Using qPCR Assays to Predict Rates of Cometabolism of TCE in Aerobic Groundwater

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Abstract

Monitored natural attenuation (MNA) is commonly used as a remedy for trichloroethene (TCE) in anaerobic groundwater; however, MNA has not been applied to TCE contamination in aerobic groundwater. Under aerobic conditions, bacteria initiate the degradation of many organic substances with oxygenase enzymes. Several of these enzymes are known to degrade TCE through a fortuitous reaction known as cometabolism. There are commercially available qPCR assays that can determine the number of gene copies of these enzymes. If the qPCR assay could be used to predict the first-order rate constant for cometabolism of TCE, the qPCR assay could be used to screen sites to determine whether MNA was a plausible remedy for TCE contamination. This study reevaluated data from water samples that were collected from 19 wells on five sites in Minnesota, New York, and Utah. Data had previously been published on the rate constant for cometabolism of TCE in the water samples as determined by a 14C-assay and the abundance of gene copies for five enzymes that cometabolize TCE as determined using a qPCR assay. The Michaelis-Menten (Haldane) kinetic parameters for cometabolism of TCE and the abundance of DNA for the five oxygenase enzymes were used to predict the rate constant for cometabolism of TCE. The predicted rate constants were evaluated and validated by comparing them to the rate constants derived from the 14C-assay. For predicted rate constants greater than 0.003 per year, the predicted rate constants agreed with the measured rate constants within a factor of three. The qPCR assay serves as a convenient screening tool to determine whether MNA is a plausible remedy for an aerobic plume of TCE.

Introduction

The most common contaminants in groundwater at CERCLA sites in the United States are halogenated volatile organic compounds such as trichloroethene (TCE) (U.S. Environmental Protection Agency [U.S. EPA] 2017). In the last 10 years, approximately 30% of new decision documents that select remedies for groundwater contamination have included monitored natural attenuation (MNA) as the remedy or part of the remedy for groundwater contamination. The U.S. EPA is more willing to accept MNA as a remedy if there is a mechanism in groundwater known to degrade the contaminant to harmless substances (U.S. EPA 1999). It is widely known that anaerobic bacteria in groundwater can mediate degradation of TCE to harmless substances (Löffler and Edwards 2006), and regulators acknowledge that biodegradation is a plausible mechanism for MNA of anaerobic groundwater. In contrast, TCE will not support the growth of microorganisms under aerobic conditions, and regulators have not recognized biodegradation as a plausible mechanism of MNA under aerobic conditions.

Although TCE does not support the growth of bacteria under aerobic conditions, TCE can be cometabolized by bacteria that produce oxygenase enzymes that are intended to initiate the metabolism of a wide variety of other organic compounds including alkanes, monoaromatic hydrocarbons, and phenols (Alvarez-Cohen and Speitel Jr. 2001; Arp et al. 2001). This fortuitous metabolism of TCE by organisms that are supported on another substrate is termed cometabolism of TCE (Arp et al. 2001). Although cometabolism of TCE is a possibility in groundwater, there is a conventional wisdom in groundwater science that TCE does not biologically degrade in aerobic groundwater because the substrates that might support it have been degraded and are no longer available.

Despite this conventional wisdom, TCE degradation has been reported in aerobic aquifers. Sturchio et al. (1998) evaluated the attenuation of TCE along a flowpath in the Northwest aquifer at the Paducah Gaseous Diffusion Plant in Kentucky. Along the entire flowpath, the concentration of dissolved oxygen (DO) was ≥1 mg/L, Fe2+ was ≤0.2 mg/L, methane was ≤2 μg/L, vinyl chloride was <3 μg/L, ethene was <30 μg/L, and the concentration of cis-1,2-dichloroethene (DCE) was never greater than 20% of the concentration of TCE. Despite these indications of aerobic conditions, the concentrations of TCE attenuated from 200 mg/L to 0.1 mg/L. The attenuation could not be explained by dilution.

At the Paducah site, the ratio of the stable isotopes of chlorine (δ18Cl) changed along the flowpath. The residual TCE was enriched in the heavier isotope, as would be
expected if the TCE was removed by biodegradation. The attenuation of TCE concentrations and the change in the ratio of isotopes (δδ13C) followed the Rayleigh equation. The apparent isotopic enrichment factor (ε′) for chlorine was −0.525‰. Sturchio et al. (1998) demonstrated the fractionation of isotopes of chlorine as evidence for aerobic biodegradation of the TCE at the site.

The same plume was investigated in detail by the Kentucky Research Consortium for Energy and Environment (KRCEE 2008). They used data on attenuation of chloride and 99Tc to correct data on TCE for attenuation due to dilution along the flowpath. They then used time-of-travel along the flowpath and the corrected data on TCE concentrations to extract first-order rate constants for TCE cometabolism. The rate constants ranged between 0.06 and 0.21 per year.

They also measured the ratio of stable isotope of carbon (δ13C) in TCE between well pairs in the plume. In eight of ten well-to-well comparisons, the apparent isotopic enrichment factor (ε′) for carbon between the well pairs was equal to or more negative than −1.1‰, a value for ε′ obtained for cometabolism of TCE from a culture of Methylosinus trichosporium OB3b growing on methane (Chu et al. 2004). These data support and confirm the claim of Sturchio et al. (1998) that TCE is degraded in aerobic groundwater in the plume.

Sorensen et al. (2000) and Wymore et al. (2007) evaluated TCE attenuation in a large plume of TCE in a basalt aquifer on the Test Area North (TAN) site in Idaho. In the plume, DO ranged from 2 to 10 mg/L, Fe2+ was <0.1 mg/L in all but one location, and methane varied from 0.003 to 0.062 mg/L. They used data on tritium in the groundwater and the tracer correction method to extract a first-order rate constant for TCE cometabolism of 0.05 per year.

Lee et al. (2008) used enzyme activity probes to show that significant densities of native bacteria in the groundwater at the TAN site expressed oxygenase enzymes that can cometabolize TCE. The probes are chemicals that react with the oxygenase enzymes to produce a fluorescent product that accumulates in the cells. The cells were filtered from the groundwater, the filter was exposed to water containing the probe for several minutes, and then the filter was examined using epifluorescent microscopy to quantify the fluorescent cells. In groundwater from many of the wells at the TAN site, from 10% to 60% of the native groundwater bacteria had oxygenase enzymes that could cometabolize TCE. Lee et al. (2008) offered the high abundance of bacteria that reacted with the enzyme activity probe as a line of evidence that the native bacteria in the groundwater were responsible for the attenuation of TCE.

The rate constants at the Paducah and TAN sites were relatively slow compared to rate constants for aerobic degradation of nonchlorinated hydrocarbons or rate constants for degradation of TCE under anaerobic conditions as provided in Suarez and Rifai (1999). However, at these large sites with dilute plumes of TCE, aerobic cometabolism made a substantial contribution to natural attenuation and might have provided a basis for a MNA remedy.

Lebrón et al. (2015) developed a decision support system to evaluate the prospects that MNA could be used as a remedy for groundwater contaminated with tetrachloroethylene, TCE, DCE, and vinyl chloride. The decision support system was designed to provide the lines of evidence for MNA as specified by the U.S. EPA in the OSWER Directive on MNA (U.S. EPA 1999). The first line of evidence requires “… data that demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at appropriate monitoring or sampling points.” The decision support system uses a computer model to extract a first-order rate constant for TCE degradation. It then uses the model to predict whether that rate constant will allow the plume to meet regulatory expectations at the site. If it does, the field-scale rate constant provides the first line of evidence.

The second line of evidence requires “Hydrogeologic and geochemical data that can be used to demonstrate indirectly the type(s) of natural attenuation processes active at the site, and the rate at which such processes will reduce contaminant concentrations to required levels. For example, characterization data may be used to quantify the rates of contaminant sorption, dilution, or volatilization, or to demonstrate and quantify the rates of biological degradation processes occurring at the site.”

Lebrón et al. (2015) use the abundance of gene copies for the 16S-rRNA gene in Dehalococcoides mccartyi to test whether anaerobic biodegradation of TCE is a plausible explanation for the field-scale rate constant. The test is quantitative. The abundance and rate constant at the site of interest are compared to the range of values at a set of benchmark sites where anaerobic biodegradation is accepted as the mechanism for MNA. If the site under evaluation falls within the range of the benchmark sites, anaerobic biodegradation is a plausible explanation for the rate constant and provides the second line of evidence.

There is no equivalent comparison and no second line of evidence for aerobic cometabolism of TCE. As a result, the decision logic in Lebrón et al. (2015) cannot be used to evaluate sites where aerobic cometabolism could provide a basis for MNA. Wiedemeier et al. (2017) sought to remedy the deficiency. They developed a 14C-assay that directly measured the rate constant for TCE cometabolism in a sample of groundwater and applied the assay to 19 wells from five sites in Minnesota, New York, and Utah. The assay method and the resulting rate constants are provided in Mills et al. (2018). The assay can be used to provide a second line of evidence for TCE cometabolism. The assay is commercially available from Microbial Insights, Inc. (Knoxville, Tennessee).

Wiedemeier et al. (2017) also used qPCR assays conducted by Microbial Insights, Inc. to determine the abundance of DNA in groundwater that was amplified by qPCR primers for genes for selected oxygenase enzymes. The intention was to use the qPCR assay as an alternative second line of evidence. The SMMO primer targets soluble methane monoxygenase. The RDEG primer used by Microbial Insights targets toluene-2-monoxygenase. The RMO primer targets the toluene-3-monoxygenase and the toluene-4-monoxygenase. The TOD primer targets the toluene-2,3-dioxygenase, and the PHE primer targets phenol hydroxylase (PHE). Presumably, the RDEG and RMO primers amplify the gene that is responsible for the initial biochemical reaction with the primary substrate and the...
PHE primer amplifies the gene that further metabolizes the phenolic compound produced from the initial reaction.

The SMMO, RDEG, RMO, TOD, and PHE qPCR primers amplify DNA for oxygenase enzymes that have been associated with cometabolism of TCE (Sun and Wood 1996; Alvarez-Cohen and Speitel Jr. 2001; Arp et al. 2001; Futama et al. 2001; Parales et al. 2008). Xylene monooxygenase, which is amplified by the TOL primer, does not cometabolize TCE (Hori et al. 2005; Clingenpeel et al. 2012).

It is possible that many or most of the bacteria in the groundwater samples are dead or inactive. If that is the case, the presence of DNA coding for an enzyme would not necessarily mean that the bacteria express the enzyme. Under such circumstances, the detection of mRNA transcripts for the enzymes would indicate that the enzymes were being produced in the bacteria. However, the absence of a detectable abundance of mRNA transcripts does not indicate that the enzymes are absent in the bacteria.

Wiedemeier et al. (2017) evaluated the relationship between the rate constants as measured in the 14C-assay and the abundance of DNA and mRNA for the oxygenase enzymes by comparing the abundance of the DNA and mRNA for each individual enzyme to the overall rate constant for TCE cometabolism. There was a correlation between the rate constants and the abundance of several of the qPCR markers. However, the approach taken by Wiedemeier et al. (2017) assumes that only one of the enzymes determines the rate constant, and all of the other enzymes have no effect on the rate constant. What is needed is an analysis that considers the contribution of all the oxygenase enzymes in the water sample to the rate constant for TCE cometabolism.

This paper uses a different approach to reanalyze the primary data provided in Wiedemeier et al. (2017). We assumed that Michaelis-Menten (Haldane) kinetic parameters that describe TCE cometabolism in laboratory cultures will apply to bacteria in groundwater. We used values for $v_{\text{max}}$ and $K_{\text{m}}$ from laboratory studies available in the literature to predict an enzyme-specific rate constant for each of the five enzymes described above. We then multiplied the abundance of cells containing a gene for that enzyme by the enzyme-specific rate constant to calculate a first-order rate constant for TCE cometabolism carried out by that enzyme in the groundwater. Finally, we added the rate constants for the five enzymes to predict an overall first-order rate constant for TCE cometabolism. We then evaluated and validated the rate constants predicted from the qPCR markers by comparing them to the rate constants directly measured in the 14C-assay.

**Methods**

qPCR Assays for DNA and mRNA Associated with Oxygenase Enzymes

The quantitative polymerase chain reaction (qPCR) was used to determine the abundance of deoxyribonucleic acid (DNA) and messenger ribonucleic acid (mRNA) coding for the oxygenase enzymes. The raw data on the qPCR analyses was previously published in Wiedemeier et al. (2017). Groundwater was sampled from four wells at Site A on the former Twin Cities Army Ammunition Plant in Minnesota, four wells at the Former Plattsburgh AFB in New York, four wells at the Hopewell Precision Superfund Site in New York, four wells at the Tooele Army Depot in Utah, and three wells at Operable Unit 10 at Hill AFB in Utah.

Groundwater was sampled at the well head and 1000 mL was pumped through Bio-Flo<sup>®</sup> Sterivex<sup>™</sup> filter cartridges (EMD Millipore, Billerica, Massachusetts). Filter cartridges intended for analysis of mRNA were preserved with RNA-protect Cell Reagent (QIAGEN, Inc., Valencia, California). One filter unit for DNA and one filter unit for mRNA were analyzed from each well sampled. Once the samples were collected, the cartridges were stored on water ice, and shipped on ice via an overnight carrier to Microbial Insights, Inc. in Knoxville, Tennessee.

Upon receipt, membranes were aseptically removed from the plastic cartridge. DNA extractions were performed using DNA PowerSoil Total DNA Isolation kits (MO BIO Laboratories, Inc., Solana Beach, California) according to the manufacturer’s recommendations. RNA extractions from field preserved (RNAprotect Cell Reagent, QIAGEN, Inc., Valencia, California) Sterivex<sup>™</sup> filters were performed using RNA PowerSoil Total RNA Isolation kits (MO BIO Laboratories, Inc., Solana Beach, California) according to the manufacturer’s instructions except as noted below. Firefly (Coleoptera) luciferase mRNA (Promega, Madison, Wisconsin) was added to all samples (3.7 × 10<sup>6</sup> gene copies per μL) as an exogenous internal reference mRNA (Johnson et al. 2005). RNA extracts were DNase treated using Turbo DNA-free (Ambion, Austin, Texas) according to the manufacturer’s instructions. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California) according to the manufacturer’s protocol.

All qPCR and RT-qPCR assays were performed on an ABI 7300 Real-Time PCR System (ABI Sequence Detector software v1.3) or QuantStudio 12 K Flex Real-Time PCR system (Applied Biosystems, Foster City, California). Thermocycling conditions and the primer-probe set employed to quantify the luciferase reference standard were described previously (Johnson et al. 2005). TaqMan probes and primers for qPCR quantification of PHE, ring hydroxylation toluene monooxygenase (RMO, RDEG), toluene dioxygenase (TOD), and soluble methane monooxygenase (SMMO) genes were designed and validated at Microbial Insights, Inc. The substrate specificity of ring-hydroxylation toluene monooxygenase and PHE enzymes is not always strict, with some toluene monooxygenases catalyzing both the initial oxidation and the subsequent hydroxylation of the phenols/cresols produced. Overall however, PHE assays target the large subunits of PHE genes (e.g., dmpN, ImpH, poxD, etc.) while the RMO assays target toluene-3- and toluene-4-monooxygenase genes (e.g., tofE, tubA1, etc.) and RDEG quantifies toluene-2-monooxygenases (e.g., tomA3). The TOD assay targets the large subunit of a variety of toluene/benzene dioxygenases (e.g., todC1, bedC1, etc.) and the SMMO assay quantifies mmoX.

To ensure comprehensive quantification, multiple primer/probe sets were used for each gene target. During primer/probe design, target gene sequences were retrieved for well-characterized isolates and uncultured organisms. For the PHE, RMO, RDEG, and TOD assay designs, target
gene sequences from characterized isolates spanned a broad spectrum of genera which included Pseudomonas, Burkholderia, Comamonas,Ralstonia, Thauera, Arthrobacter, Dechloromonas, Alcaligenes, Xanthobacter, and Rhodococcus among others. For SMMO, mmoX genes were included from a variety of methanotrophic genera including Methylococcus, Methylocaldum, Methylovulum, and Methylophilus.

While multiple primer/probe sets were used for each gene target, all qPCR was performed individually (no multiplex). Amplification efficiencies for PHE (96%), RMO (98%), RDEG (98%), and SMMO (99%) assays were within acceptable limits. The qPCR and RT-qPCR analyses included appropriate negative (no reverse transcription enzyme and no DNA) and positive control reactions. Positive controls for PHE, RMO, RDEG, and TOD assays were described by Baldwin et al. (2003). No amplification was detected in negative controls (Ct > total cycles).

The reporting limit for DNA and mRNA samples varied between 5 and 8.3 gene copies per mL.

14C-Assays for Rate Constants for Aerobic Cometabolism of TCE

The procedure used to conduct the 14C-assays and the results of the assays was reported in Wiedemeier et al. (2017) and Mills et al. (2018). To conduct the assay, 100 mL of groundwater was sealed in a 165 mL serum bottle. Purified vapos of 14C-labeled TCE was added to the headspace of the bottle, and the bottles were incubated at room temperature. Samples were removed seven or eight times over a period of 40 to 46 days. At each sampling event, samples were sparged with nitrogen to remove the TCE, and then scintillation counting was used to determine the accumulation of polar 14C labeled degradation products.

The assay bottles were amended with 14C-labeled TCE to provide a concentration of labeled TCE in the groundwater of approximately 280 μg/L. The concentration of TCE in the groundwater as collected was not measured. Previous analyses at the sites showed TCE concentrations less than 20 μg/L. The total concentration of TCE in the assay bottle should have been less than 0.3 mg/L.

Rate Constants for TCE Cometabolism Based on Michaelis–Menten (Haldane) Kinetics

The goal was to develop a relationship between the abundance of DNA that is amplified by selected qPCR primers and the experimentally derived rate constants for TCE cometabolism as determined by Mills et al. (2018). As a first approximation, the relationship between the rate of the cometabolism, the activity of the microorganisms, and the concentration of TCE can be described with the Michaelis–Menten (Haldane) Equation,

\[ v = \frac{v_{\text{max}} S}{K_m + S} \]  

where \( v \) is the specific rate of the reaction (μg of TCE cometabolized per year per mg of cell protein or mg of TCE cometabolized per year per mg of cell dry matter); \( v_{\text{max}} \) is the rate of reaction (μg of TCE cometabolized per year per mg of cell protein or mg of TCE cometabolized per year per mg of cell dry matter); and \( K_m \) is TCE concentration at which \( v = 0.5 v_{\text{max}} \) (mg/L). Note that the Haldane version of Equation 1 refers to the concentration where \( v = 0.5 v_{\text{max}} \) as \( S_{\text{max}} \).

When \( S < < K_m \), Equation 1 can be simplified to:

\[ v = \frac{v_{\text{max}} S}{K_m} \]  

and rearranged to:

\[ v = \frac{v_{\text{max}} S}{K_m} \]  

Whenever \( S < K_m \) by a factor of 10, the rate of degradation \((v/S)\) can be described within an error of ±10% by a pseudo first-order rate constant with a value equal to \( v_{\text{max}}/K_m \). The simplification in Equation 3 assumes that cometabolism of TCE in groundwater samples is faithfully described by the Michaelis–Menten (Haldane) Equation (Equation 1) and the values for \( v_{\text{max}} \) and \( K_m \) that were extracted from laboratory culture studies apply to the groundwater bacteria.

The maximum contaminant level (MCL) for TCE is 0.005 mg/L. At field sites where natural aerobic cometabolism of TCE can be expected to make a substantial contribution to MNA, the field concentrations will be within an order of magnitude of the MCL, which is to say ≤0.050 mg/L. This value is an order of magnitude lower than the values of \( K_m \) that are available for these oxygenase enzymes (Table 1), and the assumption of first-order kinetics should apply at these concentrations.

High concentrations of TCE can inhibit growth of the organisms that cometabolize TCE (Alvarez-Cohen and Speitel Jr. 2001). However, inhibition of TCE cometabolism by TCE at concentrations ≤0.050 mg/L is unlikely. This issue is discussed in more detail in Appendix S1, Supporting Information.

Protein and Dry Matter Content of Bacterial Cells in Groundwater

To express the first-order rate constant in terms of the abundance of gene copies of the oxygenase enzymes, it is necessary to estimate the number of gene copies per cell and the amount of protein or organic matter per cell in the groundwater bacteria that express the oxygenase enzymes. We will assume that the groundwater community has passed through log-phase growth and has reached a state where the bacteria are growing very slowly under near starvation conditions. Under these conditions, most of the bacterial cells will have only one copy of the genome (Givskov et al. 1994). We will assume one genome per cell. We will further assume that there is only one copy of the oxygenase gene in the genome.

The protein content of bacterial cells is related to the size of the cell. Harvey et al. (1997) determined the average size of bacteria in the water table aquifer on Cape Cod, Massachusetts. In pristine water, the average length was 0.3 μm. In groundwater that was impacted by treated municipal wastewater, the average length was 0.6 μm. We used the equations of Kinner et al. (1998) to estimate the volume of the two size classes. Details of the calculations are provided in Appendix S1. The average length of 0.3 μm
would correspond to an average volume of 0.0122 μm³ and the average length of 0.6 μm would correspond to an average volume of 0.085 μm³.

Simon and Azam (1989) evaluated the relationship between the volume of bacterial cells and the protein content of the cells for samples of pelagic bacteria from seawater and growing cultures of the bacteria. They fit their 24 data points to the following equation,

\[
p = 88.6 \cdot V^{0.59}
\]  

(4)

where \( p \) is the protein content of the cell (femtogram per cell) and \( V \) is the volume of the cell (μm³).

Simon and Azam (1989) also evaluated eight samples from a freshwater lake and found that the relationships were not significantly different from the equation developed for marine bacteria. We will use Equation 4 to estimate the protein content of groundwater bacteria. A volume of 0.0122 μm³ for the smaller cells in pristine groundwater and 0.0850 μm³ for the larger cells in the impacted groundwater correspond to a protein content of 6.59 femtogram/cell and 1.95 femtogram/cell, respectively.

### Table 1

| Enzyme Organism Reference | \( v_{max} \) (mg/year * mg Protein or Dry Matter) | \( K_m \) (mg/L) | \( M_{max} \) (mg Protein or Dry Matter/Cell) | \( K_{enzyme-specific} \) (L/year • Gene copy)) qPCR Primer |
|---------------------------|-----------------------------------------------|-----------------|---------------------------------|---------------------------------|
| Toluene-2-monooxygenase P. cepacia G4 (Sun and Wood 1996) | 620 protein | 0.37 | 1.1E-11 dry matter | 4.6E-10 RMO |
| Toluene-2-monooxygenase P. cepacia G4 (Landa et al. 1994) | 346 dry matter | 1.3 | 6.6E-12 protein | 1.9E-09 SMMO |
| Toluene-4-monooxygenase P. mendocina KR1 (Sun and Wood 1996) | 1400 protein | 3.3 | 1.1E-11 dry matter | 3.6E-11 PHE |
| Toluene-2,3-dioxygenase P. putida F1 (Sun and Wood 1996) | 550 protein | 0.66 | 6.6E-12 protein | 1.6E-09 PHE |
| Toluene-2,3-dioxygenase P. putida F1 (Hori et al. 2005) | 400 dry matter | 1.1 | 1.1E-11 dry matter | 1.4E-11 |
| Soluble methane monooxygenase Mixed enrichment culture (Alvarez-Cohen and McCarty 1991) | 194 dry matter | 0.37 | 1.1E-11 dry matter | 1.4E-09 |
| Soluble methane monooxygenase M. trichosporium OB3b (Kol et al. 1993) | 1100 protein | 17 | 6.6E-12 protein | 1.9E-09 |
| Soluble methane monooxygenase M. methanica 68-1 (Kol et al. 1993) | 2700 protein | 30 | 6.6E-12 protein | 1.9E-09 |
| Phenol hydroxylase C. testosteroni E6 (Futamata et al. 2001) | 48 dry matter | 1.5 | 1.1E-11 dry matter | 1.6E-09 PHE |
| Phenol hydroxylase P. putida P2 (Futamata et al. 2001) | 270 dry matter | 78 | 1.1E-11 dry matter | 1.14E-10 |
| Phenol hydroxylase P. sp. WAS2 (Futamata et al. 2001) | 62 dry matter | 47 | 1.1E-11 dry matter | 4.3E-11 |

Notes: Values in bold are used to calculate \( K_{enzyme-specific} \).
The Abundance of DNA Gene Copies Amplified by Primers for Oxygenase Enzymes and the Predicted Rate Constants (K\text{predicted}) for TCE Cometabolism in Water Samples

| Well                  | SMMO Abundance (Gene Copies/mL) | RDEG Abundance (Gene Copies/mL) | RMO Abundance (Gene Copies/mL) | TOD Abundance (Gene Copies/mL) | PHE Abundance (Gene Copies/mL) | K\text{predicted} (per year) |
|-----------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|-------------------------------|-----------------------------|
| Hill AFB U10-019      | 539                             | 28,400                          | 15,400                          | 207                             | 48,200                        | 0.36                        |
| Plattsburgh MW-02-006 | 4950                            | 6840                            | 1950                            | 387                             | 16,100                        | 0.104                       |
| Tooele D20            | 332                             | 6090                            | 1080                            | 30.9                            | 21,800                        | 0.068                       |
| Plattsburgh MW-02-019 | 1650                            | 181                             | <5                              | 166                             | 415                           | 0.012                       |
| Tooele D25            | 190                             | 1130                            | 320                             | 26                              | 1520                          | 0.013                       |
| Hill AFB U10-043      | 26.4                            | 267                             | <5                              | 34                              | 62.3                          | 0.0023                      |
| Hill AFB U10-025      | 57.7                            | 29                              | 1                               | 40.8                            | 8.2                           | 0.00077                     |
| Tooele D23            | 9.6                             | 78.2                            | <6.7                            | 36                              | 118                           | 0.00072                     |
| Hopewell EPA-16S      | 19,000                          | 2230                            | <5                              | 13.4                            | 1940                          | 0.38                        |
| TCAAP 01 U115         | 1440                            | 3920                            | 374                             | 81.7                            | 4880                          | 0.14                        |

1The abundance is above the detection limit but below the limit of quantitation.

20.7 femtogram/cell, respectively. These values are reported in Table 1 as \( M_{\text{per-cell}} \) in units of mg protein/cell.

Simon and Azam (1989) noted that the protein content of the pelagic bacteria was uniform across different size classes, at 63% of the total dry weight. This value was used to convert the estimate of the protein content of cells in the two size classes of bacteria to the content of dry matter. A protein content of 6.59 femtogram/cell and 20.7 femtogram/cell would correspond to 10.5 femtogram/cell and 32.9 femtogram/cell of dry matter. These values are also reported in Table 1 as \( M_{\text{per-cell}} \) in units of mg dry matter/cell.

Calculation of Enzyme-Specific Rate Constants

The first-order rate constant (\( K_{\text{enzyme-specific}} \)) that is specific for each enzyme is calculated as:

\[
K_{\text{enzyme-specific}} = \left( \frac{V_{\max}}{K_m} \right) \times M_{\text{per-cell}}
\]

(5)

where \( V_{\max} \), \( K_m \), and \( M_{\text{per-cell}} \) are taken from Table 1.

In the 1990s there was interest in using aerobic cometabolism for bioremediation of TCE contamination in groundwater. As a result, there is an extensive literature on aerobic cometabolism of TCE, and several good review articles are available (Alvarez-Cohen and Speitel Jr. 2001; Arp et al. 2001; Field and Sierra-Alvarez 2004). Table 1 compares values of \( K_{\text{enzyme-specific}} \) that were extracted from selected laboratory studies on TCE cometabolism. When more than one value for \( K_{\text{enzyme-specific}} \) is available, we choose to use the higher value in Equation 6.

Landa et al. (1994) and Sun and Wood (1996) evaluated TCE cometabolism by \( Pseudomonas cepacia \) G4 growing on toluene and expressing toluene-2-monoxygenase. The values of \( K_{\text{enzyme-specific}} \) that were calculated from \( V_{\max} \) and \( K_m \) reported in these separate papers agree within a factor of seven. The value of \( K_{\text{enzyme-specific}} \) calculated from the data of Sun and Wood (1996) was used in Equation 6 for the toluene-2-monoxygenase genes amplified by the RDEG primer.

Sun and Wood (1996) evaluated TCE cometabolism by \( Pseudomonas mendocina \) KR1 growing on toluene and expressing toluene-4-monoxygenase. The value of \( K_{\text{enzyme-specific}} \) calculated from the data of Sun and Wood (1996) was used in Equation 6 for the Toluene-4-monoxygenase genes amplified by the RMO primer.

Hori et al. (2005) and Sun and Wood (1996) evaluated TCE cometabolism by \( Pseudomonas putida \) F1 growing on toluene and expressing toluene-2,3-dioxygenase. The values of \( K_{\text{enzyme-specific}} \), that were calculated from \( V_{\max} \) and \( K_m \) reported in these separate papers agree within a factor of three. The value of \( K_{\text{enzyme-specific}} \) calculated from the data of Sun and Wood (1996) was used in Equation 6 for the toluene-2,3-dioxygenase genes amplified by the TOD primer.

Kol et al. (1993) and Alvarez-Cohen and McCarty (1991) evaluated TCE cometabolism by several cultures grown on methane and expressing a methane monoxygenase. The values of \( K_{\text{enzyme-specific}} \) calculated from the data of Kol et al. (1993) for \( Methylosinus trichosporium \) OB3b and for \( Methylococcus marina \) 68-1 expressing soluble methane monoxygenase agree within a factor two. However, the value of \( K_{\text{enzyme-specific}} \) calculated from the data of Alvarez-Cohen and McCarty (1991) for a mixed enrichment culture growing on methane is larger by a factor of 10.

There are two distinct methane monoxygenase enzymes, a soluble methane monoxygenase and a particulate monoxygenase. The soluble methane monoxygenase has much greater activity against TCE (Alvarez-Cohen and Speitel Jr. 2001; Arp et al. 2001; Field and Sierra-Alvarez 2004). Alvarez-Cohen and Speitel Jr. (2001) tabulated values of a parameter \( k \) that is equal to \( \frac{V_{\max}}{K_m} \) divided by \( K_m \) (their Table 1). In some of these cultures, formate was added to supply the reducing power needed to operate the monoxygen-
enzyme under conditions where the growth substrate was not available. The addition of formate would not be representative of natural groundwater. They evaluated 16 cultures where formate was not added; the highest value of \( k \) was provided by the mixed culture evaluated by Alvarez-Cohen and McCarty (1991). The value of \( K_{\text{enzyme-specific}} \) calculated from the data of Alvarez-Cohen and McCarty (1991) was used in Equation 6 for the soluble methane monoxygenase genes amplified by the RDEG primer.

Futamata et al. (2001) provide values for \( v_{\text{max}} \) and \( K_m \) for cometabolism of TCE by 13 cultures of phenol degrading bacteria expressing phenol hydroxylase. Table 1 presents data for the three cultures that had the maximum, minimum, and median values for \( K_{\text{enzyme-specific}} \). The highest value of \( K_{\text{enzyme-specific}} \) was used in Equation 6 for the phenol monoxygenase genes amplified by the PHE primer. The highest value of \( K_{\text{enzyme-specific}} \) for phenol hydroxylase was an order of magnitude lower than the value of \( K_{\text{enzyme-specific}} \) selected for the toluene oxygenase enzymes or soluble methane monoxygenase.

Calculation of Overall Rate Constants

Table 2 presents the abundance of gene copies in the samples of groundwater that were amplified by the SMMO, RDEG, RMO, TOD, and PHE primers. A first-order rate constant associated with each enzyme was calculated by multiplying the associated value of \( K_{\text{enzyme-specific}} \) by the abundance of the gene copies for that enzyme in the groundwater. First-order rate constants are additive. The overall rate constant predicted from the abundance of the qPCR primers is the sum of the enzyme-specific rate constants. We will refer to this predicted overall first-order rate constant for TCE cometabolism as \( K_{\text{predicted}} \) (per year),

\[
K_{\text{predicted}} = \sum (K_{\text{enzyme-specific}} \cdot A_{\text{enzyme-specific}} \cdot 1000) \quad (6)
\]

where \( K_{\text{enzyme-specific}} \) is the enzyme-specific rate constant associated with enzymes amplified by the SMMO, RDEG, RMO, TOD, and PHE primer, respectively (L/(year \cdot gene copy)) and \( A_{\text{enzyme-specific}} \) is the abundance of DNA amplified by the SMMO, RDEG, RMO, TOD, and PHE primer, respectively (gene copies per mL).

Table 2 provides two values of \( K_{\text{predicted}} \) for each sample of groundwater based on the two representative size classes of bacterial cells in groundwater.

Results

We shall use the term \( K_{\text{measured}} \) to identify the measured rate constant previously published by Mills et al. (2018). Figure 1 compares \( K_{\text{predicted}} \) against \( K_{\text{measured}} \) in the eight groundwater samples where cometabolism of TCE was detected (i.e., where the rate constant was greater than the filter sterilized control at 95% confidence). The value of \( K_{\text{measured}} \) is the net rate of degradation, where the abiotic degradation in the control has been subtracted from the rate in the experimental microcosm.

Panel A of Figure 1 compares values of \( K_{\text{measured}} \) and \( K_{\text{predicted}} \) to the line of equivalence for \( K_{\text{measured}} \) and \( K_{\text{predicted}} \) in groundwater samples where cometabolism was detected and samples where cometabolism was not detected. There was a useful agreement between the values of \( K_{\text{measured}} \) and \( K_{\text{predicted}} \) in samples where cometabolism was detected. The correlation coefficient between the natural logarithm of \( K_{\text{measured}} \) and the natural logarithm of \( K_{\text{predicted}} \) was 0.90.

In Panel B of Figure 1, a power function was fit to the data and a regression of \( K_{\text{predicted}} \) on \( K_{\text{measured}} \) was fit to the logarithm-transformed data. On average, the regression line based on a cell length of 0.3 μm underestimated the line of equivalence by a factor of five, and the regression that was based on a cell length of 0.6 μm underestimated the line by a factor of two.

![Figure 1. Relationship between the rate constants for TCE cometabolism in groundwater predicted from the abundance of genes for oxygenase enzymes (\( K_{\text{predicted}} \)) and the rate constants that are measured with a \( ^{14} \)C-assay (\( K_{\text{measured}} \)). The error bars are the 95% confidence interval on \( K_{\text{measured}} \) after correction of degradation in a filter-sterilized control. Panel B has the same data as Panel A except that the range plotted is reduced, nondetects are not shown, and trend lines are included.](https://doi.org/10.1111/1529-0147.12487)
Except for the two lowest values of $K_{\text{predicted}}$, the values of $K_{\text{predicted}}$ based on a cell length of 0.6 μm were within a factor of three of the values of $K_{\text{measured}}$ (Panel A of Figure 1). We consider the values of $K_{\text{predicted}}$ based on a cell length of 0.6 μm as the best estimate to interpret the relationship between the abundance of DNA and the first-order rate constant for cometabolism of TCE in groundwater.

Notice that with one exception, the measured rate constants exceeded the predicted rate constants. While it is possible that groundwater bacteria cometabolized TCE more readily than bacteria in laboratory cultures, it is more likely that there were bacteria in the groundwater that cometabolized TCE but did not contain DNA that was amplified by the qPCR primers used in this study. If this is the case, the predicted rate constants will tend to underestimate the true rate constant for TCE cometabolism. Also note that the greatest divergence between the two rates were for the lowest values of $K_{\text{predicted}}$. The groundwater samples with a low abundance of gene copies that were amplified by the qPCR primers contained a low abundance of total bacteria (Wiedemeier et al. 2017). These sparse oligotrophic groundwater bacterial communities may contain a greater proportion of bacteria with oxygenase enzymes that are not expressed using the available qPCR primers.

Panel A of Figure 1 also compares $K_{\text{predicted}}$ and $K_{\text{measured}}$ for the 11 groundwater samples where TCE cometabolism was not detected (i.e., where the measured rate constant was not significantly greater than the filter-sterilized control). In this case, the value of $K_{\text{measured}}$ is not corrected for the rate of degradation in the filter-sterilized control. For nine of the eleven water samples where cometabolism was not significantly greater than the control, both $K_{\text{predicted}}$ and $K_{\text{measured}}$ were less than 0.02 per year. Relatively high rate constants were predicted in two samples, but measured rate constants were not detectable (Figure 1). Groundwater from well EPA-16S at the Hopewell site had a $K_{\text{predicted}}$ of 0.38 per year and groundwater from well O1U115 at the TCAAP had a $K_{\text{predicted}}$ of 0.14 per year (Table 2). There is nothing in the relative abundance of DNA gene copies in these wells that would explain the lack of TCE cometabolism.

Table 3

| Site                        | SMMO | RDEG | RMO | TOD | PHE |
|-----------------------------|------|------|-----|-----|-----|
| Hill AFB U10-019            | 19.6 | 203  | 3 $^*$ | <8.3 | 284 |
| Plattsburgh MW-02-006       | 37.7 | <7.1 | <7.1 | 115 | <7.1 |
| Tooele D20                  | <5   | <5   | <5   | 5   | 48.4 |
| Plattsburgh MW-02-019       | <5   | <5   | 51$^*$ | 12 | 117 |
| Tooele D25                  | <5   | <5   | 5   | 48.4 | <5 |
| Hill AFB U10-043            | <5   | <5   | 16$^*$ | 1$^*$ | 62.1 |
| Hill AFB U10-025            | <5   | <5   | <5   | <5   | <5 |
| Tooele D23                  | <5   | <5   | 29$^*$ | <5   | <5 |
| Measured rate constant not significant at 95% confidence |
| Hopewell EPA-16S            | 514  | 1460 | <5   | 0$^*$ | 78.2 |
| TCAAP 01 U115               | 23.6 | 5    | <5   | 48$^*$ | 88.2 |

$^*$The abundance is above the detection limit but below the limit of quantitation.

to the abundance in wells where TCE cometabolism was detected. The mRNA data did not explain the failure to detect TCE cometabolism in the groundwater samples from well EPA-16S and O1U115.

If a gene is used to produce an enzyme, that gene is said to be expressed. Copper ions inhibit the expression of the gene for soluble methane monooxygenase in Methylosinus trichosporium OB3b at very low concentrations (Tsien et al. 1989; Murrell et al. 2000). In cultures actively growing in a chemostat, copper concentrations of 0.5 μM (32 μg/L) essentially prevent TCE cometabolism, concentrations of 0.25 μM (16 μg/L) reduce TCE cometabolism to less than half, while concentrations of 0.1 μM (6.3 μg/L) have no discernable effect on TCE cometabolism (Tsien et al. 1989). Green et al. (1985) showed that copper ions are actively taken up by Methyllococcus capsulatus (Bath), and that the copper ions irreversibly damage one of the components of soluble methane monooxygenase.

The average concentration of copper ions in shallow groundwater at TCAAP is 8.8 μg/L (Trojan 1999). This concentration is slightly above the concentration of no discernable effect. It is possible that over prolonged periods of exposure, copper can damage soluble methane monooxygenase enzymes in the native groundwater bacteria. Inhibition by copper may have contributed to failure of bacteria in groundwater from well O1U115 at the TCAAP site to cometabolize TCE.

**Discussion**

For the 19 wells in this study, whenever the rate constant for TCE cometabolism in the 14C-assay was greater than 0.02 per year, the rate constant was greater than the rate constant in the filter sterilized controls at 95% confidence (Figure 1). This can be considered an effective detection
limit for the assay. At this rate constant, the required exposure time in the aquifer is extensive for relatively low concentrations of TCE. It would take 34 years to bring 10 μg/L to the MCL and 55 years to bring 15 μg/L to the MCL. The C-assay has adequate sensitivity for practical application at most sites of concern.

In six of the seven wells, the predicted rate constant and the measured rate constant agreed with each other within a factor of three, and this agreement extended over a range of predicted rate constants extending from near 0.01 per year to 1 per year (Figure 1). The association of the predicted rate constants with the measured rate constants has adequate sensitivity for application at most sites of concern.

Figure 2 can be used to evaluate a rate constant for TCE cometabolism within the regulatory framework of a site of interest. This figure predicts the residence time along a flowpath in groundwater that is required for cometabolism of TCE to bring the concentration of TCE to the MCL. Figure 2 only applies to the residence time after the groundwater has moved away from a perennial source of TCE contamination. The predicted time to reach the MCL should be multiplied by the expected seepage velocity of the groundwater to predict a distance that the plume will travel before the TCE reaches the MCL. To complete the evaluation, the distance that the TCE contamination might travel should be compared to the distance from the contaminated well to the site boundary or a sentinel well.

As shown in Figure 1, the rate constants in three of the 19 wells in this survey were >0.12 per year. These rate constants would bring concentrations of TCE ≤50 μg/L to the MCL within 20 years of residence time in the aquifer (Figure 2). In our judgment, when TCE ≤50 μg/L and Kpredicted ≥ 0.12 per year, MNA based on cometabolism of TCE could be a plausible remedy in most large dilute plumes. The rate constants for two additional wells were >0.06 per year. These rate constants would bring 30 μg/L TCE to the MCL in 30 years. These rate constants might be useful in plumes where the distance to a site boundary or a sentinel well is large.

The number of sites and wells in this survey are too small to provide a robust depiction of the distribution of rate constants at sites where TCE cometabolism may potentially contribute to MNA as a remedy. Figure 3 compares Kpredicted calculated from 427 water samples that were submitted to Microbial Insights, Inc. for qPCR analysis where RDEG was one of the primers used in the assay. Not every primer was used on every water sample. In the Microbial Insights data, 90% of the samples that were assayed for RDEG were also assayed for the PHE primer, 88% for the RMO primer, 25% for the SMMO primer, and 26% for the TOD primer. In the surveyed wells used in this study, PHE was the most abundant marker on average, followed by RDEG, then SMMO, then RMO, then TOD in descending order (compiled from data in Table 2). The Microbial Insights data provided a reasonable mix of the same markers. Of the most abundant primers, only SMMO was underrepresented in the Microbial Insights database.

Values of Kpredicted were calculated for the water samples in the Microbial Insights database (Figure 3). Only 27% of the samples had a Kpredicted > 0.12 per year and 34% had a Kpredicted > 0.06 per year. The distribution of Kpredicted in the larger data set was not markedly different from the distribution of Kmeasured provided by Mills et al. (2018), which was 18% >0.12 per year and 26% >0.06 per year.

Conclusions and Recommendations

Rate constants predicted from qPCR assays of oxygenase enzymes had a useful correspondence to rate constants that were directly measured using the C-assay of Mills et al. (2018). In the range of rate constants that might have application to MNA, they agreed within a factor of three.

In general, the aerobic cometabolism rate constants predicted from the qPCR assays will be slow compared to rate constants for natural attenuation of TCE under anaerobic conditions. As a result, cometabolism of TCE as a mechanism for an MNA remedy will be most appropriate for plumes with low concentrations of TCE and a long travel time from the contaminated well to a property boundary, sentinel well, or some other receptor. However, for some very large aerobic plumes, MNA may be the only practicable remedy if an adequate rate constant can be documented.

The predicted rate constants were false in two of the wells in the survey; the predicted rate constants were in a
range that would be useful for MNA at some sites, but cometabolism was not detected in the \(^{14}\text{C}\)-assay. The possibility of a false positive means that the predicted rate constants should only be used to screen sites for the applicability of MNA. If the results are encouraging, the predicted rate constant should be confirmed using the \(^{14}\text{C}\)-assay.

Sampling of water from monitoring wells frequently blends water produced from different screened intervals. It is possible that the TCE in a sample of well water came from an anaerobic flowpath in the aquifer and the oxygen from another flowpath that did not have TCE. If this is the case, oxygen may not be available to the bacteria in the aquifer, leading to the erroneous conclusion that the TCE can be cometabolized. Meckenstock et al. (2015) noted that bacteria can reach high abundance levels at an interface between anaerobic groundwater containing organic compounds that can support their growth and aerobic groundwater with oxygen to support aerobic metabolism. In this situation, the active bacteria would not be uniformly distributed in the groundwater. If active bacteria and DO are not uniformly distributed in the groundwater that is contaminated with TCE, the rate constants derived from the laboratory \(^{14}\text{C}\)-assay or the rate constant predicted from the qPCR assay will overestimate the true rate constant for TCE cometabolism at field scale.

To guard against this error, groundwater samples should be analyzed for geochemical parameters, and the parameters should be interpreted to determine whether it is reasonable to assume that the active bacteria and adequate supplies of DO are uniformly distributed in the groundwater that is contaminated with TCE. Wiedemeier et al. (2017) devised criteria to recognize water that had adequate oxygen uniformly distributed in the aquifer to support aerobic metabolism. The criteria are DO >0.1 mg/L, Fe\(^{2+}\) <0.5 mg/L, and methane <0.005 mg/L. If these criteria are met, it is reasonable to extrapolate the rate constants to describe the field-scale plume. If these criteria are not met, the predicted rate constants from the qPCR assay or the rate constant from the \(^{14}\text{C}\)-assay should not be used uncritically to model MNA at a site. It will be necessary to characterize the aquifer at a finer resolution to determine whether active organisms and DO are available to support cometabolism of the TCE.

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**Supporting Information**

**Appendix S1.** Supporting Information for details on the calculation of the volume of cells of bacteria in groundwater, and conversion of values of the kinetic parameters for TCE cometabolism from the units in the original references to the units used in this manuscript.

**References**

Alvarez-Cohen, L., and P.L. McCarty. 1991. A cometabolic biotransformation model for halogenated aliphatic compounds exhibiting product toxicity. *Environmental Science & Technology* 25: 1381–1386.

Alvarez-Cohen, L., and G.E. Speitl Jr. 2001. Kinetics of aerobic cometabolism of chlorinated solvents. *Biodegradation* 12, no. 2: 105–126.

Arp, D.J., C.M. Yeager, and M.R. Hyman. 2001. Molecular and celluar fundamentals of aerobic cometabolism of trichloroethene. *Biodegradation* 12, no. 2: 81–103.

Baldwin, B.R., C.H. Nakatsu, and L. Nies. 2003. Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. *Applied and Environmental Microbiology* 69, no. 6: 3350–3358.

Chu, K.-H., S. Mahendra, D.L. Song, M.E. Conrad, and L. Alvarez-Cohen. 2004. Stable carbon isotope fractionation during aerobic biodegradation of chlorinated ethenes. *Environmental Science & Technology* 38, no. 11: 3126–3130.

Clingenpeel, S.R., J.L. Moan, D.M. McGarth, B.A. Hungate, and M.E. Watwood. 2012. Stable carbon isotope fractionation in chlorinated ethene degradation by bacteria expressing three toluene oxygenases. *Frontiers of Microbiology* 3, article 63: 1–7.

Field, J.A., and R. Sierra-Alvarez. 2004. Biodegradability of chlorinated solvents and related chlorinated aliphatic compounds. *Reviews in Environmental Science & Bio/Technology* 3: 185–254.

Futamata, H., S. Harayama, and K. Watanabe. 2001. Diversity of kinetics of trichloroethylene-degrading activities exhibited by phenol-degrading bacteria. *Applied Microbiology and Biotechnology* 55: 248–253.

Givskov, M., L. Eberl, S. Möller, L.K. Poulsen, and S. Molin. 1994. Responses to nutrient starvation in *Pseudomonas putida* KT2442: Analysis of general cross-protection, cell shape, and macromolecular content. *Journal of Bacteriology* 176, no. 1: 7–14.

Green, J., S.D. Prior, and H. Dalton. 1985. Copper ions as inhibitors of protein C of soluble methane monooxygenase of *Methylococcus capsulatus* (Bath). *European Journal of Biochemistry* 153: 137–144.

Harvey, R.W., D.W. Metge, N. Kinner, and N. Mayberry. 1997. Physiological considerations in applying laboratory-determined buoyant densities to predictions of bacterial and protozoan transport in groundwater: Results of in-situ and laboratory tests. *Environmental Science & Technology* 31: 289–295.

Hori, K., J. Mii, Y. Morono, Y. Tanji, and H. Unno. 2005. Kinetic analyses of trichloroethylene cometabolism by toluene-degrading bacteria harboring a tod homologous gene. *Biotechnical Engineering Journal* 26: 59–64.

Johnson, D.R., P.K.H. Lee, V.F. Holmes, and L. Alvarez-Cohen. 2005. An internal reference technique for accurately quantifying specific mRNAs by real-time PCR with application to the tecA reductive dehalogenase gene. *Applied and Environmental Microbiology* 71, no. 7: 3866–3871.

Kentucky Research Consortium for Energy and Environment (KRCEE). 2008. PGDP trichloroethene biodegradation investigation summary report regional gravel aquifer & northwest plume. Prepared by the TCE Fate and Transport Team.
through University of Kentucky. http://www.ukrcee.org/Challenges/Documents/GroundwaterTCE_Fate_and_Transport/KRCEE_22.7_2008d.pdf (accessed March 6, 2019).

Kinner, N.E., R.W. Harvey, K. Blakeslee, G. Novarino, and L.D. Meeker. 1998. Size-selective predation on ground-water bacteria by nanoflagellates in an organic-contaminated aquifer. Applied and Environmental Microbiology 64, no. 2: 618–625.

Kol, S.-C., J.P. Bowman, and G. Sayler. 1993. Soluble methane monoxygenase production and trichloroethene degradation by a type 1 Methanotroph, Methylo monas methanica 68-1. Applied and Environmental Microbiology 59, no. 4: 960–967.

Landa, A.S., E.M. Sipkema, J. Weijma, A.A.C.M. Beenackers, J. Weijma, and E.M. Sipkema. 1994. Cometabolic degradation of trichloroethylene by Pseudomonas cepacia G4 in a chemostat with toluene as the primary substrate. Applied and Environmental Microbiology 60, no. 9: 3368–3374.

Lebrón, C., T. Wiedemeier, J. Wilson, F. Löffler, R. Hinchee, and M. Singley. 2015. Validation of a quantitative framework and management expectation tool for the selection of bioremediation approaches at chlorinated solvent sites. Final Report ER-201129. https://www.serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-ontamination/ER-201129/ER-201129 (accessed March 6, 2019).

Lee, M.H., S.C. Clingenpeel, O.P. Leiser, R.A. Wymore, K.S. Sorenson Jr, and M.E. Watwood. 2008. Activity-dependent labeling of oxygenase enzymes in a trichloroethene-contaminated ground-water site. Environmental Pollution 153: 238–246.

Löffler, F.E., and E.A. Edwards. 2006. Harnessing microbial activities for environmental cleanup. Current Opinion in Biotechnology 17: 274–284.

McKenstock, R.U., M. Elsner, C. Griebler, T. Lueders, C. Stumpf, J. Aamand, S.N. Agathos, H.-J. Albrechtsen, L. Bastiaens, P.L. Bjerg, N. Boon, W. De fonghe, W.E. Huang, S.I. Schmidt, E. Smolders, S.R. Sørensen, D. Springael, and B.M. van Breukelen. 2015. Biodegradation: Updating the concepts of control for microbial cleanup in contaminated aquifers. Environmental Science & Technology 49: 7073–7081.

Mills, J.C., J.T. Wilson, B.H. Wilson, T.H. Wiedemeier, and D.L. Freedman. 2018. Quantification of TCE co-oxidation in ground-water using a 14C-assay. Groundwater Monitoring & Remediation 38, no. 2: 57–67.

Murrell, J.C., I.R. McDonald, and B. Gilbert. 2000. Regulation of expression of methane monoxygenases by copper ions. Trends in Microbiology 8, no. 5: 221–225.

Parales, R.E., J.V. Parales, D.A. Pelletier, and J.L. Ditty. 2008. Diversity of microbial toluene degradation pathways. In Advances in Applied Microbiology, Vol. 64, ed. A.I. Laskin, S. Sariaslani, and G.M. Gadd, 1–73. Burlington, MA: Academic Press.

Simon, M., and F. Azam. 1989. Protein content and protein synthesis rates in planktonic marine bacteria. Marine Ecology Progress Series 51: 201–213.

Sorenson, K.S., L.N. Peterson, R.E. Hinchee, and R.L. Ely. 2000. An evaluation of aerobic trichloroethene attenuation using first-order rate estimation. Bioremediation Journal 4, no. 4: 337–357.

Sturchio, N.C., J.L. Clausen, L.J. Heraty, L. Huang, B.D. Holt, and T.A. Abrajano Jr. 1998. Chlorine isotope investigation of natural attenuation of trichloroethylene in an aerobic aquifer. Environmental Science & Technology 32, no. 20: 3037–3042.

Suarez, M.P., and H.S. Rifai. 1999. Biodegradation rates for fuel hydrocarbons and chlorinated solvents in groundwater. Bioremediation Journal 3, no. 4: 337–362.

Sun, A.K., and T.K. Wood. 1996. Trichloroethene degradation and mineralization by pseudomonads and Methylosinus trichosporium OB3B. Applied Microbiology and Biotechnology 45: 248–256.

Trojan, M.D. 1999. Baseline water quality of Minnesota’s principal aquifers – Region 6, Twin Cities Metropolitan Area, Minnesota Pollution Control Agency. https://www.pca.state.mn.us/sites/default/files/metro-rpt.pdf (accessed March 6, 2019).

Tsen, H.-C., G.A. Brusseau, R.S. Hanson, and L.P. Wackett. 1989. Biodegradation of trichloroethylene by Methylosinus trichosporium OB3b. Applied and Environmental Microbiology 55, no. 12: 3155–3161.

U.S. Environmental Protection Agency (U.S. EPA). 1999. Use of monitored natural attenuation at superfund, RCRA corrective action, and underground storage tank sites. Office of Solid Waste and Emergency Response Directive Number 9200.4-17P. Washington, DC: U.S. Environmental Protection Agency.

U.S. Environmental Protection Agency (U.S. EPA). 2017. Superfund remedy report, 15th Edition. EPA-542-R-17-001. Washington, DC: U.S. Environmental Protection Agency, Office of Land and Emergency Management.

Wiedemeier, T.H., J.T. Wilson, D.L. Freedman, B. Lee. 2017. Providing additional support for MNA by including quantitative lines of evidence for abiotic degradation and co-metabolic oxidation of chlorinated ethylenes. Final Report ER-201584. https://serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201584/ER-201584 (accessed March 6, 2019).

Wymore, R.A., M.H. Lee, W.K. Keener, A.R. Miller, F.S. Colwell, M.A. Watwood, and K.S. Sorenson Jr. 2007. Field evidence of intrinsic aerobic chlorinated ethene cometabolism by methanotrophs expressing soluble methane monoxygenase. Bioremediation Journal 11, no. 3: 125–139.

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