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A STATISTICAL APPROACH TO UNDERSTANDING MICROCOSM METHODS FOR MICROBIALLY MEDIATED DECHLORINATION OF TRICHLOROETHENE IN BEDROCK AQUIFERS

BY

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DISSERTATION

Submitted to the University of New Hampshire

in Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

in

Civil Engineering

May, 2007

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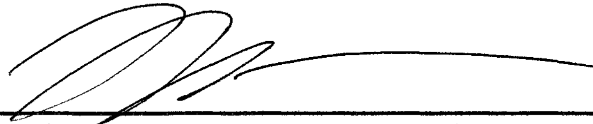

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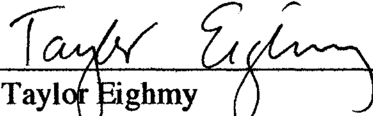

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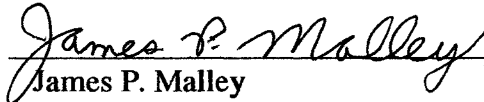

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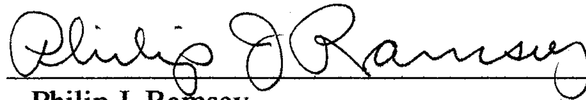

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DEDICATION

This work is dedicated to my family, represented in the past, present and future: Henry and Barbara who instilled in me a love of learning and willingness to try until I succeed; Margo, my wonderful partner and best supporter; and Maggie and Henry, who reflect so much exuberance about the world. I am surrounded in your love and support.

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ACKNOWLEDGEMENTS

True learning is hard work, let there be no doubt, but good teachers and supportive colleagues make the burden lighter. The Bedrock Bioremediation Center graciously provided support for sample collection, materials, incubation, and chemical analysis, funded under USEPA Grant CR 827878-01-0. Assistance and advice was provided by Professors Philip Ramsey, Tom Ballestero, Wally Bothner, James Malley, Taylor Eighmy, Elise Sullivan, Sterling Tomellini and Joseph Liccardi. Gonzalo Pulido provided hydrogeologic information. Field and laboratory support was provided by Jen Jencks, Don Dubois, Joe Cunningham, Michelle Mills, Jeanne Spear and Michelle Majko. Dr. Evan Griffiths provided input on early concepts. Students Whitney Blanchard, Hillary Schmidle, Maureen Lewis, Tim Puls, Jenna Wallis and David Frye provided laboratory efforts. Technical support was provided way above and beyond contract requirements by Perkin Elmer Corporation and Resource Laboratory, Inc. Business support was provided by Kelly Hinton of the CEPS Business Service Center. Microcosm advice was graciously provided by Dr. Sam Fogel of Bioremediation Consulting, Inc., Dr. Paul Bradley, USGS, and Professor Susan Pfiffner, University of Tennessee. Special places for writing were freely offered by the Hamilton-Wenham Public Library, the City of Beverly Public Library, the Atomic Café of Beverly and School's Out, Inc. of Hamilton.

Professor Nancy Kinner challenged me to be better every day of this effort, dramatically strengthening the results, for which I am truly grateful.

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ABSTRACT

A STATISTICAL APPROACH TO UNDERSTANDING MICROCOSM METHODS FOR MICROBIALLY MEDIATED DECHLORINATION OF TRICHLOROETHENE IN BEDROCK AQUIFERS

by

Stephen J. Druschel

University of New Hampshire, May, 2007

Microcosms were evaluated using statistical methods to advance the measurement and characterization abilities for *in situ* reductive dechlorination of trichloroethene (TCE) in fractured rock aquifers. Microcosms constructed with unincubated crushed rock in groundwater provided the best microcosm model of *in situ* TCE degradation, when prepared and incubated to simulate the *in situ* environment. Microcosms constructed with only groundwater were effective at modeling the TCE degradation only when the microcosms were amended with (total) organic carbon (TOC). Incubation of crushed rock core within the residual TCE plume caused a substantial decline in TCE degradation for biotic intrinsic microcosms, suggesting an effect that is inhibitory to TCE degrading microbes. Glass beads were found to be an inadequate substitute for rock media because their corrosion (i.e., hydrolysis and ion exchange) increased pH and dissolved oxygen beyond *in situ* ranges. Addition of incubated granular material to sterilized groundwater provided insufficient microbial population or metabolic activity in the microcosms to achieve TCE degradation.

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The ability of microcosms to discern slow rates of TCE degradation was evaluated, with specific application to bedrock aquifers. A method was developed to determine whether TCE biodegradation is occurring, assuming first order kinetics, and to estimate what is the longest half-life (i.e., the smallest biodegradation rate) that can be predicted by microcosm experiments for a reasonable incubation period, an acceptable statistical confidence and the fewest replicates when evaluating natural attenuation of TCE in fractured bedrock aquifers.

A factorial experiment of biostimulated anaerobic TCE dechlorination in fractured bedrock aquifers using microcosms evaluated several potential biostimulants (i.e., nutrients, vitamins, sterile groundwater). Optimum TCE degradation occurred with biotic crushed rock microcosms with sterile groundwater that resupplied *in situ* nutrients to the microcosm.

The procedures and methods developed in this study substantially enhance the ability to evaluate biotic fate of TCE in fractured rock aquifers, providing an effective approach for remedial design at low to moderate cost.

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CHAPTER I

INTRODUCTION

Trichloroethene (TCE) (C_2HCl_3) is a halogenated aliphatic compound used as an organic solvent for cleaning metal parts associated with aircraft and machinery maintenance, electronics manufacture, and plating. Because of its chemical structure, TCE is heavier than water (density of 1.46 g/mL @ 20° C), with relatively low aqueous solubility (1100 mg/L @ 25° C) (CRC, 1999). Hence, when released into the environment, TCE often sinks below the source of the release through the geologic strata, driven by gravity, coming to rest as a dense, non-aqueous phase liquid (DNAPL) when contacting low permeability layers of soil or rock in areas with no hydraulic discontinuities. TCE will slowly dissolve from the DNAPL into the surrounding groundwater creating plumes of contamination (Parker et al., 1994).

The characteristics of TCE that make it a good solvent also make it harmful to human health. It is associated with toxicity and cancer of the liver and kidneys, as well as reproductive and developmental toxicity and perhaps neurotoxicity (National Academy of Sciences, 2006). TCE can be ingested or inhaled when the contaminated groundwater is used for drinking and bathing. USEPA (2006) and most states have adopted a maximum contaminant level (MCL) for TCE in groundwater of 5 ug/L, a value about 1 / 220,000 of its aqueous solubility. While air stripping or activated carbon adsorption can remove TCE from water, such treatment is costly, inconvenient and requires frequent maintenance; all of which can be substantial burdens to most

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homeowners or small community plant operators. Fortunately, microbially-mediated treatment (biodegradation) of TCE in groundwater can occur, under certain conditions, to reduce concentrations and protect human health.

Biodegradation has the potential to effectively remediate TCE in bedrock aquifers, as *in situ* anaerobic TCE half-lives range from 80 to 800 days, with longer half-lives at locations without amendment or enhancement (i.e., under biotic intrinsic conditions) (Aziz et al., 2002). With suitable amendments (i.e., electron donor or acceptors to stimulate reducing conditions, nutrients), the half-life can be reduced to 0.18 days (Parsons Corp., 2000). Microcosms are needed to evaluate the potential for biodegradation in fractured rock aquifers because there typically are few monitoring wells drilled into rock, so little *in situ* data is available (Wiedemeier et al., 1998).

Microcosms do not oversimplify an evaluation, unlike a single species or pure culture experiment that may not incorporate effects due to nutrient cycling, trophic level interaction, or variations such as pH, dissolved oxygen (DO), redox potential, or organic content (Pritchard and Bourquin, 1984). Their small size (20 to 500 mL) also permits replication, simplified dosing mechanics, control over inputs and outputs from the bottles, adequate mixing and control of mass transfer effects, and variation in treatments (Pritchard and Bourquin, 1984). However, scaling of microcosm results from the laboratory to the field is considered difficult (Sturman et al., 1995). Furthermore, wall or other boundary effects, ecosystem biogeochemistry, and trophic level interactions of the natural system are not incorporated; and results are limited to specific zones within the ecosystem (Pritchard and Bourquin, 1984).

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Microcosms cannot be used as the sole proof of *in situ* biodegradation because they are unable to replicate the delicate and intricate balance of chemical, physical, and biological relationships that can change rapidly in response to environmental factors (e.g., DO, water, pH, nutrients, minerals) (Madsen, 1991). Rather, three types of evaluations demonstrate the potential for *in situ* biodegradation (Madsen, 1991): comparison of biotic and abiotic treatments distinguishes biologically-mediated activity in excess of abiotic loss; a decrease in contaminant concentrations in the field greater than the losses in the abiotic microcosms confirms indigenous activity; and biological activity in biotic contaminated microcosms should be compared to biotic pristine microcosms to demonstrate ecological adaptation, if there is more than one contaminant compound involved.

Fractured rock aquifers are a challenging environment in which to evaluate microbial activity because of mineral distribution, fracture spacing and orientation, porosity, seasonal geochemical cycling, and hydrogeologic heterogeneity. These factors affect microbial growth and distribution by influencing nutrition, habitat and trophic interactions. Therefore, estimation of microbial dechlorination rates for fractured rock aquifers requires that microcosms address impacts of rock surfaces, formation minerals, rock porosity, trophic interactions, nutritional cycles, and slow rates of growth and metabolism. To date, only four studies (Yager et al., 1997; Hohnstock-Ashe et al., 2001; Byl and Williams, 2000; Lenczewski et al., 2004) have been published in the technical literature evaluating microbial dechlorination in fractured rock aquifers with microcosms, none of which have addressed *in situ* rates of dechlorination, only relative activity between locations or under different effects. [N.B., Studies have been done evaluating

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microbial dechlorination in fractured rock aquifers with microcosms, but have not been published (Fogel, pers. comm., 2005.)]

Objectives

Three main questions are addressed in this study to advance the measurement and characterization abilities for *in situ* reductive dechlorination of TCE in fractured rock aquifers.

- How well do microcosms model *in situ* reductive dechlorination of TCE in fractured rock aquifers and are there preparation techniques that can improve the model?
- How well do microcosms resolve very slow rates (half-lives slower than 300 days) of *in situ* reductive dechlorination of TCE in fractured rock aquifers, and what is the limit of a monitored natural attenuation (MNA) determination?
- How well do microcosms function in factorial evaluations of potential biostimulants for *in situ* reductive dechlorination of TCE in fractured rock aquifers?

These questions are addressed in Chapters 3, 4 and 5, respectively. Chapter 2 is a review of the pertinent literature on TCE as a pollutant, the development of microcosms for TCE, and application of microcosms to fractured rock.

In Chapter 3, microcosm methods used to predict the *in situ* anaerobic reductive dechlorination of TCE in bedrock aquifers were evaluated. Biotic intrinsic (i.e., microcosm conditions that simulate *in situ* conditions without amendments) and biotic amended (i.e., microcosm conditions that are amended with organic carbon to stimulate

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degradation) preparations were compared to abiotic preparations (controls) using field site conditions as a model. The microcosm method of Wilson et al. (1996) was selected for this study as the technique more appropriate for the goal of modeling *in situ* conditions within bedrock aquifers, emphasizing the elucidation of slower rates and modeling of *in situ* conditions without amendment or enhancement. The effect of rock media in the microcosms as a source of surface area and nutrients was also assessed. Microcosm conditions (e.g., surface area : volume ratio, initial TCE concentration, incubation temperature) were selected to simulate *in situ* conditions. The effect of a 45 day pre-microcosm *in situ* incubation of rock media in a groundwater well within a TCE plume was assessed in comparison to use of sterile rock media.

In Chapter 4, the research evaluated the ability of microcosms to discern slow rates of TCE degradation (half-lives longer than 300 days), with specific application to bedrock aquifers. The source and amount of experimental variation within the microcosm process was evaluated, and factors for improvement were considered including the effect of higher sample numbers, increased replication, and decreased statistical confidence. A method was developed to determine whether TCE biodegradation is occurring in a microcosm, assuming first order kinetics (Chapelle et al., 2003), and to estimate what is the longest half-life (i.e., the smallest biodegradation rate) that can be predicted by microcosm experiments for selected experimental conditions (i.e., incubation period, statistical confidence, replication) when evaluating natural attenuation of TCE in fractured bedrock aquifers. This evaluation calculated a detectable difference (E) that represents the minimum decrease in TCE concentration that must be

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observed in the biotic treatments to insure with a given statistical confidence that the effect is not solely abiotic (Box et al., 2005).

In Chapter 5, the use of factorial microcosm experiments to assess the effects of biostimulants and *in situ* factors on anaerobic TCE degradation in fracture rock aquifers was explored. This approach provided substantial cost savings and resource efficiency because: (1) factorial designs require relatively few microcosms per factor evaluated; (2) the analyis of the results involves simple arithmetic and computer graphics; (3) promising directions for further experimentation and causative relationships are indicated; and (4) designs can be suitably augmented when a more thorough local exploration is needed (Box et al., 2005). In this dissertation research, a factorial experiment of biostimulated TCE dechlorination in fractured bedrock aquifers evaluated several potential biostimulants (i.e., nutrients, vitamins, sterile groundwater). TCE degradation was evaluated using three methods of data analysis: analysis of covariance (ANCOVA) between biotic and abiotic treatment trend line slopes; calculation of biodegradation half-life; and effects screening by model fitting.

The procedures and methods developed in this study substantially enhance the ability to evaluate biotic fate of TCE in fractured rock aquifers, providing an effective approach for remedial design at low to moderate cost.

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CHAPTER II

LITERATURE REVIEW ON MICROCOSMS TO ASSESS THE TRICHLOROETHENE BIODEGRADATION RATE IN BEDROCK AQUIFERS

TCE is an organic solvent used by industry and the military that has been linked to significant human health effects when it is a groundwater contaminant (Moran, 2006). While microbial dechlorination of TCE contaminated groundwater has been directly demonstrated in several environments, it has only been indirectly observed in bedrock aquifers (i.e., through geochemical assessment or gene identification evidence) (Yager et al., 1997; Lenczewski et al., 2003; Lehman et al., 2004). The rate of dechlorination impacts the evaluation of human health risk at sites with TCE contamination (Pope et al., 2004). Microcosms consisting of groundwater placed into airtight glass bottles have become a highly effective tool for demonstrating the potential for *in situ* biodegradation, assessing the efficacy of amendments, and estimating degradation rates (Weidemeier et al., 1998).

TCE as a Pollutant

TCE (C_2HCl_3) is a halogenated aliphatic compound used as an organic solvent for cleaning metal parts associated with aircraft and machinery maintenance, electronics manufacture, and plating. Because of its chemical structure, TCE is heavier than water (density of 1.46 g/mL @ 20° C), with relatively low aqueous solubility (1100 mg/L @ 25° C) (CRC, 1999). Hence, when released into the environment, TCE often sinks below

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the source of the release through the geologic strata, driven by gravity, coming to rest as a dense, non-aqueous phase liquid (DNAPL) when contacting low permeability layers of soil or rock in areas with no hydraulic discontinuities (Figure 2). TCE will slowly dissolve from the DNAPL into the surrounding groundwater to create a contaminant plume (Parker et al., 1994).

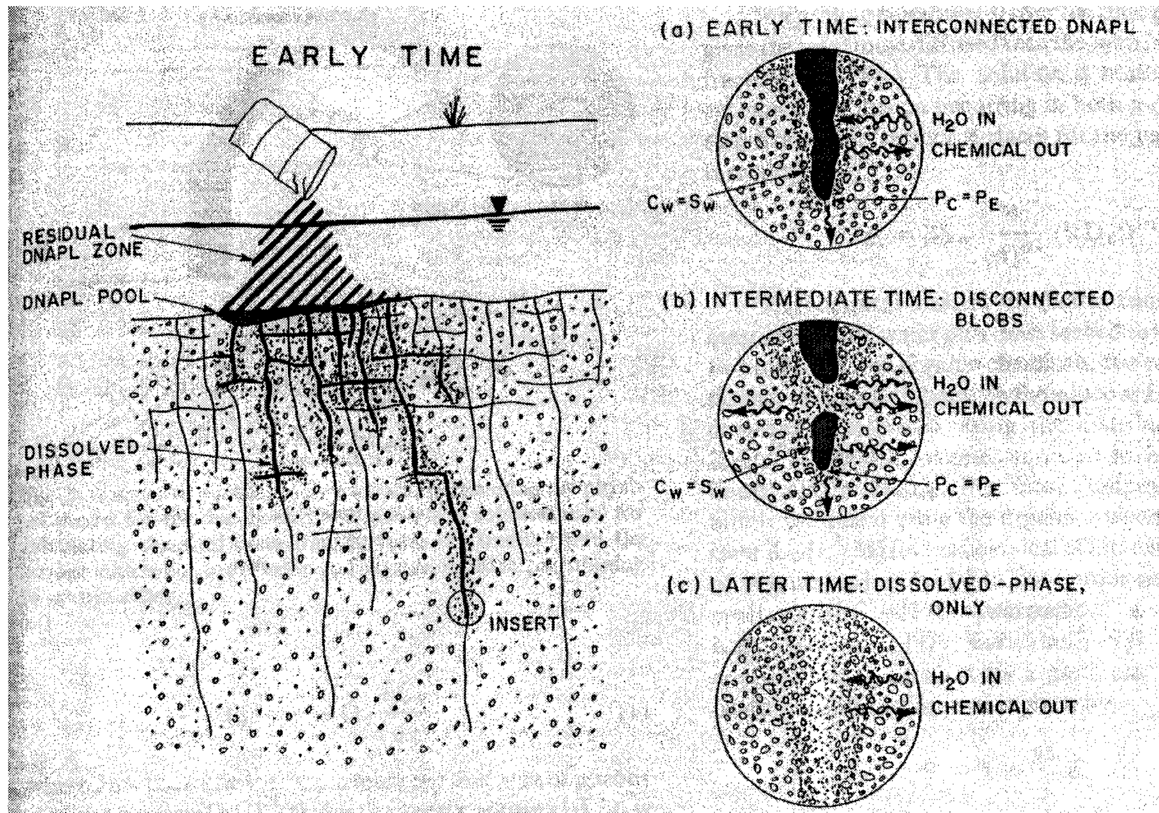

Figure 1. Conceptual model for DNAPL distribution in fractured rock aquifers (from Parker et al., 1994).

The characteristics of TCE that make it a good solvent also make it harmful to human health. It is associated with toxicity and cancer of the liver and kidneys, as well as reproductive and developmental toxicity and perhaps neurotoxicity (National Academy of Sciences, 2006). TCE can be ingested or inhaled when the contaminated groundwater is used for drinking and bathing. USEPA (2006) and most states have

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adopted a MCL for TCE in groundwater of 5 ug/L, a value about 1 / 220,000 of its aqueous solubility. While air stripping or activated carbon adsorption can remove TCE from water, such treatment is costly, inconvenient and requiring frequent maintenance; all of which can be substantial burdens to most homeowners or small community plant operators. Fortunately, microbially-mediated treatment of TCE in groundwater can occur, under certain conditions, that lead to reductions in concentrations and protection of human health (Pope et al., 2004).

While chloroethene degrading microorganisms are now a recognized part of the biogeochemistry of chlorinated compounds, prior to 1980 the consensus view was that these compounds had no natural homolog and that biodegradation was impossible (Bradley, 2003). In addition, it was accepted that groundwater was sterile, in spite of previous discovery and documentation of microbial activity related to subsurface petroleum deposits (Chapelle, 2001). The period 1967 to 1981 provided several high profile demonstrations of microbial activity after marine oil spills, including the T/V Torrey Canyon and T/V Exxon Valdez accidents, in which pollutant mass was reduced with limited human intervention (Atlas and Bartha, 1998). Documentation of this beneficial response led to a growing awareness that microbes could degrade organic contaminants. However, chloroethenes were thought to be highly recalcitrant, because of: (1) the occurrence and persistence of chloroethene compounds in groundwater at former release sites (e.g., Love Canal, Niagara Falls, NY) where the release was shown to have ceased 30 years prior to the evaluation (Bradley, 2003); and (2) the mistaken belief that organochlorine compounds were strictly anthropogenic and an indigenous degrading population could not have evolved.

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Bouwer and McCarty (1983), Vogel and McCarty (1985) and Vogel et al. (1987) were among the first to report that TCE can be microbially-degraded in the environment to DCE via reductive dechlorination, a process in which chlorine is replaced by a hydrogen. Sequential dechlorination from tetrachloroethene (perchloroethene, PCE) to TCE to DCE to VC to ethene can be observed in certain situations where the kinetics are favorable (He et al., 2002) (Table 1). The Gibbs free energy (ΔG) is the amount of useful energy liberated or used during a reaction, with energy consuming (anabolic) reactions having $+\Delta G$ and energy releasing (catabolic) reactions having $-\Delta G$ (Chapelle, 2001). The energy released by catabolic reactions supports anabolic reactions that allow a cell to grow and function.

Bouwer (1994), Chapelle (1996), Gossett and Zinder (1996), McCarty (1996), McCarty and Semprini (1994), and Vogel (1994) have shown that microbial reductive dechlorination is ubiquitous in anaerobic, TCE contaminated aquifers. However, the extent of microbial reductive dechlorination is highly variable (Fennell et al., 2001; Hendrickson et al., 2002) based on the presence of 16S ribosomal DNA from *Dehalococcoides*, one of the few microbes with a demonstrated ability to reductively dechlorinate, in samples from chloroethene-dechlorinating sites analyzed with polymerase chain reaction (PCR).

The original reductive dechlorination discovered was an anaerobic co-metabolism resulting from accidental interaction of chloroethenes with enzymes produced by microorganisms for other metabolic purposes (McCarty and Semprini, 1994). Reductive dechlorination (chlororespiration) was subsequently discovered (Mohn and Tiedje, 1992),

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as an energy conserving, respiratory process in which the chlorinated compounds are used as terminal electron acceptors (TEAs) and hydrogen is the electron donor (Table 1).

Table 1. Microbial reactions and the corresponding Gibbs free energy changes (He et al., 2002).

Microbial Reactions	Gibbs Free Energy, ΔG (kJ) *
$C_2Cl_4 + H_2 \rightarrow C_2HCl_3 + H^+ + Cl^-$ (PCE) (TCE)	-191.48
$C_2HCl_3 + H_2 \rightarrow C_2H_2Cl_2 + H^+ + Cl^-$ (TCE) (DCE)	-188.91
$C_2H2Cl_2 + H_2 \rightarrow C_2H_3Cl + H^+ + Cl^-$ (DCE) (VC)	-175.43
$C_2H_3Cl + H_2 \rightarrow C_2H_4 + H^+ + Cl^-$ (VC) (ethene)	-183.36
$C_3H_5O_3^- + 2H_2O \rightarrow C_2H_3O_2^- + HCO_3^- + H^+ + 2H_2$ (lactate) (acetate) (carbonate)	-109.52
$C_2H_3O_2^- + 4H_2O \rightarrow 2HCO_3^- + H^+ + 4H_2$ (acetate) (carbonate)	-49.57
$HCO_3^- + H^+ + 4H_2 \rightarrow CH_4 + 3H_2O$ (carbonate) (methane)	+9.88
$2HCO_3^- + H^+ + 4H_2 \rightarrow C_2H_3O_2^- + 4H_2O$ (carbonate) (acetate)	+49.57

* At 25°C and pH 7.0, with 1 mM of organic substrates, 30 mM of HCO_3^-, 1000 ppmv of CH_4, 10 ppmv of H_2, 1 mM chloride, 5 ppmv ethene, and all chloroethenes at 5 mg/L.

While reductive dechlorination can occur through the action of a single microbial species, it now appears that it is generally the result of a consortium of microorganisms involving the sequential production of less chlorinated progeny (Bradley, 2003). He et al. (2002) describe a consortium that includes chlororespiration of TCE by at least two separate populations of dehalogenating bacteria (DHB), identified by their use of either

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H_2 or acetate (CH_3COO^-) as direct electron donors, of which *Dehalococcoides* is a major genus. Gribble (1992), Asplund and Grimvall (1991), Gribble (1994), Grimvall (1995) and Keppler et al. (2002) have documented that organochlorine compounds have been common in nature for thousands of years. When coupled with the energetic potential of chlorinated compounds, the likelihood of natural selection and adaptation creating microbial mechanisms for organochlorine degradation and transformation is compelling (Bradley, 2003).

Microbially-mediated reductive dechlorination appears to be controlled by several important factors: the presence of a suitable chlororespiring microbial population or consortium; the redox character of the chloroethene contaminant; the presence of alternate and potentially competing TEAs; the reduction kinetics of the individual chloroethene compounds; the electron donor supply; and the presence of specific inhibitory compounds (Bradley, 2003). Reductive dechlorination, through chlororespiration and consumption of H_2 by DHB, is in competition with the growth of other organisms that also use H_2, including sulfate reducing bacteria (SRB) and methanogenic bacteria (MB); although DHB may have a competitive advantage over SRB at low concentrations of H_2 (Fennell and Gossett, 1998; He et al., 2002). Hoelen and Reinhard (2004) demonstrated DHB are able to use H_2 at lower concentrations than SRB or MB, although the complete degradation of TCE to ethene proceeds slowly over several years.

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Application of Microcosms

Microcosms, artificial assemblies of natural components in containers isolated from the environment, provide an opportunity to study microbial processes in a laboratory setting. Microcosms have been proposed for three purposes related to microbially-mediated reductive dechlorination: (1) evaluating *in situ* biodegradation capability (Weidemeier et al., 1998); (2) assessing effects of potential amendments (Morse et al., 1998); and (3) estimating degradation rates (Newell et al., 2002).

In situ capability must be demonstrated for selection of a monitored natural attenuation (MNA) remedial option under USEPA's Office of Solid Waste and Emergency Response Directive 9200.4, typically using a "lines of evidence" approach (Weidemeier et al., 1998). Four potential lines of evidence supporting MNA should be demonstrated for chloroethene release sites: (1) existing geochemical conditions conducive to known degradation mechanics; (2) distinct trends toward decreasing chloroethene concentrations; (3) progeny (i.e., breakdown products) formation indicative of chloroethene biotransformation; and (4) laboratory data supporting the hypothesis that there is an indigenous microbial community degrading chloroethenes. For many sites, chloroethene disappearance coupled with the production and accumulation of progeny is sufficient evidence and microcosms are optional, as they can be resource intensive (Bradley, 2003). However, where other lines of evidence are nebulous or contradictory (e.g., less than favorable geochemistry, no progeny observed), microcosms can provide a vital indicator of *in situ* biodegradation capability.

Microcosm studies can provide qualitative, and potentially quantitative, assessment of the effects that potential amendments may produce *in situ* (i.e., enhanced

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anaerobic bioremediation) (Morse et al., 1998). Uniform conditions are created in microcosms similarly prepared, across which factors can be tested. Relative effects are demonstrated through comparisons and amendments can be optimized. Microcosm studies for potential amendments are particularly useful given the apparent heterogeneity between microbial populations at chloroethene release sites (Hendrickson et al., 2002), and the difficulty in optimizing the factors controlling TCE degradation at a particular site.

Degradation rates can be estimated from microcosms by measuring the decrease in contaminant concentrations and/or the increase in $^{14}CO_2$, if radiolabeled compounds are used (Chapelle et al., 1996). Microcosms are useful in that they can mimic a wide range of hydrologic systems, the results can be obtained in time frames of weeks or months, and abiotic controls can be used. Degradation rates must be interpreted with consideration of variability from experimental sources as well as uncertainties introduced due to media (e.g., sediment, rock) disturbance and the difficulty of reproducing *in situ* conditions (Chapelle et al., 2003).

Variability in TCE degradation rates has significant effect on the estimates of human risk and subsequent selection of a remedial action. TCE microbial degradation half-lives ($t_{1/2}$) range between 80 and 800 days (ESTCP, 2001; Aziz et al., 2002) for temperatures between 20 and 25° C. Assuming an apparent groundwater velocity of 0.3 m/day (typical of medium sand; Cedergren, 1989) and requiring degradation of TCE from the maximum solubility (C_{max}, 1100 mg/L) to the allowable MCL (C_{MCL}, 5 ug/L), the safe distance away from the TCE release point for groundwater use is between 430 to 4300 m, if microbial degradation is the sole attenuation mechanism:

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$$\text{safe distance} = t_{1/2} \left[\ln \left(C_{max} / C_{MCL}\right)\right] (\text{travel velocity}) / \ln 2 \qquad \text{(Eq. 1)}$$

Microcosm Development

Microcosm development began with the work of Dr. Otto Warburg, the 1931 Nobel Prize winner in medicine, who developed techniques of respirometry to measure oxygen uptake in living tissue. Warburg enclosed experimental units in glass flasks that were sealed from the atmosphere. The Warburg respirometer can measure gas generation and provide samples for compositional analyses. During the decades that followed Warburg's initial development, respirometry has been used in many other experimental applications for the fields of biochemistry, medicine, botany and microbiology; wherever there is a need to isolate the experimental process from the natural atmosphere (*Encyclopædia Britannica,* 2006).

Pritchard and Bourquin (1984) broadly define a microcosm as a laboratory test where the natural physical integrity of the field sample is maximized and isolation from the other ecosystem components is minimized. They proposed microcosms for determination of ecological toxicology and assessment of biodegradation. While the construction of microcosms varies depending on their purpose, they can address five overarching concerns: (1) fear of oversimplification, in that single species or pure culture experiments may not incorporate effects due to nutrient cycling, species interaction, or variations in functional characteristics (e.g., pH, DO, redox potential, organic content); (2) problems associated with field studies of pollutants or xenobiotics; (3) influence of natural variations caused by seasonal and climatic events that are beyond control; (4) advantage of small size that permits replication, simplified dosing mechanics, control

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over imports and exports from the system, and adequate mixing; and (5) opportunity to vary environmental conditions to simulate a large array of perturbations.

Although microcosms that differ greatly from *in situ* conditions have little predictive value, quantitative extrapolation to the field is possible if the controlling variables that affect the fate of the compound are identified and assessed within or between microcosms (Pritchard and Bourquin, 1984). Specifically, scaling a microcosm study from the laboratory to the field requires demonstration of four key aspects for the results: (1) demonstration of interactions (i.e., single factor effects have been isolated); (2) limited decoupling of ecosystem biogeochemistry and species interactions of the natural system; (3) minimal effects of artificial surface enhancement (e.g., wall or other boundary effects); and (4) acknowledgment of limitations of microcosm rates in specialized portions or zones within an ecosystem (e.g., within contaminant free phase or at the intersection of the contaminant plume and pristine conditions).

A good microcosm should incorporate biological and physical factors (e.g., mixing, turbulence, light intensity, quality). Consideration must be given to whether the microcosms are open to outside atmospheric inputs or closed. Other factors to be considered include: the microcosm volume, container materials, inoculation, incubation prior to inoculation or amendment, temperature, duration, and chemical and biological monitoring. Pritchard and Bourquin (1984) note that increased microcosm volume and incubation prior to inoculation decrease variation in factors of nutrient cycling and in functional properties of the microcosm (e.g., pH, DO, dissolved organic carbon (DOC), conductivity).

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Qualitative microcosms screen for general trends, emphasizing consistency of methods to support remedial decisions. A screening microcosm must possess a degree of reproducibility and repeatability that will allow microbial responses to be distinguished from other processes.

Quantitative microcosms develop a statistically significant relationship or model between factors and effects. A good fit of the microcosm data to the model output suggests model assumptions were correct, without missing considerations of subtle interactions or synergisms. Quantitative microcosm results can serve as a conceptual basis for the extrapolation of trophic interactions, population dynamics and nutrient cycles to field situations. Confidence will increase with experience and, where possible, with comparisons to actual field dosing experiments and data.

Madsen (1991) described the main purpose of microcosms as supporting proof of *in situ* biodegradation activity. He noted that biotic and abiotic microcosms should be prepared in parallel and compared, to distinguish biologically-mediated and abiotic transformations. The decrease in contaminants observed in field conditions should be greater than the microcosm abiotic rates to confirm indigenous activity. Biotic activity, for pristine and contaminated conditions, should be assessed in microcosms to demonstrate ecological adaptation. Madsen (1991) warned that mass balance calculations of biodegradation using intermediary metabolites have the potential for error, since the knowledge of microbial metabolism and abiotic reactions is sometimes ambiguous.

Madsen (1991) also noted that microcosms are suggestive studies, as laboratory conditions are unlikely to replicate the intricate balance of nutritional, physical, and

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biological relationships that can change rapidly in response to *in situ* factors (e.g., DO, water, pH, nutrients). However, the convergence of several lines of evidence developed from laboratory microcosms and field measurements can provide a convincing argument that *in situ* biodegradation is occurring.

Issues of scale from laboratory microcosms to field applications for *in situ* bioremediation were developed by Sturman et al. (1995) in a review of the technical literature. Microcosms are described as either micro or meso scale experiments, depending upon whether the experiment includes trophic interactions (e.g., predation, commensalism, synergy). Reaction kinetics controlling bioremediation occur at the micro scale. Partitioning processes, including sorption and interphase transport, dominate at the meso scale, although the relative population size and growth dynamics of attached versus planktonic bacteria may also contribute. Interaction between interphase transport and microbial colonization may be significant, if contaminants or nutrients are available in micro pores of the mineral structure which are too small to be colonized by microbial populations, or if microbial colonization influences nutrient flux.

Sturman et al. (1995) described the macro scale as generally controlled by the flow related processes of advection and dispersion along with the effect of field heterogeneity. Field heterogeneity may be spatial (porosity or permeability), mineralogical, physiochemical (e.g., localized redox zones), biological (e.g., biotic plugging from gas bubbles, biomass accumulation), or biochemical (e.g., microbially-mediated chemical precipitation of nutrient byproducts within pores). Isolation of environmental samples within a laboratory experiment may cause changes in population characteristics as selective pressures on consortia symbiosis are altered.

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Weidemeier et al. (1998) described microcosm studies as a common technique to explicitly show that microorganisms capable of degrading contaminants are present *in situ*. Microcosms are useful for development of additional evidence beyond contaminant and geochemical data when site specific aquifer materials and contaminants are studied. Results of such studies are strongly influenced by the nature of the geologic material used, the physical properties of the microcosm, the sampling strategy, and the incubation period. If properly designed, implemented and interpreted, microcosm studies can provide very convincing documentation of the occurrence of biodegradation. Weidemeier et al. caution, however, that because of the time and costs required, microcosm studies should be undertaken only at sites where there is considerable uncertainty concerning the biodegradation of contaminants. Further, biodegradation rate constants determined by microcosm studies often are higher than rates achieved in the field because of laboratory conditions (e.g., warmer temperatures, increased mixing, higher concentrations, inoculation into sterile conditions), and therefore must be interpreted with caution.

Parsons Corp. (2004) suggests that microcosms constructed using site soil and groundwater coupled with molecular identification techniques can be useful in determining whether biodegradation will be successful at a site. They note that microcosm studies provide information on the potential for indigenous microbial populations to degrade contaminants to acceptable end products. However, the artificial conditions under which microcosms are conducted means their results may not be indicative of what will be accomplished in the field. Further, the benefits associated with microcosms may not outweigh the costs of performing them when existing field

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biogeochemical data are favorable. However, they argue that when site selection indicators are marginal or questionable, microcosms can provide useful information for determination of *in situ* biodegradation potential.

Minimum requirements for a useable microcosm study are: (1) use of representative site soil and/or groundwater samples collected by reasonably aseptic and anaerobic collection procedures; (2) use of appropriate concentrations of contaminant and substrate; (3) analysis of substrate and contaminant data over time, including progeny, intermediates (e.g., volatile fatty acids), and H_2; (4) use of relevant temperatures, media formulations, and controls; and (5) sufficient time for microbial acclimation and growth (6 months minimum) (Parsons Corp., 2004).

Further, the confidence in extrapolating microcosm results to the field increases when the tests are performed using aquifer material from several promising locations at a site with incubation at field temperatures. Microcosms may be capable of answering several questions regarding biodegradation at a site: (1) Are indigenous microbial populations capable of biodegradation? (2) What are the primary biodegradation pathways used by the indigenous microbial populations for differing substrates (e.g., acetate, lactate, butyrate, and H_2)? (3) Will an acclimation period occur prior to the onset of biodegradation? (4) Can non-indigenous (but natural) species enhance the biodegradation rate, and if so, which ones are the optimum for the site conditions? (5) Are there toxicity or inhibitory effects on biodegradation related to very high contaminant concentrations?

Microcosms are generally unable to answer the following questions (Parsons Corp., 2004): (1) What is the rate of dechlorination in the field? (2) What is the

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efficiency of substrate use that will occur in the field? (3) What is the acclimation period in the field? (4) Are there any secondary effects that will occur in the field during biodegradation, (e.g., increased contaminant dissolution, transport)?

According to Parsons Corp., the primary disadvantage of microcosms is that they may differ from exact simulations of subsurface conditions. Microcosm testing must also address the heterogeneous distribution of microbial populations in natural aquifer systems. Nonetheless, Parsons Corp. supports microcosms as an effective method for determining the potential for complete dechlorination when existing data are insufficient to support remedial decisions. This is often true in bedrock aquifers where data on *in situ* conditions is limited by the cost of drilling boreholes and the complex hydrogeology.

Anaerobic Bottle Microcosms

Microcosms to assess anaerobic biodegradation were developed by Owen et al. (1979), who used assay techniques described in McCarty et al. (1963) and Wolin et al. (1963), combined with the serum bottle culturing method of Miller and Wolin (1974).

McCarty et al. (1963) and Wolin et al. (1963), working in parallel on different aspects of microbiology, extended Warburg respirometry through techniques of vacuum evacuation of the natural atmosphere and pressure flooding with anaerobic growth gases. Wolin et al. (1963) applied this technique to evaluation of methanogenesis by bacterial extracts in experiments that used a side arm chamber to hold amendments during evacuation and flushing prior to combination with the material of interest (Figure 3). Parameters investigated included atmospheric constituent proportions, amendment

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concentration, and pH. McCarty et al. (1963) used the technique to study changes in volatile fatty acids during anaerobic digestion of aqueous wastes.

Owen et al. (1979) recognized the need for a simple and inexpensive procedure for bioassays, in which biodegradation and possible toxicity of constituents in anaerobic treatment processes could be monitored. They described the limitations of the Warburg respirometer: (1) the technique is costly and requires some degree of skill to operate; (2) the instrument is limited in the number of samples that can be incubated at one time; (3) sample volume is limited, hindering the number of analyses that can be performed on

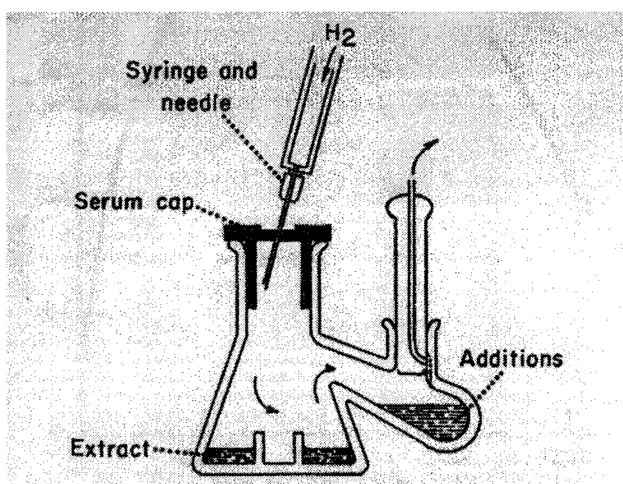

Figure 2. Warburg flask fitted with a rubber serum cap as used by Wolin et al. (1963).

the samples; (4) it is difficult to sample the gas and liquid phases during the assay; and (5) extended incubation times are impractical and produce inconsistent results.

Owen et al. (1979) replaced Warburg respirometry with serum bottle incubation techniques, developed by Miller and Wolin (1973) for cultivation of anaerobes. In their technique, media are prepared under nonsterile conditions in 5 to 1000 mL serum bottles closed with butyl rubber stoppers that are held tight with crimped metal seals. The serum

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bottles are composed of borosilicate glass and have a shape that minimizes the neck diameter above the whole body volume of the bottle. The bottles are purged with oxygen-free gases during sealing, then autoclaved to create sterility. Inoculation is done with a sterile syringe that has a 25-gauge or finer needle. Pressure measurements and sampling are performed using sterile syringes to penetrate the rubber seal and access the bottle contents. [They used a nominal bottle size of 250 mL (measured mean volume of 264 ± 1 mL) filled with 160 mL of media, although some experiments changed these volumes.] Gas production and composition are measured from the bottle headspace and used to indicate biodegradability. By using the serum bottle technique, they demonstrated relatively rapid and repeatable methods for anaerobic bioassays that offered flexibility for screening and in depth evaluation of constituents.

Shelton and Tiedje (1984) applied the Owen et al. serum bottle technique to determination of anaerobic biodegradation potential for over 100 chemicals in wastewater treatment. They suggested standardized methods including: use of 160 mL serum bottles with 100 mL liquid volume; inoculation with a 10% solution of primary anaerobic sludge in water; incubation for at least 2 months; weekly measurement of gas production; an initial compound concentration of 50 mg C/L; and triplicate unamended preparations for comparison. They evaluated two mineral and metal nutrient media and found differences to be modest, then suggested that the sludge inocula likely had sufficient mineral and metal nutrients for biodegradation. They recommended that use of prepared nutrient media would eliminate possible growth limitations.

Further, they evaluated the variability and reproducibility of the serum bottle technique in an application for anaerobic biodegradation potential. Relative standard

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deviations (RSDs) of triplicate preparations were generally less than 10%; they suggested that 15% be considered the maximum allowable variation. Results were reproducible with little difficulty for sludge inocula taken over a two year period. Preparations in which the seal leaked and allowed oxygen intrusion showed a reduction in CH_4 and CO_2 production as a result of aerobic respiratory activity using the organic substrates.

Boethling & Alexander (1979), working with aerobic cultures, found that concentrations of 2,4-dichlorophenoxyacetate (2,4-D) and 1-napththyl-N-methylcarbamate (Sevin) had a significant effect on the rate and amount of organic compound biodegradation in microcosms. In their experiments, the trends were inconclusive since increases in concentration from 2.2 to 22,000 ug/L caused both increased and decreased biodegradation, depending upon the compound; the effect was significant in all cases. They hypothesized that the cause was either a mass transfer and distribution effect, or a population effect in which a minimum threshold concentration is required to sustain growth of the active microbes relative to other populations. They concluded that the use of concentrations in microcosm experiments that are higher than those found *in situ* can lead to results that will not be representative of the field (emphasis added).

After the publication of Shelton and Tiedje (1984), the serum bottle technique for anaerobic biodegradation was applied to wastewaters containing organic compounds by Parsons et al. (1984), Battersby and Wilson (1988), and Birch et al. (1989). The serum-bottle microcosm procedures have been standardized as ASTM Standard E2170-01 (2001).

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Gibson and Sulfita (1986) applied the serum bottle technique to evaluate biodegradation in groundwater aquifers, analyzing 20 different chlorinated and non-chlorinated phenol, benzoate, and phenoxyacetate compounds. Working in an anaerobic glovebox, they placed 100 g wet weight of sediment or soil in a 160 mL serum bottle. Solids had been collected in sterile mason jars from four different habitats: the benthic zone of a pond; a 1 to 1.8 m deep test pit adjacent to a municipal landfill at a location where CH_4 emission was observed; a second test pit, similar to the first, but at a location where CH_4 emission was not observed; and primary anaerobic digester sludge. No other inocula were used. Bottles were capped with 1 cm thick butyl rubber septa, held in place with aluminum crimp seals. After capping, bottles were flushed with a mixture of 90% N_2 – 10% CO_2, while 50 mL of filter sterilized groundwater was added to reach a final volume of 100 mL. In addition, each bottle received 1 mM of Na_2S as an oxygen reductant and 0.0002% resazurin as a redox indicator. Bottles were incubated at room temperature (37° C for the digester sludge) in the dark. Experiments were performed in duplicate, with autoclaved samples as controls. Substantial differences in biodegradation were noted as a function of the inoculum, suggesting location-specific ecological differences for biodegradation.

Gibson and Sulfita's (1986) method has been used by a number of researchers for microscale studies of microbial respiration and nutrition including: Wilson et al. (1986, 1990); Tandoi et al. (1994); Fennell and Gossett (1998); Loeffler et al. (1999); Fennell et al. (2001); He et al. (2003); Griffen et al. (2004); and Shen and Sewell (2005). Other researchers have reduced the microcosm size to 20 mL, but kept the liquid and headspace ratios generally constant (e.g., Gibson and Sewell, 1992; Bradley and Chapelle, 1999; He

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et al., 2002). An intermediate size of 120 mL has been used by Aulenta et al. (2002) and Heimann and Jakobsen (2006). Variations in method among these studies have included liquid volume, solid to liquid ratios, sampling method, headspace gas replacement, nutrient or substrate replenishment, incubation temperature, incubation atmosphere, and degree of mixing. In general, if a microcosm is to be sampled repetitively, headspace samples are taken, whereas if the microcosm is to be sacrificed, the samples are liquid.

Yagi et al. (1992) measured PCE and TCE degradation rates for three soil environments, and assessed the effects of initial contaminant concentration and incubation temperature. Microcosms for this study were constructed in 68 mL serum bottles capped with Teflon® septa, to which 5 g dry weight of soil and distilled water were added until a final slurry volume of 50 mL was obtained. Sterile controls were made by autoclaving soil and water prior to assembly. All preparations were made in duplicate. Different biodegradation rates were measured for each soil source, though these were not correlated to organic content. This agreed with the findings of Gibson and Sulfita (1986) that *in situ* microbial degradation is location specific.

Incubation temperature is a significant factor for biodegradation rate, with the half-life for TCE decreasing from 44 to 18 to 8 days at 10° C, 20° C and 30° C, respectively, a behavior that is similar to the doubling in growth rate observed for a temperature increase of approximately 10° C (Metcalf and Eddy, 2003). Similar results were shown for PCE, although the half-lives were about 15% shorter for each temperature, perhaps reflecting PCE's higher energy potential. Initial concentration (C_0) is also a significant factor for the degradation rate, as PCE and TCE half-lives increased by a factor of two and three, respectively, with a ten-fold increase in C_0. Interestingly,

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the mass of chlorinated solvent degraded increased with increasing C_0, but the rate decreased, suggesting mass transfer effects (e.g., increased sorbed compound) or a modest Haldane (microbial toxicity) effect occur.

Temperature effects on TCE, cDCE and VC degradation were investigated by Bradley et al. (2005) in 10 mL microcosms inoculated with 10 g saturated sediment from cold temperature-adapted sediments obtained at two locations within TCE plumes: aquifer and river sediments from Soldotna, Alaska; and aquifer sediments taken from Fairbanks, Alaska. The annual mean groundwater temperature was 5 to 7° C and 3 to 5° C at the Soldotna and the Fairbanks sites, respectively. Sediments at the Fairbanks site were also obtained from a pristine area. Biodegradation of radiolabeled TCE, cDCE and VC was measured in microcosms after incubation at 4° C for up to 200 days. Substantial biodegradation of TCE (>80% dechlorination, $t_{1/2} = 81$ d) was observed for the aquifer sediments from the Soldotna site, which had redox characteristics favorable to dechlorination. Limited or no TCE biodegradation was observed for the Soldotna River or Fairbanks aquifer sediments that both had relatively oxidized character. They concluded that the presence of psychrotolerant and psychrophilic microorganisms can support significant TCE biodegradation at < 5° C, if the redox conditions are favorable for reductive dechlorination.

Bradley and Landmeyer (2006) investigated temperature effects on biodegradation of methyl tert-butyl ether (MtBE) in shallow groundwater aquifers that experience annual temperatures < 10° C and are thought to host psychrophilic and psychrotolerant microbial communities. Biodegradation of radiolabeled MtBE was measured in 10 mL microcosms inoculated with 5 to 6 g of saturated bed sediment

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incubated at 4° C, 14° C, 24° C, and 34° C. Sediment was obtained from sites in northern Kansas and northwestern Montana where the annual range of *in situ* groundwater temperature ranged from 7 to 18° C and 5 to 14.5° C, respectively. For both sediment sources, biodegradation was most efficient at 24° C, well above the maximum *in situ* groundwater temperatures and in the range of psychrotolerant and mesophilic microbial activities. The sediment with the higher *in situ* groundwater temperature demonstrated more MtBE biodegradation and a more pronounced temperature inhibition. MtBE biodegradation occurred at incubation temperatures of 4° C in amounts approximately 20% and 65% of the maximum for the Kansan and Montanan sediments, respectively, suggesting that less temperature inhibition may be experienced by microbial communities with greater psychrotolerance.

Hutchins (1997) evaluated the effect of solid:liquid ratio within a serum bottle microcosm in an experiment determining the biodegradation of toluene in groundwater. Aquifer sediment was placed in 12 mL serum bottles at wet weights ranging from 1 to 15 g, and the bottles filled with groundwater to zero headspace. These preparations corresponded to solid:liquid ratios ranging from 0.039:1 to 1.9:1. Little difference was observed in the biodegradation rate or final mass degraded. Additionally, the effect of C_0 of the toluene was evaluated using a thousand-fold variation, and increased the biodegradation half-life by a factor of two, a minor shift in light of the large range of experimental conditions. Hutchins suggested that the rate of degradation was only limited by the bioavailability of toluene (i.e., that sorbed to aquifer solids within the microcosms). These results suggest that microbial growth in microcosms (which controls degradation) is relatively insensitive to proportional changes in initial conditions.

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Wilson et al. (1996) suggested standardization of microcosm construction and interpretation methods for evaluation of biodegradation of chlorinated solvents in groundwater. Many of their recommendations (Table 2) concern two particular susceptibilities of anaerobic microcosms for chlorinated solvent contamination in groundwater: maintenance of anaerobic conditions to protect oxygen-sensitive microbes, and prevention of inadvertent substrate loss due to the high volatility of the contaminant. They suggested microcosms be constructed with zero headspace to limit the potential for diffusion of substrate compounds through the septum caps. Weidemeier et al. (1998) reference the methods of Wilson et al. (1996), and include their text in an appendix (although unattributed). Because Wilson et al. were all employees of USEPA at the time of publication, and Weidemeier et al. (1998) was published by USEPA, their combined work is often termed "The EPA Microcosm Method".

Alternatively, Morse et al. (1998) presented a method specific to reductive anaerobic biological *in situ* treatment technology (RABITT) for the remediation of chloroethenes. Their method used many of the techniques of Gibson and Sulfita (1986) with the evaluation results of Hutchins (1997): the use of a solid:liquid ratio of 1:1; inoculum by inclusion of unconsolidated material (sediments) collected from the aquifer to mimic the indigenous ecology; and headspace sampling. Because the research represented in Morse et al. was funded and promoted by the U.S. Air Force Center for Environmental Excellence (AFCEE), their recommended method is often termed "The Air Force (or AFCEE) Microcosm Method" (Table 2).

Morse et al. (1998), in the Air Force microcosm method, generally use higher contaminant concentration, incubation at laboratory ambient temperatures, analysis of

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Table 2: Comparison of Anaerobic Chloroethene Microcosm Methods.		
Method Name	**EPA Microcosm Method**	**Air Force Microcosm Method**
Reference	Wilson et al. (1996)	Morse et al. (1998)
Purpose	Estimate rate constants for site specific transport and fate models.	Evaluate amendments for site specific treatability enhancement.
Microcosm Size, Container and Closure	Size and container not specified, closure using Teflon lined butyl rubber septa, attachment method not specified.	160 mL serum bottles closed with Teflon lined butyl rubber septa and aluminum crimp caps.
Contents	*In situ* groundwater and aquifer material, collected in manner that excludes air, cooled and stored in the dark. Includes sufficient aquifer material to achieve same solid:liquid ratio as *in situ.*	50 g dry (drained but not dried) weight soil from aquifer location of interest, collected in manner that avoids exposure to air and placed on ice for storage and transport. 50 mL groundwater from aquifer location of interest, collected without volatilization or aeration, stored and transported at temperatures less than 35° C.
Preparation	Bottles filled to minimal headspace. Anaerobic glovebox, but strict aseptic techniques not necessary.	Headspace of ~90 mL, flushed with anoxic gas composed of 30% CO_2 and 70% N_2, scrubbed of O_2. Gaseous overpressure at 0 to 0.5 atm to prevent vacuum.
Initial Concentration (C_0)	Similar to *in situ* concentration representative of higher values in geochemical zone of interest. Aqueous solution of spiking chemical preferred. No alcohol or other carrier that can be metabolized under anaerobic conditions.	30 uM or about 5 mg/L, high enough for analytical ease, but without causing toxicity. Methanol or ethanol carrier for spike solution 100 to 1000 times desired final concentration.

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Table 2: Comparison of Anaerobic Chloroethene Microcosm Methods, continued.		
Method Name	**EPA Microcosm Method**	**Air Force Microcosm Method**
Nutrients and Buffers	None specified.	Yeast extract (containing wide array of nutrients) at three levels: 0, 20 and 200 mg/L. Vitamin B_{12} at two levels: 0 and 50 mg/L. $NaHCO_3$ buffer if alkalinity less than 0.05 eq/L. Resazurin at < 1 mg/L for redox indicator (colorless indicator at E_H < -110 mV and pink/purple at higher values). pH between 6 and 8, otherwise adjust by changing CO_2 content of headspace flushing gas.
Replication	Triplicates for each sample round.	Triplicate for each treatment.
Abiotic Preparation	Autoclaved microcosm materials prior to assembly or a prepared microcosm prior to chloroethene spike for long term evaluation; addition of $HgCl_2$ for short term.	Autoclaved a prepared microcosm twice prior to chloroethene spike.
Amendments	None specified.	Lactate, butyrate and benzoate, typically.
Incubation	In dark, at *in situ* ambient temperature and under anaerobic atmosphere, preferably inverted.	At ambient laboratory temperature (20 to 25° C).
Sampling	Sacrificial – one bottle for one sample. Typically, bottles sacrificed at two month intervals for 12 to 18 months.	Repetitive – one bottle sampled weekly for six months or minimum of three depletion cycles.

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Table 2: Comparison of Anaerobic Chloroethene Microcosm Methods, continued.

Method Name	EPA Microcosm Method	Air Force Microcosm Method
Analyses	None specified, although suggested to conform to groundwater monitoring program.	Gaseous volume change. H_2 by thermal conductivity detector (TCD) and reduction gas detector (RCD) in series. CH_4, chlorinated compounds and ethenes by gas chromatograph (GC), with flame ionization detector (FID) and electron capture detector (ECD) in series. Volatile fatty acids by GC-FID. Lacate and benzoate by high performance liquid chromatography with ultraviolet detection (HPLC-UV). Microbial cultures by most probable number (MPN).
Evaluation	Graph log C (concentration) versus time. Linear regression and compare slopes of biotic and abiotic for significant difference. If significant, difference is rate of biodegradation.	Compare substrate concentrations to reactant products and develop mass balance. Assess donor fermentation and chloroethene reduction pathways and develop site specific models for contaminant fate and transport.

headspace, and repetitive analyses. Their method is more sensitive to the quality and composition of seals and potential leakage, while it is relatively insensitive to other factors (e.g., temperature, C_0, light, carrier compounds, *in situ* liquid:solid ratio) concerning rate, since it is primarily a screening test for high rate degradation to evaluate and optimize amendment strategies. An emphasis is also placed on measuring a wide range of parameters to characterize the degradation.

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Wilson et al. (1996), in the EPA microcosm method, suggest matching the *in situ* conditions of concentration and temperature, while using each microcosm as a one time experimental unit without repetitive sampling. The emphasis of this method is elucidating slower rates, being less susceptible to variations in technique or material, modeling *in situ* conditions most representative of existing conditions, but not necessarily with amendment or enhancement. They advise matching the microcosm analysis and groundwater monitoring efforts, which may be narrower than the program suggested by Morse et al..

Fennell et al. (2001) used the method of Morse et al. (1998) to evaluate DNA measurements by PCR analysis as a predictive indicator of indigenous reductive dechlorinating potential. The authors concluded that a combination of field data, microcosm studies, and real time PCR for *Dehalococcoides* provided complementary information about the potential for the indigenous microbial community to accomplish complete dechlorination via *in situ* electron donor addition. However, they acknowledged that the heterogeneous distribution of dechlorinating activity points to the potential weakness in using microcosms to predict responses at a given site. The time and expense for microcosm studies clearly dictate that the locations for testing must be carefully selected to represent the most current site data (e.g., plume location, groundwater flow direction, biogeochemical distribution, geologic strata).

Parsons (2004) interpreted the results of Fennell et al. (2001) as suggesting that microcosm studies for bioremediation candidate sites be as expansive as possible, and include collection of microcosm samples from a number of locations and/or compositing samples from multiple locations. However, expanding a microcosm study will increase

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costs to a point where they become equal to those of a small field pilot. A field scale pilot test may be more valuable because it evaluates a much larger and more representative volume of an aquifer, although it can only test one condition per location. Organisms that may be initially present in only a relatively small portion of the site may be able to grow during the pilot test, becoming more active and widely distributed after a substrate amendment is introduced.

The cost of a microcosm study is a function of: preparation, groundwater collection, microcosm assembly, incubation, sampling and analysis, and disposal. Preparation costs are impacted by procurement and sterilization of materials (e.g., bottles, caps, collection vessels) and cleaning and filling of facilities (e.g., anaerobic glovebox). Groundwater collection costs are set by the number of locations to be evaluated including well development; however, these costs can be minimized by collecting microcosm water at the same time as sampling for quarterly or annual monitoring. Microcosm assembly and incubation costs are directly related to the number of replicates, although thresholds will be encountered (e.g., quantity of microcosms that can be assembled within the limits of a working day by a single person or team, capacity of a single incubator) beyond which costs will escalate. Sampling and analysis costs are the most variable, depending on microcosm replicates, locations or amendments evaluated, parameters analyzed, detection limits, and quality control. Disposal costs can be substantial if $HgCl_2$ is used for abiotic control, or minimal if autoclaving is sufficient. Costs related to contingent actions (e.g., failed analyses, compromised microcosms) or other operational shortcomings must also be considered.

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Application to Fractured Rock

Fractured rock aquifers are a challenging environment in which to evaluate microbial activity because of mineral distribution, fracture spacing and orientation, matrix porosity, seasonal geochemical cycling, and heterogeneity. These factors affect microbial growth and distribution by influencing nutrition, habitat, and trophic interactions (e.g., predation, commensalism, synergy). Further, fractured rock sampling can be difficult and expensive, requiring specialized techniques (e.g., diamond core drilling, mud rotary coring, sonic drilling) to collect representative media, which can severely limit the number of locations sampled.

Eighmy et al. (2006) describe several key interactions between microbes and the environment for fractured rock aquifers, including: (1) the metabolic activity of microorganisms can affect the chemical and physical properties of the microenvironments in which they reside; (2) surfaces may very well confer numerous advantages to bacteria and can influence metabolic processes, particularly as microbes adhere where there may be nutritional benefit; (3) microbial populations that are attached may be compositionally different from those in fracture (pore) water (Lehman et al., 2001); and, (4) growth rates of microbes in the deep subsurface may be very slow, perhaps due to low concentrations of metabolites and nutrients.

Further, they note that the ability of microbes to colonize fracture surfaces is clearly constrained by the aperture size (pore throat width) of the microfracture network, hydraulic connectivity, advective or diffusive transport of groundwater, planktonic microbes, and entrained nutrients from near surface environs.

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The evaluation and modeling of microbial dechlorination in fractured rock aquifers requires that microcosms address effects from rock surfaces, formation minerals, host rock porosity, trophic interactions, nutritional cycles, and potentially slow rates of growth and metabolism.

Microcosms for Fractured Rock

Yager et al. (1997) evaluated TCE degradation in a bedrock aquifer near Niagara Falls in western New York. The release occurred during the 1950s and 1960s from an unlined evaporation pond penetrating through glacial tills and into the Lockport Group bedrock, a petroliferous dolomite containing gypsum, metal sulfides and trace amounts of naturally occurring bitumen. The effective porosity of the aquifer is ~3%. Groundwater flow in the vicinity of the site is dominated by interconnected fractures and vugs with a velocity calculated between 0.2 to 0.9 m/d. Chloroethene degradation was evaluated by preparing microcosms using 36 or 120 mL serum bottles with 28 or 52 mL, respectively, of a 1:1 mixture of groundwater and a nutrient solution. Yeast extract was added to each bottle for a final concentration of 200 mg/L. Five TEAs were added to model carbonate reduction (methanogenesis), sulfate reduction, iron reduction, manganese reduction and nitrate reduction, respectively. 10 umoles of TCE were added to each bottle to achieve C_0 ~0.2 uM, equivalent to the maximum site concentration. Abiotic controls were prepared using 2 mL of an acidic solution of 0.2 M $HgCl_2$. Bottles were incubated in an inverted position on a rotary shaker operating at 50 rpm in the dark at 22 ± 2 °C. Samples were taken from the headspace of the microcosms and analyzed by GC-FID for chlorinated ethenes, methane and ethene.

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They observed dechlorination of TCE to cDCE, VC and ethene only in microcosms prepared with groundwater from the contaminated portion of the site. Microcosms prepared with groundwater from the uncontaminated portion were inactive and exhibited no TCE dechlorination. They interpreted this behavior as adaptation, suggesting that growth of microorganisms able to dechlorinate TCE occurred because the dechlorination is a physiologically beneficial process. In addition, dechlorination was observed only in the carbonate reduction treatments (each bottle amended with $NaHCO_3$ and Na_2S to concentrations of 0.1% and 2 mM, respectively), and was substantially increased by 60 g/L sterile pulverized dolomite added during assembly. Yager et al. (1997) hypothesized a physiological effect related to the presence of the dolomite media (e.g., an electron donor contributed from the dolomite or traces of naturally occurring bitumen within the dolomite, the effect of additional surface area within the microcosm).

Hohnstock-Ashe et al. (2001), in a follow-up study to Yager et al. (1997), used the same materials and methods to further evaluate the dechlorination activity in the aquifer. Six electron donor materials were added to TCE spiked microcosms: yeast extract, lactate, H_2, hexadecane, sterile pulverized dolomite, and sterile pulverized dolomite that had been combusted at 500° C to remove organics. Triplicate microcosms of groundwater from within the contaminant plume and upgradient were used for each treatment, mixed 1:1 with a nutrient solution. Groundwater served as the microbial inoculum. Controls were made using filter sterilized water and abiotic preparations contained $HgCl_2$. All three triplicates with yeast extract and two of the non-combusted dolomite in plume groundwater exhibited dechlorinating activity. The rates varied, but no specific information about the range or amount of variation was provided. They

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concluded that the organic material in the dolomite was providing the electron donor for dechlorinating activity. Further, dechlorination only occurred where microorganisms had adapted to plume conditions: non-plume groundwater microcosms were incapable of supporting dechlorinating activity under conditions similar to the successful plume groundwater microcosms.

In the second phase of the experiment, also described in Hohnstock-Ashe et al. (2001), dolomite chips were used as the inoculum (2.5 g of chips were placed in 45 mL of nutrient solution with no groundwater). The proportions approximated the dolomite concentration of 60 mg/mL as with the pulverized dolomite of the first phase. The dolomite chips were collected from a fracture face of a core sample of rock, obtained from within the plume, using a sterile chisel in an anaerobic glove box. No physical description (e.g., size or shape of the chips) was provided. No dechlorinating activity was measured in the microcosms prepared with the dolomite chips, indicating that the chips provided an insufficient microbial population for inoculation, in spite of the intrinsic organic material.

Microcosms were used by Byl and Williams (2000) in an evaluation of the chlorinated ethene biodegradation potential in a Marshall County, TN karst aquifer contaminated with TCE. The microcosms were constructed in 40 mL vials with Teflon® lined septa caps, and consisted of groundwater with no aquifer solids, filled to a zero headspace. They believed that groundwater only microcosms were more representative of conditions in the aquifer. Twelve total biotic treatments were prepared, each representing four groundwater sources. Four replicates were prepared for each treatment, with one replicate being sacrificed and sampled at each of four time periods (0, 3, 17 and

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23 weeks) after incubation inverted in the dark at an unspecified temperature. Two sterile treatments were also constructed, although one set of sterile microcosms did not remain sterile, apparently affected by cross contamination during TCE spiking. Although results varied by groundwater source, there was generally greater dechlorination in the biotic samples than the sterile controls. No treatment rate or loading factor calculations were provided.

To assess anaerobic biodegradation of TCE in an Oak Ridge, TN saprolitic rock, Lenczewski et al. (2004) evaluated TCE degradation in aqueous flows through laboratory columns of rock samples. The saprolite was derived from *in situ* weathering of the underlying sedimentary bedrock, composed of interbedded shales and sandstones, from which the carbonates had been leached to leave a high porosity (15% to 58%) detrital matrix. Saprolite was excavated from an uncontaminated location and carved into two columns (23 cm in diameter by 25 cm long). The columns were fitted into 25 cm diameter PVC pipe and the annulus filled with TCE resistant epoxy. Deaerated groundwater was pumped through the cores at a rate of 0.2 mL/min, which corresponded to a specific discharge rate of 0.7 cm/day. One column was inhibited for microbial growth by the addition of NaN_3 and $HgCl_2$ (0.65 g/L and 0.33 g/L, respectively). After 56 days, dissolved phase TCE was added to the influent to achieve an average concentration of 1780 ug/L.

Effluent concentrations increased until equaling the influent concentration at 57 and 100 days for the biotic and abiotic columns, respectively, a difference attributed to physical factors in the saprolite (e.g., size or frequency of fractures, root holes). The abiotic column effluent was similar to the influent throughout the experiment, while the

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biotic column effluent decreased to approximately 50% of the influent TCE concentration at 250 days. No biodegradation or loading factors were reported. cDCE was observed in the biotic column effluent beginning 31 days after TCE addition, increasing with time to a stable level of 25 to 100 ug/L after 50 days. Additionally, VC was observed in the biotic column effluent after 234 days, although at concentrations < 10 ug/L. The authors interpreted the results as demonstrating microbial dechlorination activity from groundwater and saprolite of uncontaminated areas, suggesting indigenous capability and population adaptation to TCE as a new electron acceptor. Because the effluent contained only 76% of the input TCE (by mass), the authors suggested the remaining TCE was sorbed by the saprolite. Non-chlorinated progeny (e.g., ethane, CO_2) were not measured and could have been a factor.

Conclusions

Microcosms are diagnostic tools and representations of *in situ* conditions, developed over the last 40 years from closed cell respirometers that have evolved into anaerobic serum bottles. Microcosms can be used for several assessments including: *in situ* biodegradation potential and/or rate; nutrient or toxicity effects; metabolic pathways; comparison of activity between aquifer locations; the extent of acclimation necessary; and the effect of temperature. The degree to which a microcosm models the field condition will depend on how well the *in situ* conditions are known and can be represented (e.g., solid:liquid ratio for geologic strata and groundwater; inoculation population; trophic interactions such as predation and symbiosis; nutrient cycling).

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For assessment of anaerobic reductive dechlorination, microcosms can use higher contaminant concentrations, incubation at laboratory ambient temperatures, analysis of headspace, and repetitive analyses (after Morse et al., 1998). Their method is more sensitive to the quality and composition of seals and potential leakage, while it is relatively insensitive to minor factors (e.g., temperature, C_0, light, carrier compounds, *in situ* liquid:solid ratio) concerning rate since it is primarily a screening test to evaluate and optimize amendment strategies. Wilson et al.'s (1996) protocol matches the *in situ* conditions for concentration and temperature, while using the microcosms as a one time experimental unit without repetitive sampling. This method elucidates slower rates to model *in situ* conditions without amendment or enhancement and is less susceptible to variations in technique or material.

Microcosms have been evaluated for experimental effects on biodegradation rates. Yagi et al. (1992) evaluated the effect of increasing C_0 for anaerobic dechlorination of TCE and observed that degradation rates decreased, although not substantially. They also found that degradation rates approximately double for each increase of 10° C. Bradley et al. (2005) demonstrated that the presence of psychrotolerant and psychrophilic microorganisms can support significant TCE biodegradation at temperatures $< 5°$ C, if the redox conditions are favorable for reductive dechlorination. Bradley and Landmeyer (2006) evaluated biodegradation of MtBE in aquifers with annual temperatures $< 10°$ C, and found that less temperature inhibition may be experienced by microbial communities with greater psychrotolerance. Hutchins (1997) evaluated the effect of solid:liquid ratios (from 0.039:1 to 1.9:1) for toluene biodegradation in serum bottle microcosms, and found little difference in the rate or final degraded amount. While this test was performed using

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toluene, it suggests that microbial growth and the degradation in microcosms is relatively insensitive to proportional changes in initial conditions.

Fractured rock aquifers are a challenging environment in which to evaluate microbial activity because of mineral distribution, fracture spacing and orientation, porosity, seasonal geochemical cycling, and hydrogeological and biological heterogeneity. These factors affect microbial growth and distribution by influencing microbial nutrition, habitat and trophic interactions. Therefore, estimation of microbial dechlorination rates for fractured rock aquifers requires that microcosms address impacts of rock surfaces, formation minerals, rock porosity, trophic interactions, nutritional cycles, and slow rates of growth and metabolism. To date, only four studies have been published in the technical literature evaluating microbial dechlorination in fractured rock aquifers with microcosms, none of which have addressed *in situ* rates of dechlorination, only relative activity between locations or different conditions. [N.B. Additional studies have been done evaluating microbial dechlorination in fractured rock aquifers with microcosms, but have not been published (Fogel, pers. comm., 2005.)]

It is likely that high exploration costs, poorly defined contaminant distribution and transport mechanisms, and the uncertainty of laboratory studies to dependably represent field conditions have created a sense of limited gains for the investment when considering microcosms to evaluate microbial dechlorination in fractured rock aquifers. Perhaps the complexity of anaerobic dechlorination and the difficulty in defining chlorinated solvent transport in fractured rock discourage many site owners because of the method difficulty and "foreign" vocabulary. Overall, very few evaluations are being done of anaerobic dechlorination rates in fractured rock aquifers, in spite of a need to

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define degradation rates and contaminant fate at many TCE release sites (Pope et al., 2004).

Microcosms should follow the general method of Wilson et al. (1996) to best model and evaluate the *in situ* rates of anaerobic dechlorination in fractured rock aquifers because conditions are matched and they are considered one time experimental units without repetitive sampling. Inoculum should be made by inclusion of sediment and groundwater from the aquifer. Incubation should be done at temperatures representative of the *in situ* condition.

This dissertation attempted to answer three questions to advance the measurement and characterization abilities for *in situ* reductive dechlorination of TCE in fractured rock aquifers.

- How well do microcosms model *in situ* reductive dechlorination of TCE in fractured rock aquifers and are there preparation techniques that improve the model?
- How well do microcosms resolve very slow rates of *in situ* reductive dechlorination of TCE in fractured rock aquifers, and what is the limit of a degradation determination?
- How well do microcosms function in factorial evaluations of potential biostimulants for *in situ* reductive dechlorination of TCE in fractured rock aquifers?

By answering these questions, perhaps microcosms can better serve to define degradation rates and contaminant fate at TCE release sites with fractured rock aquifer.

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CHAPTER III

ROCK FRAGMENTS IN TRICHLOROETHENE MICROCOSMS FOR BEDROCK AQUIFERS

Introduction

Bedrock aquifers are important natural resources that many people use as their drinking water source. Trichloroethene (TCE), an organic solvent used by industry and the military for cleaning metal parts, is heavier than water, recalcitrant to degradation in most environments, and can migrate down into fractured bedrock aquifers. Biodegradation has the potential to effectively remediate TCE in bedrock aquifers, as *in situ* anaerobic TCE half-lives range from 80 to 800 days, with the longer half-lives from locations without amendment or enhancement (i.e., under biotic intrinsic conditions) (Aziz et al., 2002). Moreover, with suitable amendments (i.e., electron donor or acceptors to stimulate reducing conditions, nutrients), TCE half-life can be reduced to 0.18 days (Parsons Corp., 2000). Microcosms are needed to evaluate the potential for biodegradation in fractured rock aquifers because there typically are few monitoring wells drilled into rock, so little *in situ* data is available (Wiedemeier et al., 1998).

Four microcosm studies involving TCE biodegradation in bedrock aquifers have been reported in the peer-reviewed literature (Table 3 and Appendix B). Yager et al. (1997) documented TCE dechlorination in microcosms consisting of contaminated plume groundwater and nutrient media (mixed 1:1 by volume), and pulverized site dolomite (sterilized; 60 g/L). The petroliferous dolomite had an effective porosity of 3% and the

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groundwater velocity was 0.2 to 0.9 m/d underlying the Niagara Falls, NY site where TCE had been released in the subsurface from an unlined industrial evaporation pond during the 1950s and 1960s. Organic material in the dolomite was the likely source of electron donor necessary for dechlorination (Hohnstock-Ashe et al., 2001). In addition, dechlorination could only be accomplished when microorganisms from the plume were present. Hohnstock-Ashe et al. (2001) documented that rock chips alone (without site groundwater) did not provide sufficient microbes. Byl and Williams (2000) used groundwater microcosms to evaluate chlorinated ethene biodegradation potential in a karst aquifer contaminated with TCE (Marshall County, TN). Although results varied by groundwater source, there was generally greater dechlorination in the biotic samples than the sterile controls, interpreted by the authors as indicative of *in situ* chlorinated ethene biodegradation potential.

Table 3. TCE degradation rates measured in previous microcosm or column studies of bedrock aquifers.

Reference	Aquifer Matrix	Incubation Temperature (°C)	Shortest Sustained TCE Degradation Half-life (d)	Comments
Yager et al. (1997)	Petroliferous dolomite (NY)	22	66	Groundwater only
			27	Groundwater with pulverized dolomite
Hohnstock-Ashe et al. (2001)	Petroliferous dolomite (NY)	22	23	Groundwater with pulverized dolomite
			25	Groundwater with yeast extract
Byl and Williams (2000)	Karst (TN)	22	4	Groundwater only
Lenczewski et al. (2004)	Saprolite (TN)	12	35	Flow through columns

Lenczewski et al. (2004) evaluated the potential for indigenous TCE degradation in aqueous flow-through laboratory microcosm columns of fractured saprolite collected

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from an uncontaminated location in Oak Ridge, TN. After 250 days of TCE spiked flow, the 1780 ug/L of TCE decreased approximately 50% as it passed through the column. Concurrently, some TCE biodegradation progeny, cis-dichloroethene (cDCE) and vinyl chloride (VC), were observed in the effluent, although at relatively low concentrations. The data suggested indigenous microbial populations in the saprolite were capable of degrading the TCE. All four of these previous microcosm or column studies reported TCE degradation half-lives substantially faster than the 80 to 800 d range of *in situ* TCE half-lives reported by Aziz et al. (2002). None of the four previous studies attempted to relate the microcosm or column half-lives to *in situ* rates of the respective study sites.

The Bedrock Bioremediation Center (BBC) at the University of New Hampshire specializes in multi-disciplinary research on bioremediation of organically-contaminated aquifers (www.bbc.unh.edu). The BBC's field research is conducted at Site 32 of the Pease International Tradeport (formerly Pease Air Force Base; Portsmouth, NH). The site contains a contaminant plume that originated from an underground TCE waste storage tank. A variable thickness sand layer, with an embedded lens of marine clay, overlies a tightly folded, biotite-grade metasandstone and metashale crosscut by numerous porphryitic diabase dikes. The plume, which extends downward and laterally northeast ~0.5 km through the weathered and competent bedrock (Figure 4), contains cDCE (250-440 μg/L) with some trans-DCE (tDCE) (26-49μg/L), TCE (24-59μg/L), and VC (8-22μg/L) at the location from which sample water was obtained for this research (BBC-7, Figure 4). Field measurements of groundwater collected from the bedrock aquifer include: water temperature of 10 to 13° C, pH = 8.17, conductivity = 619 μS, dissolved oxygen (DO) = 0.7 mg/L, Fe^{+2} = 0.25 mg/L, and a redox potential of –191 mV

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(Well BBC7, 22.6 – 24.2 m bgs, April 5, 2004). Laboratory analyses of samples from the same well interval on the same date resulted in determination of alkalinity = 170 mg/L as $CaCO_3$, $Cl^- = 12$ mg/L, $SO_4^{-2} = 160$ mg/L, $NO_3^- < 0.1$ mg/L, $Br^- < 0.1$ mg/L, $S^0 < 0.04$ mg/L, and total Fe = 0.17 mg/L.

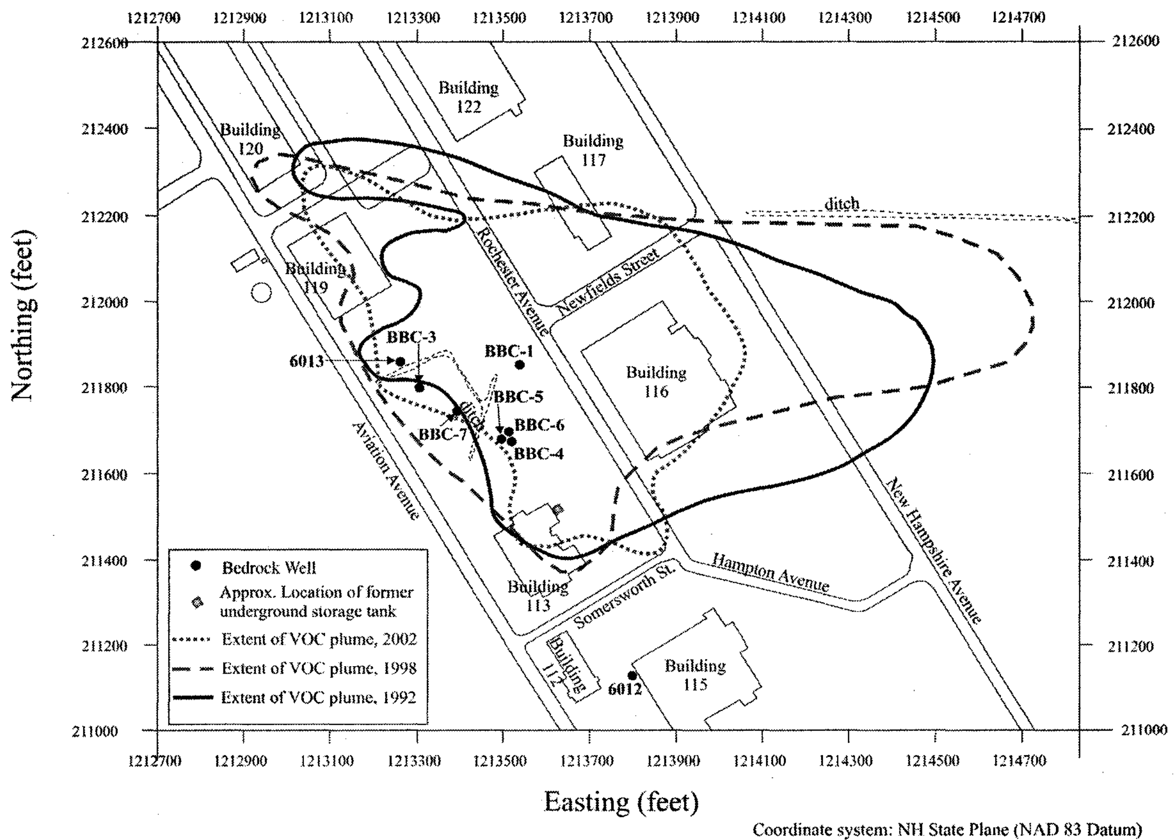

Figure 3. Location of bedrock wells and contamination by TCE and its progeny in the soil and weathered bedrock aquifer at BBC Field Research Area, Site 32, Pease International Tradeport, Portsmouth, NH, in 1992, 1998 and 2002 (Eighmy et al., 2006). (Note: insufficient data to map bedrock aquifer contamination).

Since 1997, the overburden and weathered bedrock contamination has been managed using a sheet pile containment system coupled with a pump and aboveground granular activated carbon (GAC) treatment system. The competent (less fractured)

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bedrock, with an hydraulic conductivity of 1.4 x 10^{-5} cm/s, is considered a technical impracticability (TI) zone.

Microbes capable of anaerobic reductive dechlorination of TCE are present in the bedrock microfractures at Site 32 (Eighmy et al., 2006). Evaluation of TCE concentrations in two competent bedrock wells (6012 and 6013), showed TCE degradation half-lives of 263 and 204 days, respectively (Table 4), using the point decay rate method of Newell et al. (2002) in which groundwater measurements in a single monitoring well over a time interval are evaluated by linear regression (Appendix B). Although the point decay rate method encompasses sorption and dispersion as well as biodegradation, these degradation half-lives predominantly represent biodegradation as little organic matter exist within the competent bedrock to cause sorption and the wells are on the distal reaches of the TCE plume where dispersal geometry is minimal.

Table 4. Half-life estimates of TCE natural attenuation from groundwater monitoring well measurements.

Monitoring Well	Date of Measurement [1]	C_o (ug/L)	First Order Degradation Constant (days $^{-1}$) [2]	Half-life (mean ± standard deviation) (days) [3]
6012	9/25/91 to 11/10/94	270	-0.002641 ± 0.000471	263 (223 to 319)
6013	5/17/94 to 4/11/96	630	-0.003392 ± 0.000665	204 (171 to 254)

Notes: 1. Dates of measurements are prior to pumping system operation.
2. First order degradation constant calculated from linear regression of ln C / C_0 versus time. C_0 was determined from the maximum measured TCE concentration after cessation of contaminant release and prior to initiation of hydraulic source control measures in accordance with the recommendations of Newell et al. (2002).
3. Half-life mean calculated as ln 2 / first order degradation constant.

The purpose of this study was to evaluate microcosm methods that can be used to predict the *in situ* anaerobic reductive dechlorination of TCE in bedrock aquifers with and without amendments. Biotic intrinsic (i.e., microcosm conditions that simulate *in*

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situ conditions without amendment) and biotic amended (i.e., microcosm conditions that are amended to stimulate degradation) preparations were compared to abiotic preparations using Site 32 as a model. For these types of microcosm studies, Wilson et al. (1996) recommended matching *in situ* conditions of concentration and temperature, using the microcosms as a one-time experimental unit without repetitive sampling. The emphasis of their method is elucidating slower rates and modeling *in situ* conditions without substantial amendment or enhancement (i.e., the case at the BBC site).

The effect of rock media in the microcosms as a source of surface area and nutrients was also assessed. Comparisons were made to TCE degradation rates in the literature and at Site 32 (Tables 3 and 4), and conclusions drawn about the advantages and disadvantages of rock media for the microcosms. Surface area to volume comparisons were made to bedrock aquifer conditions at Site 32. Microcosm conditions (e.g., surface area : volume ratio, initial TCE concentration (C_0), incubation temperature) were selected to simulate *in situ* conditions at the BBC site. The effect of a 45 day pre-microcosm *in situ* incubation of rock media in a groundwater well within a TCE plume, for colonization of the rock media with a microbial population representative of *in situ* conditions, was assessed in comparison to use of sterile media.

Methods and Materials

Experimental Design The experiment was a 2^2 x 3^1 factorial evaluation. TCE degradation was assessed through comparisons of three treatments (representing different microbial (biotic) conditions) in each of four microcosm preparations (representing different modes for modeling *in situ* conditions) (Table 5). Comparison of results

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between treatments within a single preparation allowed isolation of biotic effects, while comparison of results between preparations provided evaluation of the effects of *in situ* conditions. Triplicate microcosms of each preparation and treatment combination were sampled at approximate 30-day increments over 155 days, the duration selected to provide sufficient time for a statistically significant determination of a potentially long biodegradation half-life.

Table 5. Microcosm conditions for TCE biodegradation assessment and experimental nomenclature.

Preparation	Treatment	Fluid Media		Granular Media		Microbial Source
		Volume	Source	Volume	Source	
Groundwater Only (GW)	Abiotic (A)	254 mL	Sterilized Groundwater	none	Not Applicable (N/A)	None
	Biotic Intrinsic (BI)	254 mL	Groundwater	none	N/A	Groundwater
	Biotic Amended (BA)	254 mL	Groundwater with Lactate Amendment	none	N/A	Groundwater
Unincubated Crushed Rock Core in Groundwater (Rocks Autoclaved, RA)	Abiotic (A)	~215 mL	Sterilized Groundwater	60 mL loose measure (~ 39 mL solid volume)	Sterilized Crushed Rock Core	None
	Biotic Intrinsic (BI)	~215 mL	Groundwater	60 mL loose measure (~ 39 mL solid volume)	Sterilized Crushed Rock Core	Groundwater
	Biotic Amended (BA)	~215 mL	Groundwater with Lactate Amendment	60 mL loose measure (~ 39 mL solid volume)	Sterilized Crushed Rock Core	Groundwater
Incubated Crushed Rock Core in Groundwater (Rocks Incubated, RI)	Biotic Intrinsic (BI)	~215 mL	Groundwater	60 mL loose measure (~ 39 mL solid volume)	Crushed Rock Core Incubated *In Situ*	Groundwater and Granular Media
	Biotic Amended (BA)	~215 mL	Groundwater with Lactate Amendment	60 mL loose measure (~ 39 mL solid volume)	Crushed Rock Core Incubated *In Situ*	Groundwater and Granular Media
	Rock Media Population (RMP)	~215 mL	Sterilized Groundwater	60 mL loose measure (~ 39 mL solid volume)	Crushed Rock Core Incubated *In Situ*	Granular Media
Incubated Glass Beads in Groundwater (Glass Beads, GBI)	Biotic Intrinsic (BI)	~215 mL	Groundwater	60 mL loose measure (~ 39 mL solid volume)	Glass Beads Incubated *In Situ*	Groundwater and Granular Media
	Biotic Amended (BA)	~215 mL	Groundwater with Lactate Amendment	60 mL loose measure (~ 39 mL solid volume)	Glass Beads Incubated *In Situ*	Groundwater and Granular Media
	Rock Media Population (RMP)	~215 mL	Sterilized Groundwater	60 mL loose measure (~ 39 mL solid volume)	Glass Beads Incubated *In Situ*	Granular Media

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Microcosm Preparation Microcosms were prepared using the method and general recommendations of Wilson et al. (1996). 250 mL sterile amber borosilicate serum bottles with PTFE-faced silicone septa held in place by phenolic screw caps were filled under anaerobic conditions (i.e., no headspace). Groundwater was collected from Well BBC7 at Site 32 from 22.6 – 22.4 m bgs using a Grundfos Redi-Flo2 submersible pump (Grundfos Pump Corp., Olathe, KS) within an interval defined by inflatable packers [N.B., BBC7 was being used for hydrogeological tests at the time as part of another study]. The pump was equipped with PTFE tubing and decontaminated with anionic detergent (Alconox, Inc., White Plains, NY) and clean water rinse prior to placement in the well. Prior to sampling, a quantity of water greater than three volumes of the well interval was purged and temperature, pH and conductivity were stable.

Water was collected in sterile 4L borosilicate glass bottles by using low-aeration techniques in which the pump discharge (sterilized with 10% hypochlorite solution and rinsed) was submerged to the bottom. The bottles of groundwater were capped immediately upon filling (no headspace), then stored at a laboratory temperature of 18 to 20° C until use (maximum hold time of 3 days) with the assumption that minimal TCE degradation would occur during storage because no TCE spike had yet been added. Abiotic microcosms cointained groundwater and media (Table 5) that had been sterilized separately by autoclaving (All autoclave sterilization in this study: Tuttnauer, Hauppauge NY, Model 3870M, 121° C, 15 psig, 60 min) in 4L beakers covered with foil. After sterilization, the beakers of groundwater or media were allowed to cool to room temperature while covered for at least 24 hr, during which time reaeration with atmospheric oxygen occurred. The DO was allowed to remain in the microcosms as an

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added check on the sterility of the abiotic treatment; DO would be quickly consumed if biotic activity occurred.

A 100 mm diameter rock core was obtained between 28.7 and 31.8 m bgs during the drilling of Well BBC3, and was considered expendable at the time of this study as all logging had been completed and verified. While the core had initially been obtained under anaerobic conditions, after initial sampling and logging it had been stored outdoors for approximately 4 years prior to use. The rock core was crushed in a Bico Braun (Burbank, CA) VD Chipmunk jaw crusher powered by a 1.5 kW electric motor (selected to overcome the high compressive strength of the rock) to 100% passing a 12.5 mm sieve (small enough to freely pass the opening and neck of the microcosm bottles). The spherical lime soda glass beads ranging in diameter from 0.59 to 0.84 mm (Ballotini® Impact Beads; Potters Industries, Inc.; Apex, NC) and half of the crushed rock core were incubated in BBC3 using 90 kg test fishing line (Spectron Blackspot Braided Line; Cortland Line Company; Cortland, NY) at 29 to 35 m bgs (rock core origination depth), after placement in knitted polypropylene bags (Wigwam Gobi Liner, Size XL; Wigwam Mills, Inc.; Sheboygan, WI). The crushed rock core, glass beads, polypropylene bags and fishing line were all sterilized by autoclaving prior to incubation. The bags were retrieved after 45 days (anticipated to be sufficient for biofilm stasis but not checked) and placed in 4L beakers of groundwater (obtained from BBC7 concurrent with the microcosm groundwater), then quickly transported to the laboratory (3 min) and placed under nitrogen atmosphere. BBC3 was used because no other operations were anticipated for that well during the incubation. Purging of BBC3 was not done prior to either deployment or retrieval of bags to limit potential mobilization of sediment within

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the well. Unincubated rock core was placed in foil-covered 1L beakers and autoclaved then cooled to room temperature prior to transport to the field laboratory.

Microcosms were prepared under 100% nitrogen atmosphere in a double-station vinyl glove box (Flexible Vinyl Type B Anaerobic Chamber, Coy Laboratory Products, Inc.; Grass Lake, MI), which was triple purged, disinfected with 10% sodium hypochlorite solution and dried prior to use. All microcosms were initially filled approximately half full with the appropriate type of groundwater (Table 6). For those microcosms receiving granular media, a sterile 60 mL VOA vial was filled with the rock or glass beads and tamped gently to remove large voids. This volume of media was then placed into the bottle being prepared, rinsing the transfer vial with a stream of the appropriate water to insure all of the pieces entered. The crushed rock media had an average surface area of 0.083 ± 0.039 m^2/g (n=3), as determined using a Micrometrics Tri-Star 3000 porosimeter with the Brunauer-Emmet-Teller (BET) equation (Spear, 2004). With a porosity estimated at 35% based on the crushed rock media grain size distribution and particle shape (Lambe and Whitman, 1969), and a measured specific gravity of 2.65 (Eighmy et al., 2006), 60 mL of granular media (loose measure) had a total surface area of 8.6 m^2. Assuming the remainder of a 254 mL serum bottle volume was occupied by groundwater, the surface area:volume ratio (A:V) was 40.0 m^2/L (Appendix C). This A:V corresponded to a calculated fracture width of 50 um, assuming smooth sides, which approximates the values obtained from core samples at the BBC site (Eighmy et al., 2006).

The TCE spike solution was prepared in 60 mL vials using approximately 5 mL of pure phase TCE (100.0% purity; JT Baker; Phillipsburg, NJ) and 55 mL of reverse

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osmosis (RO) water, sealed with no headspace for a minimum of 5 days to reach equilibrium at the saturation concentration. 100 μL of the spike was collected in a 100 uL air-tight glass syringe, first triple rinsed with pentane, then rinsed with the aqueous solution prior to use and dedicated to the spiking procedure. The spike was delivered to the appropriate microcosms with the syringe cannula submerged in the fluid when the bottle was approximately half filled, with separate syringes used for abiotic and biotic preparations.

Biotic amended microcosms were created by the addition of a 5 mL spike of sodium lactate solution at a concentration of 2 gC/L. This solution was created by mixing sodium lactate ($C_3H_5NaO_3$) (Fisher Scientific; Fair Lawn, NJ) at 60% purity into sterilized RO water. The spike was measured and delivered using an bottle top dispenser (2 to 10 mL, Brinkmann Instruments; Westbury, NY), sterilized prior to use by soaking in a 10% sodium hypochlorite solution for 48 hours, rinsed with sterile RO water, and set to deliver 5.0 mL. To keep all treatments at equivalent concentrations, biotic intrinsic microcosms received a 5.0 mL spike of groundwater while abiotic microcosms received a 5.0 mL spike of sterile groundwater, delivered from separate bottle top dispensers that were sterilized prior to use.

Assembly of the microcosms took place on December 16, 2004 (microcosms with groundwater only and microcosms with unincubated crushed rock core), December 17 (microcosms with incubated crushed rock core) and December 18 (microcosms with incubated glass beads). This 72 hr period was required because each microcosm took ~7 min to prepare, with 360 total microcosms prepared. Completed microcosms were temporarily stored within the anaerobic glove box at a temperature of 18 to 20° C, then

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all were moved on December 19 to two anaerobic incubators (Forma Scientific Model 3140, Thermo Electron Corporation; Marietta, OH) which had pure nitrogen atmosphere, greater than 98% relative humidity at 12 ± 0.1° C. This temperature matched the *in situ* groundwater temperature, to minimize the possibility of temperature-enhanced growth rates (Yagi et al., 1992). Bottles were stored upright for greater stability in case of inadvertent jarring. Bottles were removed approximately every two weeks; caps checked and tightened, as necessary; gently agitated by tipping over and back once; and replaced in the incubator.

Microcosm Sampling and Analysis An initial sampling round occurred when groundwater only and unincubated crushed rock microcosms were 7 days old (incubated crushed rock microcosms and glass bead microcosms were 1 and 2 days newer, due to preparation order). Thereafter, microcosms were sampled at approximately 30 day intervals (37, 65, 96, 125, 155 d). Sampling was done in a single day, using randomly selected triplicate microcosms of each treatment and preparation (36 total microcosms sampled per round). A method blank of 0.5 L consumer-grade bottled water (Poland Spring Water Company; Greenwich, CT) was sampled at the start of each round and after every 12 microcosms to provide a check on contamination.

After a microcosm was opened under a fume hood in the laboratory, the following steps were completed: (1) DO measured within the bottle; (2) 10 mL glass syringe rinse with ~1 mL of contents; (3) twin 10 mL aliquots removed for gas chromatography (GC) samples; (4) 50 mL glass syringe rinse with ~5 mL of contents; and (5) 50 mL aliquot removed for pH measurement (5 mL), total organic carbon (TOC) analysis (22 mL), and archive sample (22 mL).

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The DO measurement was made using pre-measured, colorimetric self-filling vials (Chemets; CHEMetrics, Inc.; Calverton, VA). To make the DO measurement, a vial was submerged in the bottle to an approximate depth of 40 mm below surface, then opened by crushing the point with stainless steel forceps. After filling with water, the Chemet was then removed and mixed by inverting and color comparison made within 30 sec. The Chemet colorimetric self-filling vials were of two types: the Rhodazine D® method for 0.005 to 0.040 or 0.05 to 1.0 mg DO/L and the Indigo Carmine method for 1 to 12 mg DO/L concentrations. [N.B., DO measurements were made immediately upon opening the bottle, as they were affected by collection of aliquots for GC analysis, but the GC results were not significantly affected by taking the DO measurement first.]

The GC samples were placed in 23 mL glass vials (precleaned by heating to 550° C for 1 hr), injected with 50 uL of a quality control (QC) surrogate (chloroprene) and sealed with aluminum crimp caps and PTFE-lined silicone septa. pH was measured in 13 mm diameter glass test tubes using a Beckman (Fullerton, CA) F71 pH meter with a VWR (West Chester, PA) SymPHony probe, calibrated at pH = 4 and 10 (Fisher Scientific International, Inc.; Hampton, NH). TOC samples in 22 mL glass vials were acidified with 2 drops of 50% 1N H_2SO_4 solution, capped with PTFE-lined silicone septa and stored at 4° C (maximum hold time of 6 months) prior to non-purgeable organic carbon (NPOC) measurement on a Shimadzu (Columbia, MD) 5000A TOC analyzer, using a platinum catalyst at regular sensitivity, ultra high purity (UHP) air as carrier gas at a flow rate of 150 mL/min, with a 20 min sparge.

TCE, cDCE and tDCE were analyzed using a Perkin Elmer (Wellesley, MA) Autosystem GC equipped with a TurboMatrix 110 static headspace sampler system, a

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Restek (Bellefonte, PA) RTX 502.2 fused silica capillary column (105 m long and 0.53 mm diameter with a 3.0 um film thickness), and a Ni-63 electron capture detector [N.B., VC produced insufficient response to be measured]. Samples were stored at 4° C for no longer than 14 days prior to GC analysis, except as noted below for the zero, 30 and 60 day sample rounds.

UHP helium was the GC carrier gas at a pressure of 14 psi and a measured flow of 72 mL/min. P-5, an UHP mixture of 95% argon and 5% methane, was the make-up gas at a pressure of 40 psi. UHP helium was also the headspace pressurization gas. The headspace sampler was operated with a vial warming step of 90° C for 20 min, then pressurization to 40 psi for 1.0 min, followed by injection for 0.25 min. The GC program was 10 min at 35° C, then a ramp of 6° C/min to 155° C, followed by a ramp of 12° C/min to 240° C. Information on the GC method, calibration and evaluation of precision and accuracy is provided in Appendix D.

TCE, cDCE and tDCE measurements were made on GC samples obtained as described above for the 96, 125 and 155 day sample rounds (Appendix E). GC detector malfunction for the 7, 37 and 65 day sample rounds required analysis of archived samples. These samples were stored at 4° C in 22 mL borosilicate amber glass vials capped with PTFE septa caps. Specimens were obtained for GC analysis by opening the archive vial, rinsing a 10 mL glass syringe with a 1 mL aliquot, then measuring a 10.0 mL volume and transferring it to a GC headspace vial. 50 uL of the GC surrogate were added to the vial and it was capped as describe previously. Results are separated into two age ranges, microcosms incubated between 0 and 65 days in which measurements were

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made of the archive samples, and microcosms incubated between 90 and 155 days in which measurements were on fresh samples.

Biodegradation of TCE was assessed using the method of Weidemeier et al. (1995) in which the slopes of abiotic and biotic microcosm treatment trend lines were compared using a repeated measures analysis of covariance (ANCOVA), with time as a continuous covariant and biodegradation being determined by statistically significant difference with a probability of false positives (p value) < 0.20 (Appendix F). The ANCOVA method for determination of biodegradation was particularly important in this study because of the inability to measure VC, ethene or CO_2 progeny from the TCE degradation.

TCE degradation half-lives were developed using a normalized linear regression method in which the natural log of a normalized degradation response (i.e., a ratio created by the mean values of a sampling round divided by the corresponding mean value of the abiotic control set that represented a biotic C_0) were regressed as a function of microcosm age. This approach made the archive step of three measurement rounds inconsequential, and provided a means for comparison across sample rounds. The normalized degradation responses were regressed across all sample rounds, as the archive sample step in the 0 to 65 day sample rounds was made irrelevant by evaluating the ratio of TCE in biotic to abiotic microcosms within each sample round. The regression slopes were used to estimate the degradation rate assuming first order kinetics for decreasing TCE concentration. This approach follows the time of stabilization method of Chapelle et al. (2003), which allows the separation of biotic from abiotic effects, and proposes assuming

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first order degradation kinetics if contaminant concentrations are less than 1 mg/L and the microbial population is stable.

Results and Discussion

Biodegradation Evaluation TCE biodegradation (decrease in TCE concentration in comparison to abiotic controls) was only statistically significant in the 0 to 65 day groundwater only microcosms and in the 0 to 65 day and 96 to 155 day unincubated crushed rock in groundwater microcosms amended with lactate (ANCOVA, $p \leq 0.20$) (Tables 6, 7 and 8). Identification of statistically significant biodegradation was likely constrained by the low power statistics of the ANCOVA with limited sample rounds (3) in each comparison because of the additional archive step for the first half of the sample rounds. The microcosm results matched the behavior observed for the groundwater at the BBC site in that there was no significant increase in cDCE or tDCE concentrations related to TCE decrease during the 155 days of incubation (VC was unable to be measured using our GC methods). The trends of TOC consumption in the microcosms amended with lactate, 28 ± 3 and 37 ± 5 ug C/d for groundwater only microcosms and unincubated crushed rock in groundwater microcosms, respectively, occurred concurrently with the biodegradation of TCE. These TOC consumption rates were significantly higher (ANCOVA, $p < 0.0001$) than the abiotic consumption rates of 5 ± 3 and 1 ± 0.3 ug C/d for groundwater only microcosms and unincubated crushed rock in groundwater microcosms, respectively. Microcosms amended with lactate also were consistently 1.0 to 1.4 pH units lower and significantly different than the corresponding abiotic treatments (Student's t test, $p = 0.05$), with net (amended less abiotic) pH

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Table 6. Triplicate TCE concentrations in microcosm (mean ± standard deviation, ug/L, relative standard deviation, %) by sampling round, preparation and treatment.

Preparation	Treatment	Sampling Round					
		With Archive Step			No Archive Step		
		7 Days	37 Days	65 Days	96 Days	125 Days	155 Days
Groundwater Only (GW)	Abiotic (A)	302 ± 49 (16.0%)	379 ± 103 (27.0%)	400 ± 111 (27.8%)	448 ± 118 (26.3%)	438 ± 104 (25.4%)	520 ± 154 (24.0%)
	Biotic Intrinsic (BI)	389 ± 138 (35.4%)	246 ± 104 (42.0%)	372 ± 82 (21.8%)	367 ± 62 (17.1%)	401 ± 89 (22.2%)	487 ± 104 (21.2%)
	Biotic Amended (BA)	371 ± 19 (4.9%)	338 ± 100 (29.8%)	271 ± 32 (12.1%)	265 ± 7 (3.0%)	301 ± 46 (15.2%)	350 ± 58 (16.9%)
Unincubated Crushed Rock Core in Groundwater (Rocks Autoclaved, RA)	Abiotic (A)	609 ± 23 (3.9%)	541 ± 89 (16.5%)	559 ± 42 (7.5%)	543 ± 87 (16.2%)	617 ± 70 (11.3%)	634 ± 20 (3.1%)
	Biotic Intrinsic (BI)	480 ± 47 (9.8%)	386 ± 55 (14.2%)	399 ± 78 (19.4%)	323 ± 38 (11.7%)	314 ± 46 (14.6%)	331 ± 26 (6.9%)
	Biotic Amended (BA)	579 ± 26 (4.5%)	405 ± 80 (19.7%)	368 ± 63 (17.1%)	348 ± 62 (18.0%)	397 ± 21 (5.3%)	348 ± 61 (17.7%)
Incubated Crushed Rock Core in Groundwater (Rocks Incubated, RI)	Biotic Intrinsic (BI)	586 ± 45 (7.6%)	532 ± 49 (9.1%)	507 ± 43 (8.5%)	550 ± 36 (6.7%)	554 ± 21 (3.8%)	624 ± 39 (6.3%)
	Biotic Amended (BA)	528 ± 4 (0.7%)	503 ± 50 (9.9%)	430 ± 79 (18.5%)	455 ± 71 (15.6%)	453 ± 75 (16.5%)	484 ± 36 (7.6%)
	Rock Media Population (RMP)	582 ± 14 (2.5%)	520 ± 18 (3.5%)	538 ± 51 (9.0%)	515 ± 51 (9.9%)	544 ± 49 (9.2%)	608 ± 81 (13.4%)
Incubated Glass Beads in Groundwater (Glass Beads, GBI)	Biotic Intrinsic (BI)	605 ± 17 (2.8%)	436 ± 136 (30.9%)	565 ± 56 (10.0%)	596 ± 92 (15.4%)	483 ± 78 (16.3%)	577 ± 33 (5.7%)
	Biotic Amended (BA)	507 ± 88 (17.3%)	441 ± 43 (9.8%)	353 ± 110 (31.2%)	348 ± 112 (32.0%)	450 ± 89 (19.8%)	476 ± 47 (9.9%)
	Rock Media Population (RMP)	378 ± 55 (14.5%)	380 ± 49 (13.1%)	389 ± 105 (26.9%)	373 ± 78 (20.7%)	405 ± 107 (26.5%)	409 ± 20 (4.8%)

Notes: 1. RI and GBI preparations are one and two days younger than the listed age, respectively, due to the preparation sequence.

2. Shaded results were found to have a statistically significant difference of trend over time compared to the trend of abiotic treatment of GW (for all GW microcosms) or RA (for all RA, RI and GBI microcosms) preparations, within the time period of either 7 to 65 d or 96 to 155 d (See Tables 7 and 8).

3. Archive step consisted of TCE measurement of 22 mL aliquot stored for approximately 60 days after collection during microcosm sampling.

4. No abiotic treatments were prepared of RI or GBI preparations as insufficient media was available; abiotic treatment of RA preparation was used for comparisons and control.

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Table 7. Zero order degradation trends of TCE in groundwater only microcosms.			
Preparation	**Treatment**	**TCE Trend (ug/L/d)**	
		Microcosm Ages 0 to 65 d	**Microcosm Ages 90 to 155 d**
Groundwater Only (GW)	**Abiotic (A) (Control)**	+1.70 ± 1.21	+1.23 ± 1.66
	Biotic Intrinsic (BI)	-0.34 ± 1.76	+2.04 ± 1.31
	Biotic Amended (BA)	-1.70 ± 0.81	+1.44 ± 0.55

Note: Results that represent statistically significant differences from the TCE trend of the control treatment are shown shaded, determined by analysis of covariance with $p < 0.20$. TCE trends are the slope of the linear regression line representing TCE concentrations over time.

Table 8. Zero order degradation trends of TCE in microcosms with granular media in groundwater.			
Preparation	**Treatment**	**TCE Trend (ug/L/d)**	
		Microcosm Ages 0 to 65 d	**Microcosm Ages 90 to 155 d**
Unincubated Crushed Rock Core in Groundwater (Rocks Autoclaved, RA)	**Abiotic (A) (Control)**	-0.86 ± 0.83	+1.55 ± 0.86
	Biotic Intrinsic (BI)	-1.41 ± 0.89	+0.29 ± 0.58
	Biotic Amended (BA)	-3.67 ± 0.93	-0.01 ± 0.75
Incubated Crushed Rock Core in Groundwater (Rocks Incubated, RI)	**Biotic Intrinsic (BI)**	-1.38 ± 0.60	+1.27 ± 0.48
	Biotic Amended (BA)	-1.69 ± 0.73	+0.51 ± 0.83
	Rock Media Population (RMP)	-0.77 ± 0.52	+1.59 ± 0.80
Incubated Glass Beads in Groundwater (Glass Beads, GBI)	**Biotic Intrinsic (BI)**	-0.75 ± 1.57	-0.30 ± 1.21
	Biotic Amended (BA)	-2.65 ± 1.11	+2.15 ± 1.15
	Rock Media Population (RMP)	+0.20 ± 0.97	+0.60 ± 1.00

Note: Results that represent statistically significant differences from the TCE trend of the control treatment are shown shaded, determined by analysis of covariance with $p < 0.20$. TCE trends are the slope of the linear regression line representing TCE concentrations over time.

decreases of - 0.0059 and - 0.0019 pH units/d for groundwater only microcosms and unincubated crushed rock in groundwater microcosms, respectively. These rates of pH

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change suggest biotic activity produced H^+ in the presence of lactate ($C_3H_5O_3^-$) and subsequent acetate ($C_2H_3O_2^-$) reductions (He et al., 2002):

$$C_3H_5O_3^- + 2H_2O \rightarrow C_2H_3O_2^- + HCO_3^- + H^+ + 2H_2 \quad \text{(Eq. 2)}$$

$$C_2H_3O_2^- + 4H_2O \rightarrow 2HCO_3^- + H^+ + 4H_2 \quad \text{(Eq. 3)}$$

Generation of H_2 supports TCE biodegradation through a series of dechlorination reactions, when under reducing conditions (He et al., 2002):

$$\underset{\text{(TCE)}}{C_2HCl_3} + H_2 \rightarrow \underset{\text{(DCE)}}{C_2H_2Cl_2} + H^+ + Cl^- \quad \text{(Eq. 4)}$$

$$\underset{\text{(DCE)}}{C_2H2Cl_2} + H_2 \rightarrow \underset{\text{(VC)}}{C_2H_3Cl} + H^+ + Cl^- \quad \text{(Eq. 5)}$$

$$\underset{\text{(VC)}}{C_2H_3Cl} + H_2 \rightarrow \underset{\text{(ethene)}}{C_2H_4} + H^+ + Cl^- \quad \text{(Eq. 6)}$$

In addition, carbonate is often consumed by microbes during methanogenesis or acetogenesis (He et al., 2002), removing buffering capacity:

$$HCO_3^- + H^+ + 4H_2 \rightarrow CH_4 + 3H_2O \quad \text{(Eq. 7)}$$

$$2HCO_3^- + H^+ + 4H_2 \rightarrow C_2H_3O_2^- + 4H_2O \quad \text{(Eq. 8)}$$

In the microcosms, TOC decrease (i.e., lactate consumption) was concurrent with pH decrease in lactate amended treatments of groundwater only and unincubated rock media in groundwater preparations. This decrease in pH with production of H^+ was likely further promoted by carbonate consumption. However, alkalinity measurements were not made of the microcosms, therefore changes related to buffering capacity

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consumption could not be determined in this experiment. It should be determined in future TCE biodegradation evaluations.

Microcosms with incubated crushed rock in groundwater were slower and statistically different (ANCOVA, $p = 0.12$) in TCE degradation than microcosms with unincubated crushed rock in groundwater from Day 0 to Day 65, although not statistically different (ANCOVA, $p > 0.20$) from Day 90 to Day 155. The rate of TOC consumption was 46 ± 5 ug C/d for incubated crushed rock in groundwater microcosms, which was not statistically different (ANCOVA, $p > 0.20$) from the rate for unincubated crushed rock in groundwater microcosms. The net pH increase (+0.0008 pH units/d) in incubated crushed rock in groundwater microcosms amended with lactate was statistically different (ANCOVA, $p = 0.0435$) from unincubated crushed rock core microcosms amended with lactate which decreased. The differences of initial TCE degradation and pH change while the TOC consumption was not different may indicate a shift in microbial populations (i.e., one which consumes less carbonate and therefore leaves greater buffering capacity), a change in reducing conditions (i.e., less favorable to DHB), or weathering of the crushed rock during incubation (i.e., less bioavailable carbonate and reduced acetogenesis within the microcosms at the start of the experiment).

No biodegradation was measured in microcosms with incubated glass beads in groundwater. TOC consumption in these microcosms when they were amended with lactate was 36 ± 6 ug C/d, similar to the lactate amended treatments of other preparations, and pH increased 0.0032 ± 0.0005 units/d. Without lactate amendment, pH in this preparation increased 0.008 ± 0.001 units/d, reaching a maximum value of 9.340 ± 0.042 at Day 155. The change in pH for all treatments of this preparation was much larger and

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very different than any other preparation and treatment combination, and resulted in microcosm conditions approximately 1 pH unit higher than *in situ* conditions. The lime-soda glass beads were probably undergoing glass corrosion, an ion exchange process, while in the microcosms. Glass corrosion is common when lime-soda glass is in contact with water, and results in a substantial release of OH- ions (Duffer,1986) and a concurrent increase in pH. This effect was somewhat mitigated by the microbial activity, through production of H^+ (Eq. 2 – 6).

Microcosms with incubated crushed rock core or glass beads and sterilized groundwater only had a rock media associated microbial population. None of these biotic treatments exhibited TCE biodegradation nor had statistically significant differences for pH change or TOC consumption. The two rock media associated treatments represented an inoculum different from the biotic intrinsic or lactate amended treatments, in that there is no planktonic population in the groundwater, only whatever growth has occurred on the incubated media initially. However, this inoculum source did not create a statistically significant change in any measurement of this experiment. It appears that rock media associated microbial populations were insufficient to inoculate the microcosms without the addition of groundwater associated microbial population, suggesting the importance of planktonic populations.

Degradation Rate Evaluation Because the TCE trends were increasing from Day 90 to Day 155 in 10 of 12 preparation and treatment combinations, including the abiotic controls, drift in the GC measurement was suspected even though laboratory standard recoveries were all within acceptable limits (±20%). Mean values were normalized by the corresponding mean value of the abiotic control set (units of ug/L / ug/L) (Table 9)

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and statistical difference with the abiotic control of each sampling round was assessed (Student's t test, $p = 0.05$ unless otherwise shown). The normalized means were regressed across all sample rounds (Table 10 and Figure 4), as the archive sample step in the 0 to 65 day sample rounds was made inconsequential by evaluating the ratio of TCE in biotic to abiotic microcosms within each sample round. The normalization of biotic to abiotic results for each round accounted for abiotic losses, and allowed comparison between sample rounds in spite of differences in GC analytical protocols. It assumes

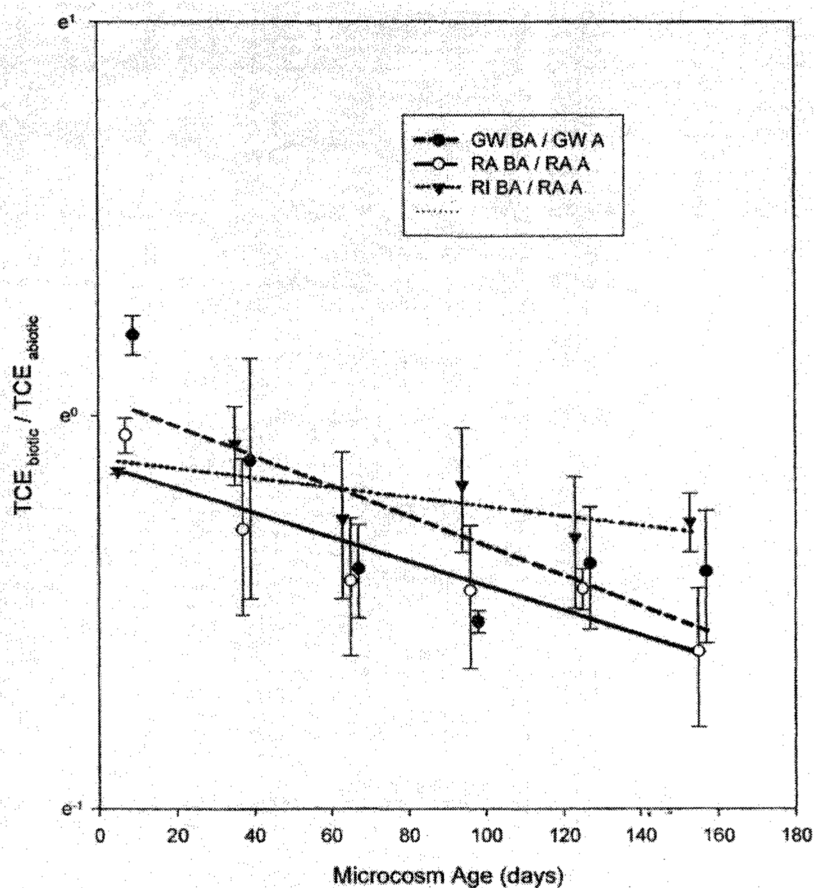

Figure 4. Normalized microcosm means of TCE concentration as a function of microcosm age for lactate amended (biotic amended, BA) treatments of groundwater only (GW), unincubated crushed rock core in groundwater (rocks autoclaved, RA), and incubated crushed rock core in groundwater (rocks incubated, RI) preparations, graphed on a natural logarithm scale with linear regression lines shown.

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only that the analysis was constant within a sample round across the treatments and preparations and that samples were selected for analysis at random from the biotic and abiotic sample populations within the round.

Table 9. Triplicate TCE concentration means normalized by abiotic mean for same sampling round.

Preparation	Treatment	Sampling Round: 7 Days	37 Days	65 Days	96 Days	125 Days	155 Days
Normalized by Sampling Round Triplicate Mean for Abiotic Treatment of GW (n=3)							
Groundwater Only (GW)	Biotic Intrinsic (BI)	129% ± 46%	65% ± 27%	93% ± 21%	82% ± 14%	92% ± 20%	94% ± 20%
	Biotic Amended (BA)	123% ± 6%	89% ± 26%	68% ± 8%	59% ± 2%	69% ± 11% **	67% ± 11% *
Normalized by Sampling Round Triplicate Mean for Abiotic Treatment of RA (n=3)							
Unincubated Crushed Rock Core in Groundwater (Rocks Autoclaved, RA)	Biotic Intrinsic (BI)	79% ± 8%	71% ± 10%	71% ± 14%	60% ± 7%	51% ± 7%	52% ± 4%
	Biotic Amended (BA)	95% ± 4%	75% ± 15%	66% ± 11%	64% ± 11%	64% ± 3%	55% ± 10%
Incubated Crushed Rock Core in Groundwater (Rocks Incubated, RI)	Biotic Intrinsic (BI)	96% ± 7%	98% ± 9%	91% ± 8%	101% ± 7%	90% ± 3%	98% ± 6%
	Biotic Amended (BA)	87% ± 1%	93% ± 9%	77% ± 14%	84% ± 13% *	73% ± 12%	76% ± 6%
	Rock Media Population (RMP)	96% ± 2%	96% ± 3%	96% ± 9%	95% ± 9%	88% ± 8%	96% ± 13%
Incubated Glass Beads in Groundwater (Glass Beads, GBI)	Biotic Intrinsic (BI)	99% ± 3%	81% ± 25% **	101% ± 10%	110% ± 17%	78% ± 13%	91% ± 5%
	Biotic Amended (BA)	83% ± 14%	82% ± 8% *	63% ± 20%	64% ± 21%	73% ± 14%	75% ± 7%
	Rock Media Population (RMP)	62% ± 9%	70% ± 9%	70% ± 19%	69% ± 14%	66% ± 17%	64% ± 3%

Notes:
1. Results that represent statistically significant differences with the abiotic control of GW (all GW preparations) or RA (all RA, RI, and GBI preparations) are shown shaded, determined by Student's t-test at $p = 0.05$, unless noted by * ($p = 0.10$) or ** ($p = 0.20$).
2. Normalized values = $C_{biotic} / C_{abiotic}$ by sampling round, with C_{biotic} and $C_{abiotic}$ representing the sampling round mean TCE concentration of biotic and abiotic measurements, respectively (units of ug/L / ug/L).

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Further, the regression slopes of the natural log of the normalized values as a function of microcosm age can be used to estimate TCE degradation half-lives (Table 10), assuming first order kinetics. This approach follows the time of stabilization method of Chapelle et al. (2003), which allows the separation of biotic from abiotic

Table 10. Half-life calculation from natural logarithm of normalized means as a function of microcosm age.

Preparation	Treatment	First Order Degradation Constant (days^{-1})	Half-life (days)
Normalized by GW-A			
Groundwater Only (GW)	Biotic Intrinsic (BI)	-0.000045 ± 0.002169	15,298 (>313)
	Biotic Amended (BA)	-0.003113 ± 0.001839	223 (140 to 544)
Normalized by RA-A			
Unincubated Crushed Rock Core in Groundwater (Rocks Autoclaved, RA)	Biotic Intrinsic (BI)	-0.002963 ± 0.000508	234 (200 to 282)
	Biotic Amended (BA)	-0.003126 ± 0.000063	222 (185 to 278)
Incubated Crushed Rock Core in Groundwater (Rocks Incubated, RI)	Biotic Intrinsic (BI)	-0.000040 ± 0.000411	17,368 (>1537)
	Biotic Amended (BA)	-0.001260 ± 0.000550	550 (383 to 976)
	Rock Media Population (RMP)	-0.000262 ± 0.000287	2640 (>1261)
Incubated Glass Beads in Groundwater (Glass Beads, GBI)	Biotic Intrinsic (BI)	-0.000438 ± 0.001188	1583 (>426)
	Biotic Amended (BA)	-0.000799 ± 0.000990	867 (>387)
	Rock Media Population (RMP)	-0.000033 ± 0.000460	21,000 (>1406)

Notes: 1. Degradation constants determined from regression of ln (C_{biotic} / $C_{abiotic}$) by age, with C_{biotic} and $C_{abiotic}$ representing the sampling round mean TCE concentration of biotic and abiotic measurements, respectively.

2. Half-lives determined by $t_{1/2} = -(\ln 2)/(\text{degradation constant})$, and are listed with range (in parentheses) determined by degradation constant estimate ± standard error of degradation constant ($p = 0.05$) (ranges with negative values listed as "greater than").

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effects, and proposes assuming first order degradation kinetics if contaminant concentrations are less than 1 mg/L and the microbial population is stable. Therefore, half-life can be calculated using the following equations:

$$C_t = C_0 e^{-kt} \qquad \text{(Eq. 9)}$$

or:

$$\ln (C_t / C_0) = - k t \qquad \text{(Eq. 10)}$$

In which:

C_0 = the mean abiotic concentration at time t

k = the degradation constant

t = the time elapsed since the assembly of the microcosms

C_t = the mean biotic concentration at time t

Linear regression of the natural logarithm of all sample round results as a function of time determines a slope equal to k:

$$k = - \ln (C_t / C_0) / t \qquad \text{(Eq. 11)}$$

Half-life, $t_{1/2}$, is defined as the time at which $C_t = 1/2\ C_0$. For the first order kinetics described above, $t_{1/2}$ may be calculated as:

$$t_{1/2} = \ln (2) / k \qquad \text{(Eq. 12)}$$

The biodegradation identified using the ANCOVA method (Tables 4, 5 and 6) had half-lives of 223 (140 to 544) and 222 (185 to 278) days for lactate amended microcosms with groundwater only or with unincubated crushed rock in groundwater, respectively calculated using the normalized linear regression method. These microcosm

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results are highly similar to the two results obtained from the analysis of groundwater monitoring well measurements from Pease Site 32 of 204 (171 to 254) and 263 (223 to 319) days (Table 5) and the range (80 to 800 d) reported by Aziz et al. (2002). However, they are an order of magnitude slower than results previously reported in the literature for TCE microcosms for bedrock sites (Table 3), which may be caused by low indigenous microbial population, low growth rates, or marginally favorable redox conditions, and may be exacerbated by incubation temperature differences.

Interestingly, using the normalized linear regression method, non-amended microcosms with unincubated crushed rock in groundwater had a half-life of 234 (200 to 282) days, similar to those microcosms that were amended with lactate and contained either groundwater only or unincubated crushed rock in groundwater. This trend was not identified as statistically significant biodegradation using the ANCOVA method (Table 8). Other lactate amended preparations of incubated crushed rock and incubated glass beads had half-lives 2 to 4 times longer while other non-amended preparations had very long half-lives estimated at 1500 days or longer. These results were similar to those of the ANCOVA method. The unweathered and uncolonized mineral surfaces of the unincubated crushed rock may provide an advantage to acetogenic microbes (Eq. 8) not attained with incubated crushed rock, who then produce H_2 without available TOC when HCO_3^- is present as it is in BBC site materials (Eighmy et al., 2006). Low concentrations of H_2 produced during acetogenesis may boost the dechlorination, as DHB have been shown to have a competitive advantage over SRB at low concentrations of H_2 (Fennell and Gossett, 1998; He et al., 2002). Additionally, Hoelen and Reinhard (2004) demonstrated DHB are able to use H_2 at lower concentrations than SRB or MB, although

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the complete degradation of TCE to ethene proceeds slowly over several years, a rate that is similar to the rates measured within this experiment. Further study with the BBC materials is recommended to evaluate this hypothesis.

In summary, microcosms constructed with unincubated crushed rock in groundwater provided degradation rate results that were closest to those estimated using the Newell et al. (2002) method for *in situ* TCE degradation for the Site 32 fractured rock aquifer, when prepared and incubated at conditions that simulated the bedrock aquifer. Microcosms constructed with only groundwater were also close to those estimated using the Newell et al. (2002) method for *in situ* the TCE degradation in the Site 32 fractured rock aquifer, although only when the microcosms were amended with TOC. Incubation of crushed rock within residual TCE plume prior to the microcosm experiments caused a substantial decline in TCE degradation observed for biotic intrinsic microcosms, suggesting an effect that is inhibitory to TCE degrading microbes. This inhibitory effect may be mitigated but not negated by the inclusion of TOC. Pre-incubation could result in poorer microbial community (e.g., SRB preferentially colonize mineral sites over DHB), media issues (i.e., glass corrosion, rock weathering), or both effects combined and should not be used.

Lime soda based glass beads are an inadequate substitute for rock media in the microcosm bottles because their corrosion (Duffer, P.F., 1986) increases pH and DO as a result of hydrolysis and ion exchange of glass when in contact with water for long periods. Inoculation provided by incubated granular material only provided insufficient microbial population or metabolic activity in the microcosms to effectively model in situ activity.

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Conclusions

Statistically significant biotic degradation of TCE was observed in microcosms with both groundwater-only and unincubated crushed rock granular media, when amended with lactate. Measured half-lives were similar to half-lives observed in field conditions.

Normalizing biotic results to abiotic results provides a method that deducts the abiotic losses and allows complete comparison between sample rounds, assuming samples were randomly selected for analysis within the sample round to avoid systematic errors or variation.

Incubation of the crushed rock core media prior to construction of the microcosms does not appear to be helpful, as the TCE degradation behavior and rate does not match the actual degradation measured at the field site through groundwater sampling and analysis over time. Similarly, changes in the water chemistry caused by incubated glass beads within the microcosms deem glass beads unsuitable for use.

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CHAPTER IV

MICROCOSM METHODS TO DISCERN SLOW RATES OF TRICHLOROETHENE DEGRADATION IN BEDROCK AQUIFERS

Introduction

Trichloroethene (TCE) is an organic solvent that has been linked to significant human health effects at low concentrations in potable water (Moran, 2006). While microbial degradation of dissolved TCE, a key step in the remediation of groundwater, has been directly demonstrated in several environments (Bradley, 2003), it has only been indirectly observed in bedrock aquifers (i.e., through geochemical assessment or gene identification evidence) (Yager et al., 1997; Lenczewski et al., 2003; Lehman et al., 2004). Direct evidence of microbial degradation is required by the United States Environmental Protection Agency (USEPA) and many state regulatory agencies as an essential component in the case for monitored natural attenuation (MNA) as a remedial option. One of the potential lines of evidence is laboratory demonstration of an indigenous microbial population capable of chloroethene degradation, typically with microcosms.

Microcosms can incorporate effects such as nutrient cycling, trophic level interactions, or variations in pH, dissolved oxygen (DO), redox potential, or organic content (Pritchard and Bourquin, 1984). Their small size (20 to 500 mL) also permits replication, simplified dosing mechanics, control over inputs and outputs, adequate mixing, and variation in treatments (Pritchard and Bourquin, 1984). Unfortunately,

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scaling of microcosm results from the laboratory to the field is considered difficult (Sturman et al., 1995). Furthermore, wall or other boundary effects, ecosystem biogeochemistry, and multi-component trophic level interactions of the natural system are not incorporated; and results are limited to specific zones within the ecosystem (Pritchard and Bourquin, 1984).

Microcosms cannot be used as the sole proof of *in situ* biodegradation because they are unable to replicate the delicate and intricate balance of chemical, physical, and biological relationships that can change rapidly in response to environmental factors (e.g., DO, water, pH, nutrients) (Madsen, 1991). Rather, three types of evaluations are needed to demonstrate the potential for *in situ* biodegradation (Madsen, 1991): comparison of biotic and abiotic microcosms distinguishes biologically-mediated activity in excess of abiotic loss; a decrease in contaminant concentrations in the field greater than the losses in the abiotic microcosms confirms indigenous activity; and biological activity in biotic contaminated microcosms should be compared to biotic pristine microcosms to demonstrate ecological adaptation, if there is more than one contaminant compound involved.

Natural attenuation of TCE appears to be controlled by several important factors: the presence of a suitable chlororespiring microbial population; the redox of the environment; the presence of alternate and potentially competing terminal electron acceptors (TEAs); the reduction kinetics of individual chloroethene compounds; the electron donor supply; presence of specific inhibitory compounds; and distribution and movement of TCE through the aquifer (Bradley, 2003). Chapelle et al. (2003) suggest that first order kinetics is an adequate approximation of TCE degradation in substrate-

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limited groundwater systems if the abundance of microbial population is not changing with time. First order kinetics are expressed as:

$$C_t = C_0\, e^{-k\,t} \quad \text{or} \quad \ln (C_t / C_0) = -k\,t \qquad \text{(Eq. 13)}$$

where: C_t = biotic contaminant concentration at time t; C_0 = contaminant concentration at t = 0 in the bottles; k = the degradation constant (units of time^{-1}); and t = the time elapsed. Abiotic losses ($\Delta C_{abiotic}$) (e.g., volatilization) from the system must be incorporated into the kinetic equation:

$$\ln (C_t / [C_0 - \Delta C_{abiotic}]) = -k\,t \qquad \text{(Eq. 14)}$$

so that:

$$C_{0\,net} = C_0 - \Delta C_{abiotic} \qquad \text{(Eq. 15)}$$

The kinetic equation can be rewritten:

$$\ln (C_t / C_{0\,net}) = -k\,t \qquad \text{(Eq. 16)}$$

$t_{1/2}$, is the time at which C_t is 1/2 $C_{0\,net}$, or for first order kinetics:

$$-\ln (1/2\, C_{0\,net} / C_{0\,net}) / k = \ln (2) / k = 0.693 / k = t_{1/2} \qquad \text{(Eq. 17)}$$

Hence:

$$k = 0.693 / t_{1/2} \qquad \text{(Eq. 18)}$$

Substituting for k in Equation 11:

$$\ln (C_t / C_{0\,net}) = -0.693\, t / t_{1/2} \qquad \text{(Eq. 19)}$$

Solving for $t_{1/2}$:

$$0.693\, t / \ln (C_{0\,net} / C_t) = t_{1/2} \qquad \text{(Eq. 20)}$$

In situ anaerobic TCE half-lives range from 80 to 800 days, with the longer half-lives from locations without amendment or enhancement (i.e., under biotic intrinsic conditions) (Aziz et al., 2002).

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Several factors complicate TCE's fate and transport in a fractured bedrock aquifer: compound mass can diffuse away from contaminated water-bearing fractures into the surrounding matrix (Parker et al., 1994); water flow may be limited by low fracture interconnectivity and geochemical precipitation (Pulido, 2003); and microbial colonization may be limited by nutrient distribution, mineral speciation, habitat availability and seasonal geochemical cycling (Eighmy et al., 2006). *In situ* temperature can also affect microbial growth rates (Yagi et al., 1992). Hendrickson et al. (2002) have shown substantial heterogeneity in the distribution of TCE-degrading microbes using molecular analysis of *Dehalococcoides* 16S ribosomal DNA from chloroethene contaminated sites throughout North America and Europe, including fractured rock.

The difficulty in determining TCE degradation is illustrated in three studies using microcosms for fractured rock [N.B., many site-specific studies have been done, but not published (Fogel, pers. comm., 2005)]. The first study, Yager et al. (1997) detected TCE dechlorination in microcosms constructed of contaminated plume groundwater and nutrient media (mixed 1:1), where pulverized site dolomite (sterilized) was added at a concentration of 60 g/L (Table 11, Appendix B). The study site was a bedrock aquifer (a petroliferous dolomite with an effective porosity of 3% and a groundwater velocity calculated at 0.2 to 0.9 m/d) near Niagara Falls, NY, into which TCE was released during the 1950s and 1960s from an unlined evaporation pond. They hypothesized that the organic material in the site dolomite provided the electron donor necessary for dechlorination (Hohnstock-Ashe et al, 2001). In the second study, Hohnstock-Ashe et al. (2001) documented that rock chips alone (without site groundwater) did not provide sufficient microbes for conducting dechlorination, leading them to conclude that the

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planktonic microbes in the plume were essential to biodegradation. In the third study, Byl and Williams (2000) used groundwater microcosms to evaluate chlorinated ethene biodegradation in a karst aquifer contaminated with TCE, located in Marshall County, TN. Although results varied by groundwater source, there was generally greater dechlorination in the biotic samples than the abiotic controls. In each study, TCE degradation was identified using microcosms, but not uniformly present in all samples across each site (TCE measured by gas chromatography in each study). Note that the shortest sustained TCE degradation half-lives for the three studies represent much faster degradation than the *in situ* ranges reported by Aziz et al (2002) of 80 to 800 d, which may have been caused by differences between laboratory and field conditions (e.g., temperature, unweathered mineral surfaces, mixing).

Table 11. TCE degradation rates measured in published microcosm studies of bedrock aquifers.

Reference	Aquifer Matrix	Incubation Temperature (°C)	Shortest Sustained TCE Degradation Half-life (d)	Comments
Yager et al. (1997)	Petroliferous dolomite (NY)	22	66	Groundwater only
			27	Groundwater with pulverized dolomite
Hohnstock-Ashe et al. (2001)	Petroliferous dolomite (NY)	22	23	Groundwater with pulverized dolomite
			25	Groundwater with yeast extract
Byl and Williams (2000)	Karst (TN)	22	4	Groundwater only

Evaluating the ability of natural attenuation of TCE in fractured bedrock aquifers requires estimating the longest half-life (i.e., the slowest biodegradation rate) using microcosm experiments with a reasonable incubation period and acceptable statistical confidence and replication. This evaluation requires determining the least significant

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difference or sensitivity (E) that represents the detectable difference necessary between the biotic and abiotic results for a specific number of replicates and confidence, to demonstrate statistical significance (Box et al., 2005). E is related to the minimum measurable TCE degradation that must be observed in biotic treatments to document that the results cannot be solely attributable to abiotic variability. For microcosms, E is actually the minimum mean decrease in TCE concentration that must be observed in the biotic treatments to insure, with a given statistical confidence level α, the effect is not solely abiotic. E can be calculated by (Box et al., 2005):

$$E = t_{\alpha,DF} \left(\frac{S_{bottle}^2}{n_{bottle}} + \frac{S_{sampling}^2}{n_{sampling} \bullet n_{bottle}} \right)^{0.5} \qquad \text{(Eq. 21)}$$

where: t = the probability point of the t distribution (t-statistic); $\alpha = 1 -$ the probability, p ; DF = analysis degrees of freedom ($n_{bottle} + n_{sampling} \bullet n_{bottle} - 2$); S_{bottle}^2 = variance component of C representing between-bottle differences; $S_{sampling}^2$ = variance component of C representing sampling differences within any microcosm bottle; n_{bottle} = number of microcosm bottles of each treatment (biotic vs. abiotic); and $n_{sampling}$ = number of TCE measurements made within each microcosm bottle. A one-tailed t-distribution is used to evaluate if the biotic TCE concentrations are significantly less than those in the abiotic bottles.

The variances associated with a microcosm (S_{bottle}^2) and the sampling and analytical methods ($S_{sampling}^2$) for biotic and abiotic microcosm populations can be estimated in experiments. Often the largest source of variation is associated with the field and sampling, which cannot be readily changed. For example, if a 95% confidence interval is required by the regulatory agency (p = 0.05), E can be made smaller by using a

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more expensive and reliable analytical method and/or accepting a $p > 0.05$. A cost-effective microcosm study can be designed for a site using Eq. 16 and an optimization process.

This research examined the ability of microcosms to discern slow rates of TCE degradation in bedrock aquifers. The source and amount of experimental variation was evaluated, and higher $n_{sampling}$, increased n_{bottle}, and decreased p was optimized to find the smallest E at which TCE biodegradation could be detected, assuming first order kinetics (Chapelle et al., 2003). This work was done by the Bedrock Bioremediation Center (BBC), a center specializing in multi-disciplinary research on bioremediation of organically-contaminated aquifers (www.bbc.unh.edu). Research was conducted using bedrock aquifer materials collected at Site 32 (Pease International Tradeport, formerly Pease Air Force Base, in Portsmouth, NH). Site 32 contains a contaminant plume of chloroethenes that was released from a TCE waste storage tank into the underlying sand and fractured bedrock.

Methods and Materials

Experimental Design Two series of microcosms were conducted. The first microcosm series used three replicate bottles (n_{bottle}) with two TCE analyses ($n_{sampling}$) made of the contents of each bottle for each of six time steps, for both biotic and abiotic treatments of both groundwater-only and crushed rock in groundwater microcosms. Groundwater-only and crushed rock + groundwater microcosms were used because they are the most common for fractured rock aquifers (Yager et al., 1997; Byl and Williams, 2000). For the second series, a hierarchical (nested) evaluation of bottle and sampling variability was

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conducted using $n_{bottle} = 7$ with $n_{sampling} = 7$, allowing for additional replication (Box et al., 2005). An n = 6 to 7 is known to provide a high statistical confidence such that greater n increases effort and cost, without the concomitant increase in confidence (Miller and Miller,1984). For the second series, abiotic treatments were selected as they exhibited the greatest variability in the first series and are the experimental treatment against which the biotic comparison is made. Twenty six day incubations represented the shortest likely duration in a study of fractured rock aquifers.

Microcosm Set Up and Incubation Conditions Microcosms consisting of full serum bottles under anaerobic conditions (DO < 0.3 mg/L for biotic microcosms) were prepared according to Wilson et al. (1996). Microcosms consisted of 250 mL amber borosilicate glass bottles with PTFE-faced silicone septa held in place by phenolic screw caps, sterilized (121° C, 15 psig, 60 min) prior to use. Groundwater used in this study was collected from Well BBC 7 at a depth of 23 m below ground surface (bgs) after purging greater than three volumes of water. Decontaminated PTFE tubing and a Grundfos Redi-Flo2 submersible pump (Grundfos Pumps Corporation; Olathe, KS) were used. Water flowed into sterile 4L borosilicate glass bottles using low-aeration techniques in which the pump discharge (sterilized prior to use in 10% hypochlorite solution and rinsed) was submerged to the bottom and the collected water allowed to purge the bottle headspace after which the tubing was removed slowly.

Granular media consisted of 100 mm diameter rock core that had been obtained from a depth of between 28.7 and 31.8 m bgs during drilling of Well BBC 3. Pieces of the rock core were crushed to 100% passing a #4 sieve. The material was oven dried at 80° C, then cooled to laboratory temperature. In the first microcosm series, 60 mL of

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crushed rock were measured and placed in each rock core microcosm after an initial placement of ~100 mL of groundwater, while in the second microcosm series, 110.0 ± 0.1 g of crushed rock core were placed in each rock core microcosm bottle prior to sterilization. Both approaches represent a surface area: volume ratio of 0.040 m^2: 1 cm^3 (i.e., the *in situ* conditions at the BBC site).

Bottles, granular media (where used), caps and groundwater (for abiotic microcosms) were autoclaved and cooled prior to use. Microcosms were prepared in a fume hood under atmospheric conditions. All microcosms were filled to ~ 175 mL with sterilized groundwater. A mass of 110 ug of TCE was spiked into each microcosm using 100 uL of an 1100 mg TCE/L water). The TCE stock was prepared in 60 mL vials containing 5 mL of pure phase TCE (100.0% purity, JT Baker; Phillipsburg, NJ) and 55 mL of reverse osmosis (RO) water in zero headspace for a minimum of 5 days. The spike was measured using the full volume of a 100 uL air-tight glass syringe, first triple rinsed with pentane, then rinsed with the aqueous stock prior to use and dedicated to the spiking procedure. The spike was delivered with the syringe cannula submerged in the microcosm fluid. Upon completion of the TCE spike, the bottles were filled using a sterile pipette to achieve zero headspace condition.

Completed microcosms were moved to an anaerobic incubator (100% nitrogen atmosphere; >98% relative humidity; 12 ± 0.1° C, the *in situ* groundwater temperature used to minimize temperature-enhanced growth rates (Yagi et al., 1992)). They were stored upright for greater stability. Bottles were removed from the incubator approximately every two weeks, caps checked and tightened if necessary, then gently agitated by rotating end-over-end and replaced in the incubator.

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Microcosm Sampling and Analysis Microcosms were opened and sampled individually in a fume hood under atmospheric conditions, beginning with the groundwater-only microcosms. Replicates were selected in a random order within a preparation. A method blank of 0.5 L consumer-grade bottled water (Poland Spring Water Company; Greenwich, CT; selected for its very low volatile organic carbon content) was also sampled before initial microcosm sampling and after every four microcosms to provide a check on cross contamination.

A 10 mL glass syringe, that had been rinsed with ~ 1 mL of sample, was used to remove seven 10 mL aliquots from the center of each bottle for $S_{sampling}$. Each sample was placed in a 23 mL glass vial (precleaned by heating to 550° C for 1 hr), injected with 50 uL of a quality control surrogate (chloroprene) and sealed with aluminum crimp caps and PTFE-lined silicone septa. The glass vials were coded prior to use, and selected without regard to label code. Samples were reorganized to disrupt patterns of sampling order, then stored together at 4° C for no longer than 6 days prior to gas chromatography (GC).

TCE was measured in a Perkin Elmer (Wellesley, MA) Autosystem GC with a TurboMatrix 110 static headspace sampler system, a Restek (Bellefonte, PA) RTX 502.2 fused silica capillary column (105 m long, 0.53 mm dia., 3.0 um film thickness), and a Ni-63 electron capture detector. Ultra high purity (UHP) helium (72 mL/min, 14 psi) was the carrier gas. P-5 gas (an UHP mixture of 95% argon and 5% methane) was the make up gas (40 psi). UHP helium was also the headspace pressurization gas. The headspace sampler was operated with a vial warming step of 90° C for 20 min, pressurization to 40 psi for 1.0 min, and injection for 0.25 min. The GC program was 10 min at 35° C, a ramp of 6° C/min to 155° C, a ramp of 12° C/min to 240° C. The GC method, calibration

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and evaluation of precision and accuracy is provided (Appendix D), and TCE measurements are presented in order of analysis (Appendices G and H).

The TCE data were analyzed using an analysis of variation (ANOVA) with the variability/gage chart graph platform in the software package JMP In Version 5.1.2 (SAS; Cary, NC). When the number of replicates was not the same for each treatment, JMP In performed a variance component analysis with residual maximum likelihood (REML) (Sall et al., 2005).

Results and Discussion

In the first microcosm series, an ANOVA for total ($S_{microcosm}^2$), bottle (S_{bottle}^2) and sample ($S_{sampling}^2$) variation and the coefficient of variation (S/C_{avg}) indicated the biotic and abiotic treatments combined had less variability (variance) (Table 12) than the abiotic treatments alone (Table 13), indicating biotic activity reduced variability in the microcosms. Similarly, the groundwater-only preparations had greater variability than the crushed rock and groundwater microcosms. A comparison as a function of incubation time (7 to 155 days) for each type of microcosm indicated that variability stabilized or was slightly reduced with increasing incubation time (Appendix G).

In the second microcosm series, the abiotic microcosm preparations with crushed rock in groundwater had substantially less total variance ($S_{microcosm}^2$) than the groundwater-only preparations (Table 14; Appendices H and I), which matched the trend from the first microcosm series (Tables 12 and 13). Overall variability was much reduced in the second series due to the increased number of replicates and analyses. Because their overall variability is smaller, microcosms with crushed rock core and groundwater are

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Table 12. ANOVA results for TCE measurement in biotic and abiotic microcosms from first microcosm series.				
		Total Microcosm	Bottle (Replicate)	Sample (Within Bottle)
Biotic and Abiotic Groundwater-only Microcosms (C_{avg} = 369 ug/L)	S (ug/L)	98.1	88.7	41.9
	S/C_{avg} x 100 (%)	26.6	24.0	11.4
	S^2 $(ug/L)^2$	9,621	7,863	1,759
Biotic and Abiotic Crushed Rock and Groundwater Microcosms (C_{avg} = 460 ug/L)	S (ug/L)	70.7	48.2	51.7
	S/C_{avg} x 100 (%)	15.4	10.5	11.3
	S^2 $(ug/L)^2$	4,997	2,323	2,674
Note: Variances (S^2) are additive, so $S_{microcosm}^2 = S_{bottle}^2 + S_{sampling}^2$				

Table 13. ANOVA results for TCE measurement in abiotic microcosms from first microcosm series.				
		Total Microcosm	Bottle (Replicate)	Sample (Within Bottle)
Abiotic Groundwater-only Microcosms (C_{avg} = 415 ug/L)	S (ug/L)	119.6	36.0	114.1
	S/C_{avg} x 100 (%)	28.9	8.7	27.5
	S^2 $(ug/L)^2$	14,302	1,295	13,008
Abiotic Crushed Rock and Groundwater Microcosms (C_{avg} = 584 ug/L)	S (ug/L)	83.9	39.5	74.1
	S/C_{avg} x 100 (%)	14.4	6.8	12.7
	S^2 $(ug/L)^2$	7,047	1,561	5,486

able to establish significant TCE biodegradation at a lower concentration difference (E) for a given p value.

The lower variability in the microcosms with crushed rock core media may be caused by better mixing or distribution of the TCE when granular media moves through

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Table 14. ANOVA results for TCE measurement in abiotic microcosms after 26 days incubation from second microcosm series.

		Total Microcosm	Bottle (Replicate)	Sample (Within Bottle)
Abiotic Groundwater-only Microcosms (C_{avg} = 505 ug/L)	S (ug/L)	25.3	15.6	19.9
	S/C_{avg} x 100 (%)	5.01	3.09	3.94
	S^2 $(ug/L)^2$	641.4	244.0	397.4
Abiotic Crushed Rock and Groundwater Microcosms (C_{avg} = 580 ug/L)	S (ug/L)	18.1	9.6	15.3
	S/C_{avg} x 100 (%)	3.12	1.66	2.64
	S^2 $(ug/L)^2$	327.0	92.6	234.5

the liquid during weekly turning, or perhaps through elimination of density currents within the closed system of the microcosm, although further analysis would be necessary to determine the cause with certainty. Sources of variability in microcosms include: the microbial population (i.e., inoculant amount, representativeness, and distribution within the microcosm); the geochemistry (i.e., groundwater differences, interaction of granular media mineralology, interaction of microcosm bottle or cap materials); the contaminant dosing (i.e., measurement variability, non-uniform distribution within the spike solution, volatile loss during transfer, microcosm container volume variation); the contaminant distribution (i.e., lack of mixing, sorption, loss during incubation); sampling (i.e., volatile loss, aliquot measurement) and analytical variability. Biotic microcosms variability over time also includes microbial metabolism and mass transfer effects, although the results of the first microcosm series indicated biotic activity reduced overall variability, perhaps by improving diffusion gradients for mass transfer through consumption during metabolism or by preferential colonization around areas of higher TCE concentration.

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Using the variances from Table 14, the longest (maximum) half-life ($t_{1/2\ max}$) (i.e., the slowest detectable degradation rate) can be calculated for a microcosm study of the same preparation and analysis for a given study duration (time, t), statistical confidence (α, represented in the determination of E in Eq. 16), abiotic concentration ($C_{t\ abiotic}$), and bottle replicate and sample scheme:

$$t_{1/2\ max} = \ln 2 \bullet t / \ln [C_{t\ abiotic} / (C_{t\ abiotic} - E)] \qquad \text{(Eq. 22)}$$

For example, if $p = 0.05$ and three bottles are used with two TCE measurements of each bottle for both abiotic and biotic treatments, the degree of freedom = 7 (degree of freedom = $n_{bottle} + n_{sampling} \bullet n_{bottle} - 2$) and E is calculated as 23 and 16 ug/L for groundwater-only and crushed rock in groundwater preparations, respectively. Assuming a microcosm incubation of 120 days and an abiotic mean concentration of 475 ug/L for the sampling event, the maximum half-life that can be resolved is 1676 and 2427 days for groundwater-only and crushed rock in groundwater preparations, respectively (Appendix J) for abiotic treatments, assuming biotic treatments have less variation as observed in the first microcosm series. Obviously, forecasting a half life of more than 4 years from a 4 month incubation would be poor practice because conditions required for biodegradation can change over the extended time which can compromise the prediction. However, the evaluation provides a limit for the degradation rate that can be resolved within the incubation period, giving statistical evidence for a biological treatment effect.

The ANOVA and subsequent calculation of maximum half-life can be entered in a cost benefit analysis for different microcosm schemes in support of MNA determinations, to optimize the sensitivity of the biodegradation determination and the number of microcosms and the number of measurements within each microcosm. As an

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illustration, consider the evaluation using the variabilities found for the microcosms with crushed rock core media (Table 14), a sample (measurement) cost of $125 each, a microcosm cost of $85 each, an initial abiotic concentration of 500 ug/L, an incubation time of 120 days, and $p = 0.05$ (Table 15). Maximum $t_{1/2}$ of 195 and 1072 d can be attained using the techniques of the first and second microcosm series, respectively, by a 2 bottle, 1 sample per bottle scheme that would cost $300, although this cost is in addition to the cost required for the replicates and measurements to do ANOVA. Other schemes could provide longer values of maximum $t_{1/2}$, but may not be justified by the increased costs.

Table 15. Evaluation of maximum biodegradation half-life ($t_{1/2}$) and cost for selected microcosm schemes.

Microcosm Schemes						Variabilities Associated with First Microcosm Series		Variabilities Associated with Second Microcosm Series	
n_{bottle}	n_{sample} per microcosm	Total Cost	DF	$t_{0.95, DF}$	p	E (ug/L)	Maximum $t_{1/2}$ (d)	E (ug/L)	Maximum $t_{1/2}$ (d)
1	2	$335	2	6.314	0.05	414	47	91	411
1	3	$460	3	2.920	0.05	170	200	38	1047
2	1	$300	2	2.920	0.05	173	195	37	1072
2	2	$550	4	2.132	0.05	99	377	22	1861
2	3	$800	6	1.943	0.05	80	477	18	2273
3	1	$630	3	2.132	0.05	103	359	22	1826
3	2	$1,005	6	1.895	0.05	72	537	16	2581
3	3	$1,380	9	1.812	0.05	61	640	14	2998
5	1	$1,050	5	1.860	0.05	70	553	15	2722
5	2	$1,675	10	1.771	0.05	52	758	11	3581
5	3	$2,300	15	1.734	0.05	45	879	10	4059

Notes:
1. Total cost = n_{bottle} (microcosm cost) + $n_{sampling} \bullet n_{bottle}$ (measurement cost) • 2, assuming that equal numbers of abiotic and biotic microcosms will be prepared. Costs do not include microcosm or measurement cost for initial variability evaluation.
2. Degree of Freedom (DF) = $n_{bottle} + n_{sampling} \bullet n_{bottle} - 2$.
3. Detectable difference (E) calculated using Equation 17, assuming $p = 0.05$.
4. Maximum biodegradation half-life ($t_{1/2}$) calculated using Equation 16.

The implications of these findings are of the utmost importance when considering the inclusion of MNA in a remedial solution. The statistical evaluation of variance

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provides a method to assess the presence of biotic degradation with a selected level of probability. The potential for a “false positive” determination (α error; Miller and Miller, 1984) for biotic degradation can thus be quantified, and appropriate contingencies developed. Additionally, the microcosm results can be assessed for evidence of biotic degradation from several parts of a contaminated site, providing spatial assessment of the potential for MNA effectiveness. A minimum rate of biodegradation, if present, can be calculated from a microcosm experiment by assessing the detectable difference through the time span of the experiment, further quantifying the evaluation of biodegradation. Operator skill that reduces experimental variability in microcosm evaluations can create a substantial improvement in the detection ability of these methods. These findings have particular importance for evaluation of dechlorination in bedrock aquifers, as previous work has shown the degradation rates in bedrock aquifers can be slow and severely limited by hydrogeological, geochemical, and microbiological factors.

Conclusions

In this work, evaluation was made of the ability of microcosm methods to discern slow rates of TCE degradation, with specific application to bedrock aquifers. An analysis of variation was performed on a bottle microcosm technique, and the source and amount of variation identified in the experimental process. Consideration was made of factors for improvement, including the effect of increased sampling, increased replication, or decreased level of probability. A method was developed to statistically conclude whether biotic degradation is occurring, and relationships developed to convert a statistically detectable difference to a maximum quantifiable degradation half-life, assuming first order kinetics.

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CHAPTER V

IMPROVEMENTS TO ANAEROBIC TRICHLOROETHENE MICROCOSM PROTOCOLS FOR BIOSTIMULATION IN BEDROCK AQUIFERS

Introduction

Bedrock aquifers are important natural resources that benefit many people as their drinking water source. Trichloroethene (TCE), an organic solvent for cleaning metal, is heavier than water, very slow to degrade in most environments (half lives of 80 to 800 d reported by Aziz et al, 2002), and commonly found in contaminated fractured bedrock aquifers. Microbial reductive dechlorination is one remedy to TCE contamination, and can be enhanced (biostimulated) by organic substrates (i.e., electron donors; Parsons Corp., 2004) or nutrients (i.e., nitrogen and phosphorus; Palumbo et al., 1995). These enhancements may be supplied to a bioreactive zone in the subsurface individually or in combination. For example, TCE dechlorination at a TCE site in South Carolina increased significantly when orthophosphate or triethyl-phosphate, or to a lesser degree from ammonia or nitrous oxide were added to microcosms (Palumbo et al., 1995). Pfiffner et al. (1997) observed increased microbial abundance and chloride concentrations after *in situ* injection of nitrous oxide or triethyl phosphate gases at the same site. With suitable amendments (i.e., electron donor or acceptors to stimulate reducing conditions, nutrients), TCE half-life can be reduced to 0.18 days (Parsons Corp., 2000).

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Laboratory microcosms using the indigenous microbial community are one of the potential "lines of evidence" to determine the rates of *in situ* microbial reductive dechlorination (Weidemeier et al., 1998). Microcosms can provide a means of assessing the effect of potential biostimulants on anaerobic TCE biodegradation (Morse et al., 1998). Unlike a single-species or pure culture experiment, microcosms can incorporate effects resulting from nutrient cycling, trophic level interaction, or variations in functional characteristics (i.e., pH, dissolved oxygen (DO), redox potential, or organic content) (Pritchard and Bourquin, 1984). Furthermore, their small size permits replication, simplified dosing mechanics, control over imports and exports, adequate mixing, and an opportunity to vary conditions over a wide array of scenarios (Pritchard and Bourquin, 1984).

Unfortunately, the artificial laboratory conditions under which microcosms are conducted means that the results may not be indicative of what will be accomplished *in situ* (Parsons Corp., 2004). Minimum requirements for a microcosm study are: representative media; groundwater samples collected using reasonably aseptic and anaerobic collection procedures; appropriate concentrations of contaminants and substrates; relevant temperatures, amendment formulations, and controls; and sufficient time for microbial acclimation and growth (6 months minimum) (Parsons Corp., 2004). Microcosms are most useful when site conditions are not well known (e.g., heterogeneous contaminant distribution, limited evidence of anaerobic dechlorination, non-optimum pH, high sulfate concentration; Parsons Corp., 2004).

Fractured rock aquifers are a particularly difficult environment in which to evaluate microbial activity because of complex mineral distribution, fracture spacing and

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orientation, porosity, seasonal geochemical cycling, and hydrogeologic heterogeneity (Eighmy et al., 2006). Hydrogeological, biogeochemical and microbiological factors can obscure evaluations of contaminant fate and transport. Contaminant mass can diffuse away from water-bearing fractures into the surrounding matrix (Parker et al., 1994); flow may be limited by low fracture interconnectivity and geochemical precipitation (Pulido, 2003); and microbial colonization may be limited by nutrient distribution, mineral speciation, habitat availability and seasonal geochemical cycling (Eighmy et al., 2006). *In situ* temperature can also affect microbial growth rates (Yagi et al., 1992). Hendrickson et al. (2002) have shown substantial heterogeneity in the distribution of TCE-degrading microbes using molecular analysis of *Dehalococcoides* 16S ribosomal DNA from chloroethene-contaminated sites throughout North America and Europe, including fractured bedrock. High exploration costs further complicate the evaluation of microbial dechlorination in fractured rock aquifers.

The difficulty in determining TCE biodegradation is illustrated by three studies using microcosms for fractured rock (Table 16). Yager et al. (1997) documented TCE dechlorination in microcosms constructed of contaminated groundwater and nutrient media (mixed 1:1) and sterilized pulverized site dolomite (60 g/L) (Table 16, Appendix B). TCE was released during the 1950s and 1960s into an unlined evaporation pond at the site and migrated into the underlying petroliferous dolomite (effective porosity = 3%, groundwater velocity = 0.2 to 0.9 m/d). Hohnstock-Ashe et al. (2001) hypothesized that the organic material in the dolomite provided the electron donor necessary for dechlorination. They documented that rock chips alone (without site groundwater) did not provide sufficient microbes for conducting dechlorination, but that yeast extract

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added to plume groundwater enhanced dechlorination similar to the enhancement of the pulverized site dolomite. Microorganisms in the plume were essential for dechlorination. Byl and Williams (2000) used groundwater microcosms to evaluate chlorinated ethene biodegradation in a karst aquifer contaminated with TCE. Although results varied by groundwater source, there was generally greater dechlorination in the biotic samples than the sterile controls. In each of the three studies, TCE degradation was identified in the microcosms, but not uniformly present across the sites.

Table 16. TCE degradation rates measured in previous microcosm studies of bedrock aquifers.

Reference	Aquifer Matrix	Incubation Temperature (° C)	Shortest Sustained TCE Degradation Half-life (d)	Comments
Yager et al. (1997)	Petroliferous dolomite (NY)	22	66	Groundwater only
			27	Groundwater with pulverized dolomite
Hohnstock-Ashe et al. (2001)	Petroliferous dolomite (NY)	22	23	Groundwater with pulverized dolomite
			25	Groundwater with yeast extract
Byl and Williams (2000)	Karst (TN)	22	4	Groundwater only

We explored the use of factorial experiments to assess the effects of biostimulants and *in situ* factors on anaerobic TCE degradation in fracture rock aquifer microcosms. This approach can provide substantial cost savings and resource efficiency because: factorial designs require relatively few microcosms per factor; the interpretation of the observations can proceed largely by common sense, simple arithmetic and computer graphics; the observations can indicate promising directions for further experimentation and causative relationships; and designs can be suitably augmented when a more in depth exploration is needed (Box et al., 2005). Morse et al. (1998) suggested a comparative

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microcosm study to evaluate biostimulation for unconsolidated aquifers that resembles a factorial experiment, although statistical interpretations were not specified.

In this study, a factorial designed experiment of biostimulated TCE dechlorination in fractured bedrock aquifers using microcosms evaluated several potential biostimulants (i.e., nutrients, vitamins, and sterile groundwater). This work was conducted at the Bedrock Bioremediation Center (BBC), a center specializing in multi-disciplinary research on bioremediation of organically-contaminated aquifers (www.bbc.unh.edu). Research was conducted using bedrock aquifer media collected at Site 32 of the Pease International Tradeport (formerly Pease Air Force Base) in Portsmouth, NH. Site 32 contains a contaminant plume of chloroethenes that was released from an underground TCE waste storage tank into underlying subsurface. TCE degradation was evaluated using three methods of data analysis: analysis of covariance (ANCOVA) between biotic and abiotic treatment trend line slopes; calculation of biodegradation half-life; and effects screening by model fitting.

Methods and Materials

Experimental Design A total of 64 microcosms were prepared using two types of *in situ* media (groundwater only, crushed rock + groundwater) and two treatments (biotic, abiotic). Biostimulants were not initially added to any of these microcosms. TCE concentrations were measured at selected intervals over the initial 81 days (t=81 d) of incubation for all preparations and treatment combinations, using 40 of these microcosms. After TCE analyses were completed and validated (t=81 to 115 d),

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degradation was proceeding uniformly for biotic preparations, indicating acclimation was occurring.

Three biostimulant conditions were created at t=115 d in microcosm pairs of each preparation and treatment combination (i.e., the remaining 24 microcosms) and incubated for 45 days (until t=160 d) to model enhanced bioremediation, using a full factorial configuration of 2^2 x 3^1 (i.e., three factors investigated, one with three settings) with replication. The factorial design assessed: (1) net biological effects by comparing biotic and abiotic treatments; (2) mineral surface interactions by comparing groundwater only and crushed rock + groundwater preparations; and (3) nutrient effects by comparing three biostimulant mixtures. Replication provided a direct check on the assumption that the error (S^2) was constant and reducing the variability of the regression coefficients in the presence of potentially large process or measurement variability (Sall et al., 2005). A 45 d incubation was selected to provide sufficient time to discern differences in the relatively slow rates of TCE dechlorination of the indigenous aquifer microbial community (Appendix B).

The three biostimulant conditions were sterile groundwater only, sterile groundwater + nutrients, and sterile groundwater + nutrients + vitamins. The nutrient mixture provided nitrogen and phosphorus plus three metals (iron, manganese and zinc) (Table 17a). The vitamin mixture included two additional metals and 12 vitamins (Table 17b).

Microcosm Set Up and Incubation Conditions Abiotic and biotic microcosms in this study were prepared using the method and general recommendations of Wilson et al. (1996), in which sterilized 250 mL amber borosilicate glass serum bottles with PTFE-

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faced silicone septa are completely filled under anaerobic conditions. Groundwater used in this study was collected from Well BBC 6 at a depth of 37 m below ground surface

Table 17. Injection mixture compounds and concentrations in microcosms.

a. Nutrients in Sterile Groundwater

Compound[1]	Microcosm Concentration (mg/L)[2,3]
Ammonia Nitrogen	3.2
Nitrate Nitrogen	0.4
Urea Nitrogen	16.4
Phosphate	30
Potash	20
Iron	0.2
Manganese	0.1
Zinc	0.1

b. Vitamins and Nutrients in Sterile Groundwater

Compound[1,4]	Microcosm Concentration (mg/L)[2,3,5]
Ammonia Nitrogen	3.2
Nitrate Nitrogen	0.4
Urea Nitrogen	16.4
Phosphate	30
Potash	20
Iron	0.32
Manganese	0.126
Zinc	0.14
Iodine	2 ug/L
Chromium	0.334 ug/L
Molybdenum	0.334 ug/L
Ethyl Alcohol	11.4
Sucrose	66.6
Vitamin A	17.4 IU/L
Vitamin B6	26 ug/L
Vitamin B12	0.08 ug/L
Vitamin C	0.8
Vitamin D	5.34 IU/L
Vitamin E	0.4 IU/L
Thiamin	20 ug/L
Riboflavin	22 ug/L
Niacin	0.266 mg/L
Biotin	4 ug/L
Pantothenic Acid	134 ug/L

Notes 1. Nutrient stock = Shultz 10-15-10 Plant Food Plus (Shultz; Bridgeton, MO).
2. Concentrations are nominal values assuming a microcosm liquid volume of 250 mL and are developed from manufacturer's literature.
3. Units are mg/L, unless otherwise noted.
4. Vitamin stock = Centrum Liquid (Wyeth Consumer Healthcare; Madison, NJ).
5. IU = International Units.
6. A blank injection consisting of sterile groundwater only was also used.

(bgs) after purging a quantity of water greater than three well volumes. A Grundfos Redi-Flo2 submersible pump (Grundfos Pumps Corporation; Olathe, KS) and PTFE

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tubing were used. Water flowed into sterile 4L borosilicate glass bottles using low-aeration techniques in which the pump discharge tubing end (sterilized prior to use in a 10% hypochlorite solution and rinsed with excess groundwater) was submerged to the bottom and the collected water allowed to purge the bottle headspace.

100 mm diameter rock core, obtained from between 28.7 and 31.8 m bgs at Well BBC 3 was crushed to 100% passing a #4 sieve. The material was oven dried at 80° C for at least 48 hr, then cooled to laboratory temperature. 110.0 ± 0.1 g was placed in each bottle designated for crushed rock + groundwater microcosms, representing a 60 cm^3 loose measure (*in situ* surface area: volume ratio of 0.040 m^2: 1 cm^3).

Bottles (i.e., empty and those with crushed rock), caps and groundwater were autoclaved and cooled. Microcosms were prepared in a single-station glove box (Cole-Parmer; Model EW-34788-10; Vernon Hills, IL) under 100% nitrogen atmosphere after three cycles of vacuum and nitrogen flushing. All microcosms were spiked with approximately 325 g of undiluted sodium lactate ($C_3H_5NaO_3$) (Fisher Scientific; Fair Lawn, NJ) at 60% purity delivered from a sterile pipette, then filled to ~ 175 mL with groundwater (N.B., sterile groundwater for abiotic). 110 ug of TCE was spiked into each microcosm using 100 uL of an aqueous stock solution (1100 mg TCE/L, 100 uL air-tight glass syringe, triple rinsed with pentane, then rinsed with the aqueous stock). The TCE stock was prepared in 60 mL vials using approximately 5 mL of pure phase TCE (100.0% purity; JT Baker; Phillipsburg, NJ) and 55 mL of reverse osmosis (RO) water with zero headspace for a minimum of 5 d. The bottles were filled with groundwater (N.B., sterile groundwater for abiotics) using a sterile pipette to achieve zero headspace. Completed microcosms were moved to an anaerobic incubator which had a pure nitrogen

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atmosphere, greater than 98% relative humidity and an internal temperature set to 12 ± 0.1° C (the *in situ* groundwater temperature at the field site). Bottles were stored upright for greater stability and were removed from the incubator approximately every two weeks to check caps and gently agitate end-over-end once.

Microcosm Sampling and Analysis Microcosms were sampled t = 4, 20, 41, 81 and 160 d. They were sampled individually in a fume hood under ambient laboratory conditions. Sample order for t = 4 through 81 d began with the groundwater-only microcosms, progressing to the crushed rock, with abiotic and then biotic treatments sampled for each preparation. The sample order for t = 160 d was completely random. A 50 mL glass syringe, that had been rinsed with ~ 2 mL of sample, was used to remove the sample aliquots from the center of each bottle. Replicates were selected in a random order within a preparation and treatment combination. A method blank of 0.5 L consumer-grade bottled water (Poland Spring Water Co.; Greenwich, CT) was also sampled before every 12 microcosms to provide a check on cross contamination.

Volatile organic analysis (VOA) followed USEPA SW-846 Method 8260B (1996) on samples placed in 40 mL VOA vials preserved with HCL. Sample vials were coded and the order mixed for randomness and so that method blanks were indistinguishable. Samples were stored at 4° C for ≤14 d prior to analysis.

Data Analysis TCE degradation was assessed in three ways: the analysis of covariance (ANCOVA) approach of Weidemeier et al. (1995) to assess biotic effects, the first-order degradation kinetics approach of Chapelle et al. (2003) to assess overall attenuation, and the effects screening/fit model approach of the National Institute of Standards and Technology (NIST) (2006) to assess factor contribution.

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ANCOVA. The TCE data (Appendix K) were analyzed for biodegradation according to Weidemeier et al. (1995) in which abiotic and biotic microcosm treatment trend line slopes are compared by an ANCOVA. Biodegradation is assumed if there is a statistically significant difference between the slopes (biotic slope > abiotic slope) with a probability of a false positive (p value) less than 0.20. The ANCOVA was performed with the fit model platform in the software package JMP In version 5.1.2 (SAS; Cary, NC).

First Order Degradation Kinetics. Chapelle et al. (2003) noted first order kinetics is an adequate approximation of TCE degradation in substrate-limited groundwater systems if microbial abundance is not changing with time. Degradation coefficients and half-life, $t_{1/2}$ (ln 2 / first order degradation constant k) were calculated for t = 4 to 81 d of each preparation and treatment combination using the first order degradation regression platform of the software package Sigma Plot version 10 (Systat Software, Inc., Richmond, CA). Net rates and $t_{1/2}$ were calculated for each preparation by subtracting the abiotic loss from the biotic degradation effect (Chapelle et al., 2003):

$$t_{1/2} = \ln 2 \, / \, (k_{biotic} - k_{abiotic}) = 0.693 \, / \, k_{net} \qquad \text{(Eq. 23)}$$

Fit Model. TCE data were analyzed using the fit model analysis recommended by NIST (2006) for analysis of factorial results using the t = 160 d results. The NIST approach: (1) checks anomalies regardless of factor levels, using the distribution platform; (2) checks time sequence and sample order effects, using the fit y by x platform with TCE response graphed by sample number; (3) assesses gross TCE response for each factor, using the fit y by x platform sorted by factor columns; (4) estimates

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interrelationships between factors using the fit model platform for the full factorial; (5) removes unnecessary terms in the model until all remaining terms or their interactions are significant ($\alpha < 0.05$); (6) tests residuals within the model to check the validity of the model assumptions of normality and a common variance; (7) evaluates optimum factor settings using the prediction profiler within the model; and (8) assesses factor interactions using the interaction profiler within the model. Upon completion of these steps, the model is rerun using estimated TCE reduction, calculated from the TCE results subtracted from an initial TCE value estimated from stoichiometry. Model validation followed the NIST steps, however, the TCE reduction values included small differences estimated for initial conditions and therefore was capable provided stronger predictions than for the TCE results only. Note that the higher statistical confidence represented in this analysis by $\alpha < 0.05$ reflects an ability to achieve greater certainty about significant factors over a single time period than in the biodegradation determination of the ANCOVA analysis which is subject to additional variation from time as a continuous covariant.

Results and Discussion

Consistent biodegradation of TCE was exhibited in groundwater-only and crushed rock microcosms (i.e., biotic treatments were significantly different covariants from the corresponding abiotic treatments [biotic > abiotic] in ANCOVA; probability of false positive $p = 0.0078$ and 0.0038 for groundwater-only and crushed rock, respectively; $t =$ 4 to 81 d; Appendix L). The net biotic $t_{1/2}$ was 276 d (95% confidence interval (CI) of 167 to 668 d) and 136 d (95% CI of 100 to 213), for groundwater-only and crushed rock,

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respectively, using the first order degradation analysis (Eq. 18) for each preparation (Appendix L). The minimum coefficient of determination (r^2) for the regression calculations was 0.9998. The $t_{1/2}$ from the crushed rock microcosms was shorter (i.e., had faster biodegradation) than the *in situ* value at Site 32 in Wells 6012 and 6013 ($t_{1/2}$ = 263 (95% CI of 223 to 319) d and 204 (95% CI of 171 to 254) d, respectively), calculated by the point decay rate method of Newell et al. (2002) using groundwater measurements over a 1142 and 695 d interval, respectively, from initial concentrations of 270 and 630 ug/L, respectively, and linear regression for a single monitoring well (Appendix B). The faster biodegradation of the crushed rock microcosms is likely to be a result of the increased organic carbon concentration (>200 mg/L) in the microcosms compared to the field. However, the biodegradation rate of the groundwater microcosms was not significantly different than the observed field rates in spite of the increased organic carbon concentration. The contradictory responses of the two preparations indicate an advantage conferred by the crushed rock media in the microcosms to the TCE biodegradation, which could be caused by habitat benefits (e.g., increased colonization sites), nutritional benefits (e.g., increased mineral and nutrient dissolution), improved buffering capacity related to the crushed rock media, or an unidentified factor. The biodegradation rate of crushed rock microcosms was not significantly faster than the biodegradation rate of groundwater only microcosms in a previous experiment (Chapter III) in which initial organic carbon concentrations were ~50 mg/L, although wider variation in the groundwater only microcosm results may have obscured the comparison.

Factorial results of TCE measurements from the t = 160 d sampling round (Table 18) had one anomaly representing an abiotic treatment of the crushed rock preparation.

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The anomaly was declared after the distribution of TCE measurements (Appendix M) suggested an outlier and the lower DO concentration was not similar to other abiotic treatments of the same preparation. No time or sampling sequence component was identified that might have affected the responses. The initial assessment of factor relationships suggested differences in TCE measurement for groundwater-only/crushed rock (preparation), abiotic/biotic (treatment) and biostimulant addition (injection). The full factorial model indicated that the three way interaction of factors (preparation x treatment x biostimulant addition) was not significant, nor was the two way interaction of

Table 18. TCE measurements and estimated decrease in concentration at t = 160 d, 45 d after biostimulant addition.

		TCE Measurement (Estimated Decrease in Concentration) for Biostimulants (ug/L) (n = 2 for all results)			Estimated Initial TCE Concentration (ug/L)
Preparation	**Treatment**	**Sterile Groundwater**	**Nutrient Mixture[1]**	**Vitamin and Nutrient Mixture[2]**	
Groundwater-Only	**Abiotic**	476 ± 21 (2 ± 20)	455 ± 35 (22 ± 35)	495 ± 21 (-18 ± 21)	477
	Biotic	371 ± 29 (137 ± 28)	421 ± 14 (88 ± 15)	396 ± 21 (112 ± 20)	507
Crushed Rock	**Abiotic**	456 ± 92 (115 ± 92 [3])	470 ± 43 (100 ± 42)	520 ± 14 (50 ± 14)	570
	Biotic	321 ± 56 (280 ± 57)	380 ± 0 (219 ± 0)	320 ± 14 (281 ± 13)	600

Notes 1. Nutrient mixture = Shultz 10-15-10 Plant Food Plus in sterile groundwater.
2. Vitamin and nutrient mixture = Centrum Liquid and Shultz 10-15-10 Plant Food Plus in sterile groundwater.
3. Value of 50 with n = 1 after removal of anomalous result; as used in factorial evaluation.
4. Estimated TCE degradation calculated from difference between measured TCE concentration and estimated initial TCE concentration.

preparation x biostimulant addition, so these combinations were removed. Biostimulant addition was not significant (p = 0.73), but the interaction of abiotic/biotic x biostimulant

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addition was significant ($p = 0.015$), so the biostimulant factor was retained. These results suggest that biostimulant effects could be obscured by the greater difference in TCE measurements between abiotic/biotic treatments, unless the interaction is evaluated. With the unnecessary terms removed and the model rerun, significant factors and interactions were abiotic/biotic ($p < 0.0001$), groundwater-only/crushed rock x abiotic/biotic ($p = 0.002$), and abiotic/biotic x biostimulant addition ($p = 0.0056$). The model residuals did not exhibit trends, suggesting the validity of the model assumptions. Optimum factor settings (i.e., those that generated the lowest TCE measurements) were associated with addition of sterile groundwater into biotic crushed rock preparations, although vitamin and nutrient addition for the same treatment and preparation combination was fairly similar. The initial TCE concentration was estimated for each treatment and preparation combination from stoichiometric relationships, from which the TCE measurements were subtracted to develop a transformed data set representing TCE degradation at $t = 160$ d (Table 18, Appendix M).

The magnitudes of the effect estimates plotted in the JMP In interaction profiler (Figure 5) suggested that treatment (abiotic or biotic) had the greatest effect on TCE degradation, while the presence of groundwater-only or crushed rock had a large, but lesser impact. The dissimilar slopes of the treatment by preparation interactions graphically demonstrated the interaction of these factors. The lack of significance for the biostimulant addition without the interaction of other factors was illustrated by the relatively small amount of difference in TCE degradation. In contrast, the significance of the interaction of biostimulant addition with treatment is illustrated by the large

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difference in TCE degradation between the treatment levels and the divergence in response slopes for the three additives.

Interaction Profiles

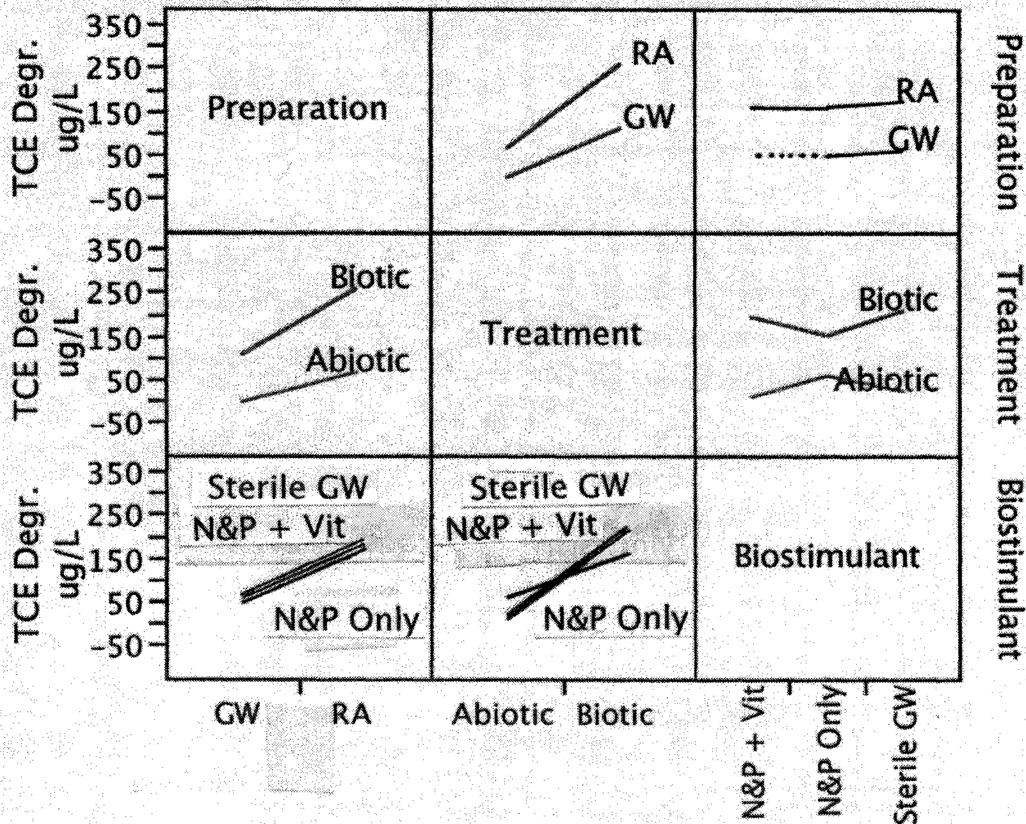

Figure 5. JMP In interaction profiler (software output) for change in TCE concentration (GW = groundwater-only, RA = crushed rock, N&P + Vit = nutrient and vitamin biostimulant, N&P Only = nutrient biostimulant, Sterile GW = sterile groundwater-only). Note: individual points at each factor setting not shown in output by software.

Optimum factors for the TCE degradation model (Figure 6) were associated with sterile groundwater-only addition into biotic treatments with crushed rock microcosms (TCE degradation of 282.3 ± a common variance of 31.7 ug/L), although the vitamin and nutrient addition for the biotic and crushed rock microcosms was nearly as optimal (TCE degradation of 270.1 ± 31.7 ug/L). Nutrient addition alone provided the least TCE

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degradation of the biostimulants (227.1 ± 31.7 ug/L). The pattern of biostimulant effects was the same for the biotic treatment of both groundwater-only and crushed rock, although the former had a TCE degradation of about 150 ug/L less than the crushed rock for all additives. These results suggested that nutrient addition slows or inhibits the degradation of TCE. Vitamin addition appeared to offset this effect. Augmenting this factorial experiment with additional studies of individual or groups of compounds from the vitamin mixture using this methodology is recommended to isolate and identify the specific factor or interaction responsible for the inhibitory compensation, then optimize TCE degradation using biostimulants.

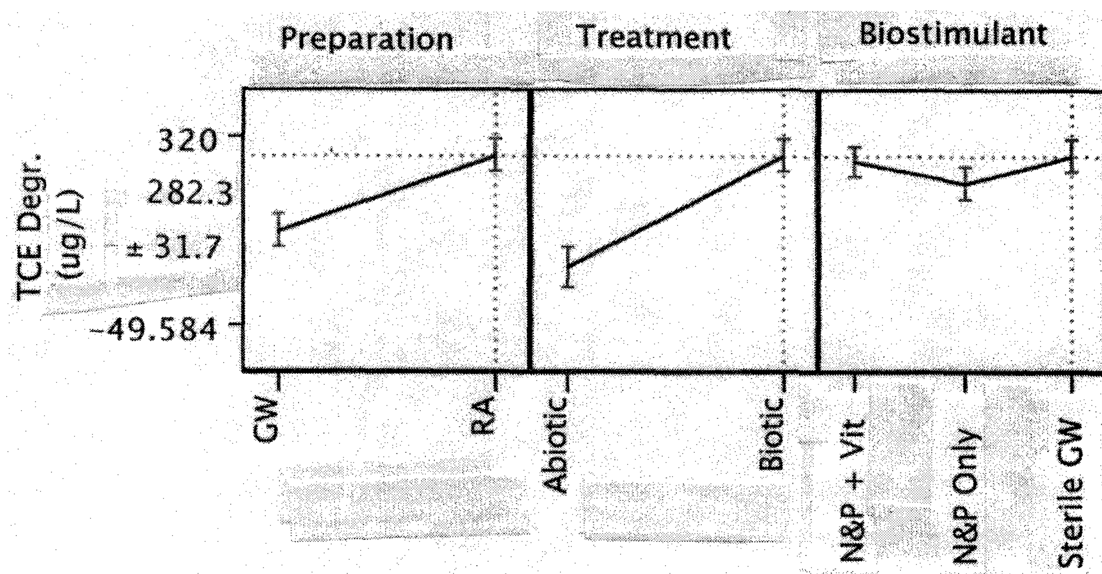

Figure 6. JMP In prediction profiler (software output) set for optimum conditions of TCE concentration change, with numerical optimum calculated by the software. Dotted lines indicate optimum factor setting.

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Conclusions

In this study, microcosms were applied to the factorial evaluation of biostimulated TCE dechlorination in fractured bedrock aquifers to assess selected compound mixtures as potential biostimulants to enhance the degradation. TCE degradation was initially evaluated (ANCOVA and first order degradation kinetic analysis) and degradation half-lives determined, to document biotic activity and characterize how well the microcosms represent the *in situ* conditions. Microcosm preparation with crushed rock in groundwater was found to more closely match the previously observed field rates than the preparation with only groundwater. Injection of nutrient and vitamin mixtures were made into microcosms that were previously aged to obtain consistent conditions, and the TCE concentration measured after incubating for 45 days. Comparison of results indicated that the nutrient mixture slows or inhibits the degradation of TCE compared to the sterile groundwater; however, the vitamin mixture offsets and nearly compensates for the inhibitory effect of the nutrient mixture. Recommendation is made to augment this factorial experiment with additional studies of individual or groups of compounds from the vitamin mixture using this methodology is recommended to isolate and identify the specific factor or interaction responsible for the inhibitory compensation.

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CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

Bedrock aquifers are important natural resources that many people use as their drinking water source. Trichloroethene (TCE), an organic solvent used by industry and the military for cleaning metal parts, is heavier than water, recalcitrant to degradation in most environments, and can migrate down into fractured bedrock aquifers. TCE has been linked to significant human health effects when it is a groundwater contaminant (Moran, 2006). While microbial dechlorination, a key step in the remediation of TCE contaminated groundwater, has been directly demonstrated in several environments, it has only been indirectly observed in bedrock aquifers (i.e., through geochemical assessment or gene identification evidence) (Yager et al., 1997; Lenczewski et al., 2003; Lehman et al., 2004). The rate of dechlorination impacts the evaluation of human health risk at sites with TCE contamination. Microcosms are needed to evaluate the potential for and rate of biodegradation in fractured rock aquifers because there typically are few monitoring wells drilled into rock, so little *in situ* data is available (Wiedemeier, et al., 1998).

Biodegradation of TCE appears to be controlled by several important factors: the presence of a suitable chlororespiring microbial population; the redox of the environment; the presence of alternate and potentially competing terminal electron acceptors (TEAs); the reduction kinetics of individual chloroethene compounds; the electron donor supply; presence of specific inhibitory compounds; and the distribution

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and movement of TCE through the aquifer (Bradley, 2003). Microcosms consisting of groundwater placed into airtight glass bottles have become a highly effective tool for demonstrating the potential for *in situ* biodegradation, assessing the efficacy of amendments, and estimating degradation rates.

Three main questions were addressed in this study to advance the measurement and characterization abilities for *in situ* reductive dechlorination of TCE in fractured rock aquifers. The results of this work are addressed for each question individually in the following sections.

How well do microcosms model *in situ* reductive dechlorination of TCE in fractured rock aquifers and are there preparation techniques that can improve the model? Microcosms constructed with unincubated crushed rock in groundwater were found to provide the best model of in situ TCE degradation for the Site 32 fractured rock aquifer, when prepared to and incubated at conditions that simulate in situ conditions. Microcosms constructed with only groundwater were also effective at modeling the TCE degradation in the Site 32 fractured rock aquifer, although only when the microcosms were amended with TOC. Incubation of crushed rock within residual TCE plume caused a substantial decline in TCE degradation for biotic intrinsic microcosms, suggesting an effect that is inhibitory to TCE degrading microbes. This inhibitory effect may be mitigated but not negated by the inclusion of total organic carbon (TOC). Glass beads are an inadequate substitute for rock media due to increases in pH and dissolved oxygen caused by glass corrosion, the hydrolysis and ion exchange of glass when in contact with water for long periods, particularly lime soda glass (Duffer, P.F. (1986). Inoculation provided by incubated granular material only provided insufficient microbial population

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or metabolic activity in the microcosms to effectively model in situ activity. These results were tempered by the inability to diagnose progeny (i.e., breakdown products) from the reductive dechlorination process. While Hoelen and Reinhard (2004) indicated that TCE can be quickly mineralized with minimal chlorinated progeny at certain H_2 concentrations, the lack of definitive proof for reductive dechlorination leaves the results of this study suggestive rather than conclusive. Concern exists about the potential for TCE to sorb to mineral or organic matter within the microcosms, although no evidence of this was noted in the abiotic microcosms. The main problem was the inability to quantify all aspects of the mass balance. Future work should include experiments with radiolabeled TCE to define the progeny compounds and increase the certainty of the results. Additional evidence for reductive dechlorination in microcosms could be developed from greater geochemical measurements (H_2 and alkalinity in particular, although sample size is a concern) and the documented presence of dehalogenating bacteria (DHB) by enumeration techniques including polymerase chain reaction (PCR) and fluorescent *in situ* hybridization (FISH) with DHB probes.

How well do microcosms resolve very slow rates (half-lives slower than 300 days) of *in situ* reductive dechlorination of TCE in fractured rock aquifers, and what is the limit of a monitored natural attenuation (MNA) determination? In the second part of this study, resolution of very slow rates was demonstrated to be primarily a function of the variability in the microcosm method, which was subsequently evaluated. The abiotic microcosm preparations with crushed rock in groundwater had substantially less total variability ($S_{microcosm}^2$) than the groundwater-only preparations, which matched the trend from the previous microcosm experiment. Overall variability was much reduced in this

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experiment due to the increased number of replications and analyses compared to the previous effort as well as the likely factor of improved operator ability. The results suggest that microcosms with crushed rock core media have less uncertainty for the determination of the degradation rate and thus can place the mean within a smaller confidence interval. This finding is significant, in that microcosms with crushed rock core media better accomplish the evaluations described by Madsen (1991) when applied to the evaluation of bedrock aquifers. First, reduced variability provides the ability to assess smaller differences between abiotic and biotic treatments to distinguish biologically mediated activity. Second, reduced variability allows greater statistical significance when comparing abiotic losses and measurements of degradation in the field. Third, reduced variability creates greater statistical confidence in determinations of ecological adaptation through comparisons of pristine to contaminated conditions. These improvements to the biodegradation evaluations are especially beneficial for fractured rock aquifers that have potentially slow degradation rates and therefore small differences between microcosm treatments.

The lower variability in the microcosms with crushed rock core media may be caused by better mixing or distribution of the TCE or perhaps improved homogeneity within the closed system of the microcosm, although further analysis would be necessary to determine the cause with certainty. Using the variances determined, the maximum half-life (i.e., slowest resolvable degradation) can be calculated for a microcosm study of the same preparation and analysis for a given study duration, statistical probability, abiotic concentration, and bottle replicate and sample scheme. The results of this study demonstrated that microcosms for reductive dechlorination of TCE may be more robust

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than previously indicated, as increased control of contaminant mass is suggested by the reduced variability of the second microcosm experiment.

How well do microcosms function in factorial evaluations of potential biostimulants for *in situ* reductive dechlorination of TCE in fractured rock aquifers? In the third part of this study, microcosms were applied to the factorial evaluation of biostimulated TCE dechlorination in fractured bedrock aquifers to assess selected compound mixtures as potential biostimulants to enhance the degradation. TCE degradation was initially evaluated (ANCOVA and first order degradation kinetic analysis) and degradation half-lives determined, to document biotic activity and characterize how well the microcosms represent the *in situ* conditions. Microcosm preparation with crushed rock in groundwater was again found to more closely match the previously observed field rates than the preparation with only groundwater, indicating an advantage conferred by the crushed rock media which could be habitat benefits (e.g., increased colonization sites), nutritional benefits (e.g., increased mineral and nutrient dissolution), or improved buffering capacity related to the crushed rock media.

Injection of nutrient and vitamin mixtures were made into microcosms that were previously aged to obtain consistent conditions, and the TCE concentration measured after incubating for 45 days. Comparison of results indicated that the nutrient mixture slows or inhibits the degradation of TCE compared to the sterile groundwater; however, the vitamin mixture offsets and nearly compensates for the inhibitory effect of the nutrient mixture.

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Recommendations for Future Research

Additional TCE release sites should be evaluated using microcosm experiments, in which rates can be compared to those *in situ*. Of great interest is the level of consistency in the comparisons of the laboratory determined rates of TCE reductive dechlorination from microcosms incubated at *in situ* temperatures to those *in situ* at different fractured rock aquifer locations.

Recommendation is made to identify the advantage conferred by crushed rock in microcosms, whether habitat, nutrition or geochemical buffer. Similarly, experiments are recommended to identify the factor that is inhibitory to TCE degrading microbes when crushed rock is incubated within the Site 32 residual TCE plume that caused a substantial decline in TCE degradation for biotic intrinsic microcosms. Experiments are also recommended to quantify the reduction in microbial population or metabolic activity that causes the decline in TCE dechlorination when microcosm inoculation is provided by incubated granular material only.

Recommendation is made to determine the cause of the lower variability in the crushed rock microcosms, assessing potential for better mixing or distribution of the TCE or perhaps improved homogeneity within the closed system of the microcosm.

As the results suggested that nutrient addition slows or inhibits the degradation of TCE while vitamin addition appeared to offset this effect, it is recommended that the factorial experiment be augmented with additional studies. Individual or groups of compounds from the vitamin mixture could be assessed using factorial methodology with the microcosms to isolate and identify the specific factor or interaction responsible for the inhibitory compensation, then optimize TCE degradation using biostimulants.

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