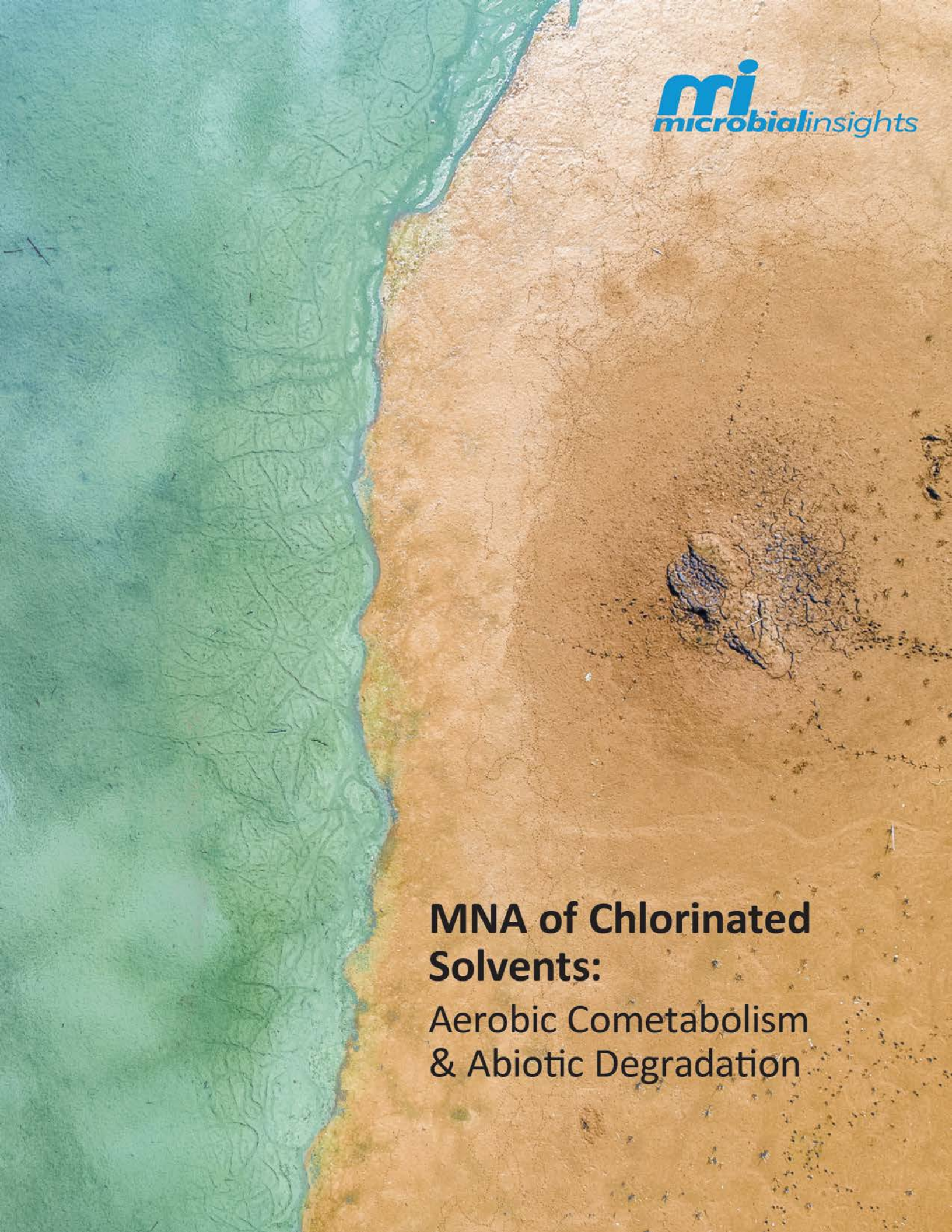

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MNA of Chlorinated Solvents:

Aerobic Cometabolism & Abiotic Degradation

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Evaluating MNA – Cometabolism and Abiotic Degradation

1.0 Overview and Purpose

Engineered remediation strategies like enhanced reductive dechlorination (ERD) are highly effective at chlorinated solvent sites, but are not always feasible for impacted areas extending further downgradient or other large dilute plumes. For large dilute plumes, monitored natural attenuation (MNA) may be the most promising risk management strategy.

Aerobic cometabolism (co-oxidation) of TCE and other chlorinated solvents can be an important component of MNA. Likewise, chlorinated compounds are also susceptible to abiotic degradation catalyzed by common iron-bearing minerals. However, quantification and conclusive assessment of cometabolism and abiotic degradation is difficult, thereby limiting the application of MNA as a site management strategy.

The purpose of this tech bulletin is to describe analyses that can be used to assess MNA at chlorinated ethene sites by answering the following questions:

Is aerobic cometabolism a meaningful component of MNA?
Is abiotic degradation contributing to MNA?
Can these processes reasonably explain the observed trends in contaminant concentrations?
What is the potential rate of TCE degradation?

2.0 Degradation Mechanisms during MNA

While attenuation includes physical processes such as adsorption, dilution, and volatilization, the discussion will focus on mechanisms that lead to contaminant destruction: aerobic cometabolism (co-oxidation) and abiotic degradation.

2.1 Aerobic Cometabolism

Microbial metabolism is the biochemical process that a microbe uses to obtain energy and carbon to live and reproduce. “Cometabolism” describes the process whereby a microorganism transforms a non-growth supporting compound such as TCE in the presence of a growth supporting substrate. The microorganism does not gain energy from cometabolism.

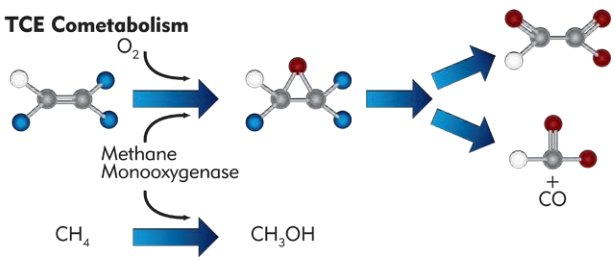

Figure 1: TCE cometabolism and methane metabolism initiated by soluble methane monooxygenase

As shown in Figure 1, cometabolism is often mediated by oxygenase enzymes with “relaxed” specificity such as methane monooxygenases that oxidize a primary, growth-supporting substrate (e.g. methane) and co-oxidize the chlorinated compound (e.g. TCE).

Chlorinated ethenes and other chlorinated solvents can be co-oxidized by a wide range of oxygenase-expressing microbes including those that utilize methane[1-3], ethene and ethane[4, 5], propane[6-9], phenol[10-12], or toluene[13, 14] as energy and carbon sources.

In general terms, the potential for aerobic cometabolism to contribute to contaminant degradation depends on a number of factors including: a growth supporting (primary) substrate, a compound that induces expression of the pathway (an inducer), bacteria with these oxygenase genes and corresponding pathways, and available oxygen. In an engineered system, a growth substrate/inducer like methane for example is added along with oxygen to stimulate growth of methanotrophs and induce expression of soluble methane monooxygenase (sMMO) which is capable of co-oxidation of chlorinated compounds. For MNA though, a growth supporting substrate and an inducer must be naturally present or produced upgradient as a result of site activities such as enhanced reductive dechlorination.

For most pathways, the primary substrate is also an inducer. However, it is important to note that other naturally occurring and anthropogenic compounds can induce expression of some pathways.

- A substantial fraction of the native bacteria in aerobic groundwater have active oxygenase enzymes.[15]
- In addition, laboratory studies have demonstrated that TCE itself can induce expression of some toluene monoxygenases.[16, 17]

Figure 2: Humic acid component of natural organic matter

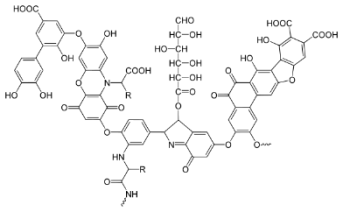

Table 2 highlights groups of microorganisms capable of co-oxidation of TCE and other chlorinated compounds as well as substrates and inducers which may be present during MNA or following enhanced anaerobic bioremediation.

Microorganisms	Primary Substrate & Other Potential Inducers	Substrate & Inducer Sources
Methane oxidizing bacteria (methanotrophs)	Methane	Methane produced during enhanced anaerobic biodegradation performed upgradient. Naturally occurring methane.
Ethene-utilizing bacteria	Ethene	Ethene produced during enhanced anaerobic biodegradation performed upgradient.
BTEX-utilizing bacteria	BTEX, phenol Natural organic matter (NOM) TCE	BTEX compounds present as co-contaminants NOM contains aromatic groups that may induce expression of toluene monooxygenase genes TCE can induce some toluene monooxygenases

2.2 Abiotic Degradation

Although not always fully considered, abiotic degradation can be a substantial or the even the primary process for chlorinated hydrocarbon destruction at sites undergoing or transitioning to MNA. A variety of iron-bearing minerals including iron sulfides (mackinawite and pyrite), iron oxides (magnetite), green rust, and iron-bearing clays are capable of complete or nearly complete degradation of PCE, TCE, and carbon tetrachloride[18]. Some iron-bearing minerals also catalyze the degradation of chlorinated ethanes and the lesser chlorinated ethenes cis-dichloroethene (DCE) and vinyl chloride. While the quantities and

types will vary, these reactive iron minerals are frequently identified in subsurface environments and the reactions can occur under oxic and anoxic conditions.

- **Magnetite** (Fe_3O_4) is a mixed valence iron mineral shown to react with PCE, TCE, and carbon tetrachloride[19, 20]. Furthermore, Ferrey, et al.[21] conclusively linked the observed degradation of cis-DCE at a former ammunition plant to magnetite in the subsurface.

- **Mackinawite** (FeS) will transform PCE and TCE primarily by elimination to acetylene[22]. Carbon tetrachloride is transformed mainly to chloroform but carbon dioxide, formate, and carbon disulfide have also been detected[20, 23]. Finally, the more heavily chlorinated ethanes including hexachloroethane, pentachloroethane, and tetrachloroethanes react to form chlorinated ethenes which can be further degraded[24].

- **Pyrite** (FeS_2) catalyzes transformation of PCE, TCE, and cis-DCE to acetylene and ethene[19]. Vinyl chloride is transformed to ethene and ethane. Pyrite is also capable of degradation of carbon tetrachloride potentially forming a number of products including chloroform, carbon dioxide, carbon disulfide, and formate depending on reaction conditions[25].

3.0 Analyses for Evaluating Degradation during MNA

Abiotic degradation and aerobic cometabolism have not always been considered as significant contributors to contaminant attenuation in part because these degradation mechanisms have been difficult to quantify and correlate to degradation rates. Unlike biological reductive dechlorination, aerobic cometabolism and abiotic degradation do not produce readily measured daughter products (e.g., DCE, vinyl chloride, ethene). Without the formation of unique products, evaluating contributions of aerobic cometabolism and abiotic degradation to MNA relies on other lines of evidence to complement traditional chemical and geochemical monitoring.

3.1 QuantArray®-Chlor or CENSUS® qPCR

Quantification of the functional genes encoding the enzymes capable of co-oxidation of TCE and other chlorinated hydrocarbons is a direct approach to evaluate the potential for aerobic cometabolism to contribute to MNA. Moreover, recent research under **ESTCP Project 201584** has demonstrated positive correlations between oxygenase gene concentrations (sMMO, RMO, and PHE) and the rate of TCE degradation[26]. For more information, the Final Report for ESTCP Project 201584 is available at https://www.serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201584/ER-201584

Submit groundwater samples from select monitoring wells for **CENSUS® qPCR** or **QuantArray®-Chlor** analysis. Quantification of the oxygenase genes included in the following table will allow site managers to evaluate the potential for aerobic cometabolism under existing site conditions.

CENSUS® qPCR and QuantArray®-Chlor are DNA-based molecular biological tools used to accurately quantify specific functional genes (e.g. methane monooxygenase) encoding enzymes capable of aerobic cometabolism of TCE and other contaminants of concern as shown in the table below.

Target Gene	Relevance
Soluble Methane Monooxygenase (sMMO)	Targets the gene encoding soluble methane monooxygenases which can co-oxidize a broad range of chlorinated compounds[2, 27-29] including TCE, *cis*-DCE, and vinyl chloride. Furthermore, soluble methane monooxygenases are generally believed to support greater rates of aerobic cometabolism [30].
Toluene Monooxygenase (RMO)	Targets a group of genes encoding ring-hydroxylating toluene monooxygenase (toluene-3- and toluene-4-monooxygenases) capable of co-oxidation of TCE. In some laboratory studies, TCE or a degradation product has been shown to induce expression of toluene monooxygenases [16, 17, 31], raising the possibility of TCE cometabolism with alternative (non-aromatic) growth substrates [32].
Phenol Hydroxylase (PHE)	While degradation rates differ, phenol hydroxylases also co-oxidize TCE [33]. As mentioned previously, TCE or a degradation product can induce expression of toluene monooxygenases[16, 17, 31] and recent research has shown positive correlations between concentrations of monooxygenase genes (sMMO, RMO, PHE) and the rate of TCE degradation [26].
Toluene Monooxygenase (RDEG)	Also targets the ring-hydroxylating toluene monooxygenase genes (toluene-2-monooxygenase). As with RMO, toluene-2-monooxygenases are capable of cometabolism of TCE [34].
Toluene Dioxygenase (TOD)	Although reports of induction by TCE differ, toluene dioxygenases are also capable of cometabolism of TCE [14] when expressed.
Ethene Monooxygenase (ETN)	Enumerates functional genes (*etnC* and *etnE*) involved in ethene utilization and vinyl chloride (co)metabolism. The ethene monooxygenase (EtnABCD) converts ethene and vinyl chloride to their respective epoxyalkanes, while epoxyalkane:CoM transferase (EtnE) mediates conjugation and breaking of the epoxide [35].

Quantitative polymerase chain reaction (qPCR) is a process whereby many copies of a specific target gene are a 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®-Chlor, please see the Microbial Insights website (www. microbe.com).

3.2 Magnetic Susceptibility

As mentioned previously, magnetite (Fe_3O_4) is a mixed valence iron mineral shown to react with a variety of chlorinated solvents including PCE, TCE, cis-DCE, and vinyl chloride. No direct chemical test is available for quantification of magnetite at the concentrations typically found in aquifer sediments. However, magnetite is the most abundant mineral in natural sediments that exhibits magnetic behavior. Therefore, magnetic susceptibility provides an inexpensive and valuable estimate of the quantity of magnetite in environmental samples.

Submit sediment core samples for Magnetic Susceptibility analysis to evaluate magnetite content and the potential for abiotic degradation. In field and laboratory studies, correlations were observed between magnetic susceptibility measurements and chlorinated ethene degradation rates.[18, 36]

Wiedemeier et al (2017) recommend using magnetic susceptibility as a second line of evidence to assess whether abiotic degradation by magnetite is a plausible explanation for observed degradation determined by groundwater monitoring data.

3.3 X-ray Diffraction (XRD)

As mentioned previously, MNA is a common follow-up to enhanced anaerobic bioremediation which not only stimulates growth of halorespiring bacteria, but also promotes growth of iron- and sulfate-reducing bacteria which produce reactive iron sulfide minerals. Reactive iron minerals may also be present in naturally reducing anoxic aquifers. Mackinawite is the most reactive of the iron-bearing minerals and a crystalline form (tetragonal FeS) can be detected by X-Ray Diffraction (XRD). Pyrite (FeS_2) capable of abiotic degradation of chlorinated ethenes and carbon tetrachloride, can also be detected by XRD.

3.4 Compound Specific Isotope Analysis (CSIA)

Compound specific isotope analysis (CSIA) is an analysis that measures the stable isotope ratio ($^{13}C/^{12}C$) of the contaminant. For many of chlorinated solvents, the ratio of stable isotopes ($^{13}C/^{12}C$) changes in a predictable manner (isotopic fractionation) as the compound is degraded. Conversely, physical processes like dilution do not appreciably alter isotopic ratios of contaminants. Thus, CSIA is increasingly being used at chlorinated solvent sites to conclusively determine whether contaminant degradation has occurred.

Contaminant	Degradation Mechanism Resulting in $^{13}C/^{12}C$ Fractionation
PCE	Abiotic degradation by mackinawite[37]
TCE	Abiotic degradation by mackinawite,[37] magnetite,[18] and pyrite[37]
TCE	Aerobic cometabolism by toluene-2-monooxygenase (RDEG)[38]
Vinyl chloride	Aerobic metabolism and cometabolism (sMMO)[39]
Carbon tetrachloride	Abiotic degradation by mackinawite and magnetite[20]

While contaminant and pathway dependent, aerobic cometabolism and abiotic degradation can result in significant isotopic fractionation and CSIA can be a strong supporting line of evidence in evaluating degradation mechanisms that contribute to MNA as shown in the table above.

Consider submitting groundwater samples from select monitoring wells along the flow path for **CSIA** as a supporting line of evidence when evaluating MNA. CSIA is powerful environmental diagnostic tools that is applicable to a broad spectrum of contaminants and remediation strategies. For more information on CSIA please visit the MI website and the MI CSIA Database.

4.0 ^{14}C TCE Degradation Rate Studies

During ESTCP Project 201584, Wiedemeier and collaborators demonstrated that concentrations of several oxygenase genes as measured by CENSUS® qPCR or QuantArray®-Chlor and magnetic susceptibility measures for magnetite abundance correlated to TCE degradation rates. However, determining rates of TCE degradation using groundwater monitoring data from the field is problematic, particularly for dilute plumes undergoing MNA. Performing **^{14}C TCE Degradation Rate Studies** in the laboratory can address this issue.

As part of Project 201584, Prof. David Freedman from Clemson University has developed and validated an innovative ^{14}C assay to determine TCE degradation rate constants from environmental samples.[40] To estimate a pseudo-first order rate constant for aerobic cometabolism of ^{14}C TCE, the study is performed on groundwater samples. To determine the rate of abiotic ^{14}C TCE degradation, the study is performed on soil/sediment samples.

In the laboratory, submitted samples are injected with a highly purified ^{14}C labeled TCE. At regular time intervals, the disappearance of ^{14}C TCE and the accumulation of ^{14}C products (e.g. $^{14}CO_2$ and ^{14}C labeled formate, glycolate, and oxalate) are quantified. The strong signal and precision allow estimation of pseudo-first order rate constants for TCE degradation of relatively short time frame (days to weeks).

Consider submitting samples to the ***Freedman Laboratory at Clemson University*** for a **^{14}C TCE Degradation Rate Study**.

To determine the rate of ^{14}C TCE aerobic cometabolism, submit three serum bottles and one glass bottle containing at least 500 mL of groundwater from each location to the *Freedman Laboratory*. For abiotic degradation, submit 250 g of soil and 500 mL of site groundwater for each location to *Microbial Insights*.

Please note, only three samples per day can be submitted per day to Dr. Freedman's laboratory for ^{14}C TCE degradation rate studies. Please notify **customerservice@microbe.com at least a week in advance of your sampling dates to coordinate the shipment(s), and email a copy of the chain of custody the day samples are shipped.*

5.0 Sample Collection Procedures

5.1 CENSUS®qPCR or QuantArray®-Chlor

Collecting samples for CENSUS® qPCR and QuantArray®-Chlor 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 (~4°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®-Chlor 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 3). 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.

Figure 3: Groundwater samples can be collected in 1 L poly bottles or using Bio-Flo filters

5.2 Soil Samples for Magnetic Susceptibility and XRD

Collecting samples for Magnetic Susceptibility and XRD is no more difficult than submitting soil samples for traditional chemical analyses. Below are guidelines and sample volume requirements to follow when collecting samples:

1. Use clean latex (or similar) gloves when collecting and handling samples
2. Fill *four* 4-oz. soil containers per sample. A minimum of 800 grams of soil per sample is required for the abiotic panel.
3. Keep samples cold (~4°C) to minimize changes
    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

For more detailed information on sample collection, complete protocols are available on the sampling page of the MI website.

5.3 Groundwater Samples for Compound Specific Isotope Analysis (CSIA)

Collecting groundwater samples for CSIA is very similar to protocols for sample collection for VOCs analysis:

1. Use clean latex (or similar) gloves when collecting and handling samples
2. Submit *four* HCl preserved 40 mL VOA vials per isotope.
3. When filling VOA vials, please leave NO HEADSPACE.
4. Keep samples cold (~4°C) to minimize changes
    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

For more detailed information on sample collection, complete protocols are available on the sampling page of the MI website.

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