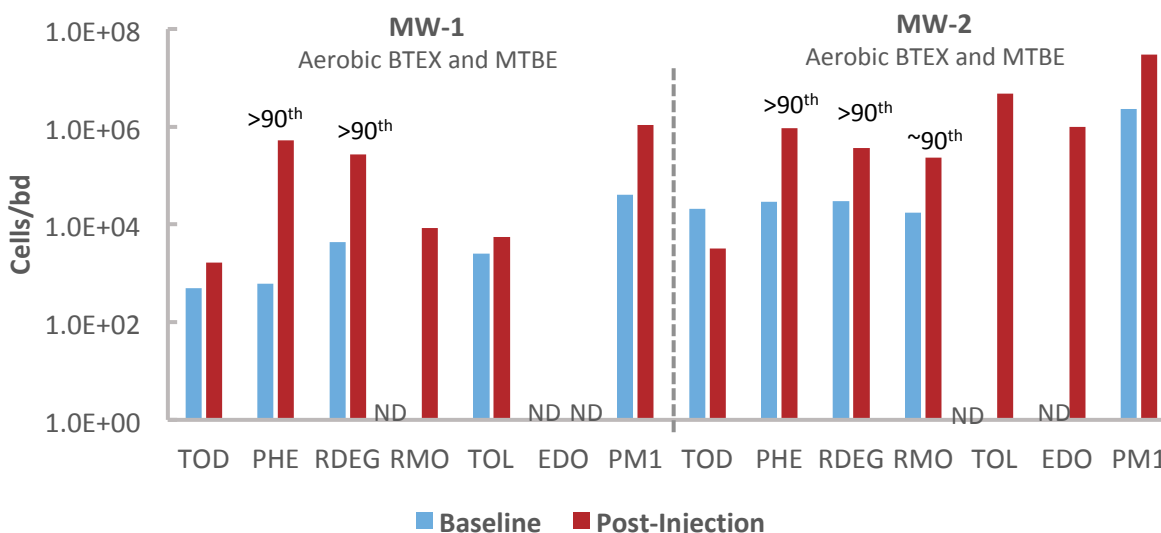
To evaluate the effectiveness of electron donor injection, QuantArray®-Petro analysis was periodically performed on Bio-Trap® samplers deployed in select monitoring wells as part of performance monitoring.

Was electron acceptor injection effective?

- Did concentrations of aerobic BTEX and MTBE degraders increase after injection?
- Were additional functional genes involved in different BTEX biodegradation pathways now detected following injection?

In Figure 3 below, QuantArray®-Petro results for monitoring wells MW-1 and MW-2 are shown from the baseline (gray bars) sampling event and the Round 1 Post-Injection sampling event (red bars).

Figure 3: QuantArray-Petro Results for Post-Injection Performance Monitoring



Overall, the QuantArray®-Petro results for the first post-injection monitoring event were very encouraging (Figure 3). Concentrations of functional genes involved in aerobic BTEX biodegradation increased substantially at both monitoring wells demonstrating growth of aerobic BTEX degraders in response to injection of the oxygen-releasing material.

- More specifically, concentrations of ring hydroxylating toluene monooxygenase (RDEG, RMO) and phenol hydroxylase (PHE) genes increased by approximately two to three orders of magnitude at MW-1 following injection. Likewise, PHE, RDEG, and RMO concentrations increased by more than an order of magnitude at MW-2, demonstrating growth of aerobic BTEX degraders.
- In fact, PHE and RDEG concentrations at both monitoring wells as well as RMO concentrations at MW-2 after the injection were very high (10⁵ cells/bd), ranking among the top 10% in the MI Database.

- Furthermore, concentrations of other aromatic oxygenase genes, which were below laboratory detection limits prior to injection, increased dramatically following injection.
- At MW-2 for example, TOL and ethylbenzene dioxygenase (EDO) genes, which were not detected during the baseline event, increased to concentrations near or greater than 10^6 cells/bd after electron acceptor injection.
- Finally, *Methylibium petroleiphilum* PM1 (PM1) which had been detected at notable concentrations prior to injection in both monitoring wells increased by an order of magnitude, demonstrating growth of a population of bacteria capable of aerobic MTBE biodegradation.



Continue monitoring - Injection of the oxygen-releasing material was successful. Concentrations of aerobic BTEX and MTBE degrading bacteria increased substantially with increased oxygen availability after injection.

5.0 CENSUS® qPCR or QuantArray®-Petro Sample Collection Procedures

Collecting samples for CENSUS® qPCR and QuantArray®-Petro analysis is no more difficult than collecting groundwater or soil samples for common chemical analyses and can be readily incorporated into a routine sampling event. Below are guidelines to follow when collecting samples for any DNA-based analysis.

1. Use clean latex (or similar) gloves when collecting and handling samples.
2. Keep samples cold ($\sim 4^{\circ}\text{C}$) to minimize changes in the microbial community.
 - a. Place samples on ice or freezer packs in a cooler after collection.
 - b. As soon as possible (preferably overnight), ship samples to the laboratory.
 - c. Include enough ice/freezer packs to ensure that samples remain cold during shipment.

Microbial Insights (MI) has been receiving field samples for DNA-based analyses for over 25 years and has performed extensive in-house testing of sample preservation and shipping requirements. Overnight shipment at 4°C combined with immediate DNA extraction upon sample receipt at the laboratory minimizes changes to the microbial community.

QuantArray®-Petro analysis can be performed on nearly any sample type including groundwater, soil, sediments and Bio-Traps®. Groundwater samples can be submitted using 1 L poly bottles or using Bio-Flo filters (Figure 4). Bio-Flo filters can be readily attached to ¼-inch tubing and are compatible with low-flow purging/sampling pumps. For more detailed information on sample collection, complete protocols are available on the sampling page of the MI website (<http://www.microbe.com/sampling-census/>).

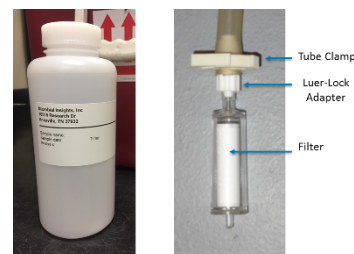


Figure 4: Groundwater samples can be collected in 1L poly bottles or using Bio-Flo filters