LABORATORY EVALUATION OF CHLORINATED ETHENE TRANSFORMATION PROCESSES IN FRACTURED SANDSTONE

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ABSTRACT

A fractured sandstone aquifer at an industrial site in southern California is contaminated with trichloroethene (TCE) to depths in excess of 244 m. Field monitoring data suggest that TCE is undergoing reduction to cis-DCE and that additional attenuation is occurring. However, vinyl chloride (VC) and ethene have not been detected in significant amounts, so that if transformation is occurring, a process other than reductive dechlorination must be responsible. The overall objective of this study was to evaluate the occurrence of biotic and abiotic transformation processes at this site for TCE, cis-DCE and VC. Anaerobic microcosms were constructed with site groundwater and sandstone core samples. $^{14}$C-labeled compounds were used to detect transformation products (e.g., CO$_2$ and soluble products) that are not readily identifiable by headspace analysis. The microcosms confirmed the occurrence of biotic reduction of TCE to cis-DCE, driven by electron donor in the groundwater and/or sandstone. VC and ethene were not detected during this part of the study. Following incubation periods up to 22 months, the distribution of $^{14}$C indicated statistically significant transformation of $[^{14}\text{C}]\text{TCE}$ and $[^{14}\text{C}]\text{cis-DCE}$ in live microcosms, to as high as 10% $^{14}\text{CO}_2$ from TCE and 20% $^{14}\text{CO}_2$ from cis-DCE. In autoclaved microcosms, significant transformation of $[^{14}\text{C}]\text{TCE}$ and $[^{14}\text{C}]\text{cis-DCE}$ also occurred; although some $^{14}\text{CO}_2$ accumulated, the predominant $^{14}$C product was soluble and could not be stripped by N$_2$ from an acidic solution (referred to as non-strippable residue, or NSR). Characterization of the NSR by high performance liquid and ion chromatography identified glycolate, acetate and formate as significant components.
The site contains minerals typical of what is found in sandstone for this region (i.e., iron sulfides, pyrite, fougerite (green rust), magnetite, biotite, vermiculite, and quartz) suggesting that these minerals may play a role in the abiotic transformations observed during the microcosm study. In order to evaluate the role of sandstone composition on the rate and extent of cis-DCE transformation, further experiments were conducted with autoclaved typical and pyrite rich sandstone from the site, as well as with pure pyrite. Since reductive dechlorination was a predominant transformation pathway in the microcosms for TCE but not for cis-DCE, these additional studies were conducted with $[^{14}\text{C}]{\text{cis}}$-DCE. The results suggest that pyrite is not responsible for abiotic transformation of cis-DCE. By contrast, the autoclaved typical sandstone was able to transform as much as 16% of the $[^{14}\text{C}]{\text{cis}}$-DCE to $[^{14}\text{C}]\text{NSR}$ and $[^{14}\text{C}]\text{CO}_2$.

During the microcosm study, the extent of cis-DCE transformation to NSR and CO$_2$ appeared to be greater in the autoclaved versus live treatments. Subsequent experiments were conducted to further test the effect of sterilization method on the rate and extent of abiotic transformation of cis-DCE in the presence of typical sandstone and groundwater. Autoclaving was compared to use of propylene oxide as a method of sterilization, both of which were compared to live microcosms. Formation of $[^{14}\text{C}]\text{NSR}$ and $^{14}\text{CO}_2$ from $[^{14}\text{C}]{\text{cis}}$-DCE was confirmed. Surface area normalized first order rates of cis-DCE transformation for typical sandstone were $1.08\times10^{-5}\pm1.1\times10^{-6}$ L/m$^2$d for the autoclaved treatment, $1.31\times10^{-5}\pm2.8\times10^{-6}$ L/m$^2$d for the live treatment, and $1.00\times10^{-6}\pm3\times10^{-9}$ for the propylene oxide treatment. Based on XPS analysis of sandstone from the different treatments, autoclaving appears to have increased the availability of magnetite
and goethite for the abiotic transformations. In the live sandstone, ferrous (FeO) and ferric oxides (Fe₂O₃) were present and may be responsible for at least a part of the cis-DCE transformation. Sterilizing with propylene oxide appeared to inhibit transformation of cis-DCE to NSR and CO₂. Minor amounts of VC, acetylene, ethene and ethane were formed in both the live and propylene oxide treated sandstone. Detection of these products was likely related to the higher initial concentration of cis-DCE that was used in these experiments versus the microcosms. The only volatile product in the autoclaved treatment was acetylene.

While the microcosm study confirmed the occurrence of TCE reductive dechlorination to cis-DCE, it did not reveal which type of microbe was responsible for this biotic transformation. A third set of experiments was conducted to evaluate which microbes is response for biotic reduction of TCE to cis-DCE. Enrichment in dechlorinating activity was achieved by repeatedly adding TCE to a microcosm that exhibited reductive dechlorination activity. A sample from the microcosm was used as inoculum for serial dilutions in anaerobic mineral medium. The composition of the microbial community in the microcosm and serial dilutions was evaluated using denaturing gradient gel electrophoreses. Individual bands from the gel were sequenced. *Pseudomonas stutzeri* was identified as the organism in the community that is most likely responsible for the reduction of TCE to cis-DCE. PCR-DGGE analysis of DNA extracted from enrichments of the microorganisms provided a sequence that matched 100% to *Pseudomonas stutzeri*. Only one other facultative anaerobe is known that can
dechlorinate TCE to $cis$-DCE under anaerobic conditions, strain MS-1. *Desulfovibrio putealis*, a sulfate reducer, was also found to be present in the community.

Overall, the results of this dissertation demonstrated that a combination of abiotic and biotic transformation processes is responsible for attenuation of TCE and $cis$-DCE in the fractured sandstone aquifer. Tracking the distribution of $^{14}C$ during the microcosm study was essential for observing these phenomena.
DEDICATION

This dissertation is dedicated to my mother, Jean Gresham. You encouraged and supported me while I pursued my dreams. Thank you for your patience and prayerful guidance.
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INTRODUCTION

1.1 Monitored Natural Attenuation

To control the risks posed by chemical contamination of the environment, monitored natural attenuation (MNA) has become a widely accepted alternative to more expensive methods such as pump-and-treat. MNA processes include biodegradation, dispersion, sorption and volatilization, and are the chosen alternative only when it can achieve remedial objectives specific to the site within a reasonable time frame. Natural attenuation, therefore, affects the fate and transport of chlorinated solvents in the environment (1).

The US Environmental Protection Agency (EPA) (1) has a “lines of evidence” approach that can be used to determine the validity of applying MNA to a site. The three lines of evidence are 1) documented loss of contaminant over time; 2) hydrogeological and geochemical data that document transformation or degradation processes and rates; and 3) direct observation of contaminant loss in laboratory or field microcosm studies. If the first two lines of evidence prove to be inconclusive, then and only then, is the third line of evidence required.

The MNA protocol of the EPA (1) focuses on anaerobic degradation of chlorinated ethenes by microorganisms. Reductive dechlorination, anaerobic oxidation and cometabolism are the processes defined in the MNA protocol. Reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE) yields unique daughter products that are easily measurable in the field. They are cis-dichloroethene (DCE), vinyl chloride (VC) and ethene. It is, therefore, easy to show that natural
attenuation is occurring by reductive dechlorination when PCE or TCE concentrations are decreasing and the daughter products are present at the site.

PCE, TCE, DCE and VC can all be degraded by reductive dechlorination under anaerobic conditions. Anaerobic oxidation occurs more readily with cis-DCE and VC. There is currently no evidence that anaerobic oxidation removes PCE and TCE. Proving that anaerobic oxidation occurs in the field is difficult because the ultimate daughter products are chloride ions and carbon dioxide. These products are not easily measured in the field unless the initial concentrations of cis-DCE and VC are very high.

Several microorganisms have been identified and/or isolated that are able to reductively dechlorinate TCE. Some of them are only able to reduce TCE to its immediate daughter product, cis-DCE (2-12), while others have the ability to reduce it to its terminal and harmless end product, ethene (13-20). The Dehalococcoides genus of microorganisms is known to dechlorinate TCE to its harmless daughter product, ethene. Dehalococcoides ethenogenes strain 195 (21) and Dehalococcoides ethenogenes strain FL2 (18) are microorganisms that have been isolated that can reduce TCE to VC metabolically, then cometabolically convert VC to ethene. Dehalococcoides strain GT (20) and strain VS (15) are capable of chlororespiring TCE to ethene. Both strain VS and GT were obtained from contaminated sites (14,20). Dehalospirillum, now referred to as Sulfurospirillum, dechlorinates TCE only to cis-DCE (6,7,10,22). Other microorganisms that can only dechlorinate TCE to cis-DCE include Dehalobacter restrictus (2), Desulfitobacterium sp. (4,23,24), Desulfuromonas sp. (3,5,25), and Geobacter lovleyi strain SZ (11,26), which is the only Geobacter currently known that can dechlorinate
TCE. Growth of these chlororespiring microbes commonly occurs under anaerobic conditions. By contrast, a facultative aerobe (Strain MS-1) has been isolated that is also capable of reductively dechlorinating TCE to cis-DCE (27). Strain MS-1 grows under strict anaerobic conditions with several electron donors, including acetate, lactate and pyruvate (27).

In order for microorganisms to grow a source of electrons, an electron acceptor and a carbon source is required. During chlororespiration TCE serves as the electron acceptor and carbon often originates from organic carbon in the sediment. Hydrogen is considered the universal electron donor for the reductive dechlorination of TCE (28,29). Several compounds can serve as carbon sources, including acetate (18), pyruvate (27), and ethanol (24). Some of the carbon sources also serve as electron donors (11,26). For sites where the microbes present can only reductively dechlorinate TCE to cis-DCE, it is important to determine if a pathway exists for the removal of cis-DCE other than via biotic reductive dechlorination.

Lee and Batchelor (30) have provided evidence in support of abiotic reactions taking place in groundwater that can assist in the degradation of chlorinated ethenes, including cis-DCE. The presence of iron-bearing minerals in subsurface sediment reportedly increases the rates of dechlorination by acting as electron donors and carriers oh and two people got fired at work on Friday and they told us today. At locations in which iron and sulfate reduction occurs, chlorinated compounds may be degraded abiotically. Iron-containing minerals, e.g., iron sulfide, pyrite, fougerite, magnetite and Fe(II)-containing phyllosilicates are potential contributors to the dechlorination reactions,
which appear to be surface catalyzed (30-32). Butler and ayes (33) found that acetylene
was a major product of abiotic reactions involving chlorinated ethenes.

The common practice when conducting abiotic studies in the laboratory is to
monitor for volatile products (and specifically acetylene) as evidence for the
transformation of the chlorinated ethenes. However, soluble compounds may also be
important products from abiotic transformation of TCE or cis-DCE. Only one study has
been found that provides evidence of the abiotic transformation of chlorinated ethenes to
soluble products, specifically glycolate and formate (34). This transformation, which
occurred during thermal remediation of TCE, also yielded CO₂ as a product (34).
Therefore, abiotic reactions may be responsible for the degradation of cis-DCE at sites
where the microorganisms needed for complete dechlorination to ethene (i.e.,
Dehalococcoides) are effectively absent.

When conducting abiotic studies in the laboratory the common method used to
sterilize the soils is autoclaving. However, several studies have shown that autoclaving
may alter the soil properties (35-37). For example, extractable manganese and pH levels
increased after autoclaving several types of soil, while extractable iron levels decreased
(36-38). Ammonia and nitrate-nitrogen increased in autoclaved sandy loam soils (38).
Depending on the mechanism of transformation and the role that soils play in abiotic
transformations, changes to soils as a result of autoclaving may alter the rate or extent of
transformations of chlorinated ethenes.
1.2 MNA Case Study

The groundwater at an industrial site in California is contaminated with TCE at concentrations ranging from 2.1 to 6,583 µg/L (41) although concentrations as high as 5,000 mg/L have been found in pore water. Most of the TCE release occurred at the surface and percolated into the underlying sandstone. cis-DCE is also present in the groundwater, at concentrations ranging from 1.9 to 2,826 µg/L although concentrations as high as 500 mg/L have been found in the pore water (39). Only minor amounts of VC have been detected. The site is underlain with fractured sandstone of a turbidite formation (sand-rich formations occur in deep water sediments due to turbidity current deposits) and is referred to as the Chatsworth Formation. The turbidite originated under the sea and was uplifted and faulted 20 million years ago. The Chatsworth Formation is primarily composed of sandstone with shale interbeds. Analysis of crushed sandstone from the site shows that it is rich in iron-bearing minerals, including iron sulfide (FeS), pyrite (FeS$_2$), fougerite, magnetite, biotite and vermiculite. Pyrite veins are also very visible in cores taken from the site. The major minerals are quartz, biotite and plagioclase feldspar. The more minor minerals are pyrite and calcite. The dominant anions present in the site groundwater are bicarbonate (200-800 mg/L), sulfate (4-810 mg/L) and chloride (38-130 mg/L). The dominant cations are calcium (25-200 mg/L) and sodium (50-225 mg/L) (39). The average pH is 7.1. The dissolved organic matter concentration in the groundwater averages 2.1 mg/L. The sandstone contains 0.53-0.83% organic matter (39).

Fractured rock makes evaluation of transport and flow paths for chlorinated ethenes very complicated. Field studies at the site provide some support for the first two
lines of evidence outlined in the MNA protocol published by the EPA. Historical concentration data from seven wells showed an increase in cis-DCE concentrations during the period from 1995 to 2002, with cis-DCE to TCE molar ratios as high as 2.75 in the groundwater and >100 in the pore water in 2002. This increase in the daughter product to parent ratio is a clear indicator of ongoing reductive dechlorination. Only minor amounts of VC and ethene are present, suggesting that biologically mediated reductive dechlorination is not a significant fate process. Nevertheless, cis-DCE concentrations have shown some decrease. The geochemical environment appears to be sulfate- and iron-reducing due to the presence of Fe(II)- and Fe(III)-bearing minerals. Also, isotope fractionation analysis of the groundwater indicated the enrichment of δ^{34}S relative to the lighter ^{32}S, which provides evidence that sulfate-reducing conditions exist, i.e., that sulfate is being converted to sulfide by sulfate-reducing bacteria, since bacteria use the lighter isotope preferentially. The main source of sulfate from the site is oxidation of pyrite. Measuring the concentration of geochemical indicators at the site indicated the absence of methanogenesis (39) due to the lack of methane production. This is somewhat consistent with the lack of major conversion of cis-DCE to VC and ethene, which often (although not always) occurs concurrently with methanogenic conditions.

Groundwater monitoring data taken over many years suggests that the chlorinated ethenes are attenuating. Consistent with this observation is the fact that TCE and cis-DCE have not appeared in surface water around the site, which led to the hypothesis that TCE, cis-DCE, and VC undergo anaerobic transformation at this site, either biotically or
abiotically, by pathways other than sequential reductive dechlorination. A major focus of this dissertation involved an evaluation of this hypothesis.

Isotopic analysis of $\delta^{13}$C$_{\text{TCE}}$ in field samples has provided further evidence that TCE undergoes biotic and abiotic degradation. During abiotic degradation, $\delta^{13}$C$_{\text{TCE}}$ becomes enriched in the range of $\varepsilon = 7$ to 22 ($\varepsilon$ is the change in the ratio of the heavy isotope to the lighter isotope from the standard value). During biotic degradation, $\delta^{13}$C$_{\text{TCE}}$ values are less enriched, from $\varepsilon = 3$ to 7. Isotopic analysis of the groundwater showed $\delta^{13}$C$_{\text{TCE}}$ enrichment in both the biotic and abiotic ranges, indicating that TCE is being degraded by both processes at the site. $\delta^{13}$C$_{\text{DCE}}$ results suggest no substantial degradation of cis-DCE by either biotic or abiotic processes at the site. Acetylene, a known product of abiotic chloroethene transformation, has been detected, but only at relatively low concentrations (3-17 µg/L) (39).

The extensive field data from this site is compelling with respect to the first two lines of evidence needed to establish MNA of TCE and cis-DCE, although not entirely conclusive. The lack of evidence for transformation of TCE and cis-DCE by a pathway other than reductive dechlorination raises some doubt as to how these compounds are attenuating. Complicating this issue is the fact that fractures in the sandstone make it difficult to locate and follow the plume. Given these uncertainties, a third line of evidence is needed to confirm the occurrence of biotic reduction of TCE to cis-DCE and to establish that natural attenuation of TCE and cis-DCE is also occurring by processes other than reductive dechlorination, such as anaerobic oxidation. Previous research has shown that chlorinated ethenes can be transformed by abiotic reactions in the presence of
minerals (30,33,40). A key question that arises for the California site is whether TCE and cis-DCE are being transformed by processes other than reductive dechlorination. Evaluation of this question was a major focus of this work. Transformation of TCE, cis-DCE and VC was evaluated using $^{14}$C-labeled compounds in various types of microcosms, prepared with groundwater and sandstone core samples obtained from the site.

1.3 Objectives

The overall objective of this dissertation was to evaluate biotic and abiotic transformations of TCE, cis-DCE and VC in fractured sandstone, with an emphasis on identification and quantification of soluble transformation products. The specific objectives were:

1) To confirm the occurrence of biotic reductive dechlorination of TCE to cis-DCE in fractured sandstone in field samples from the southern California industrial site. This objective will be addressed in Chapter 2.

2) To evaluate the rate and extent of biotic and abiotic transformation of TCE, cis-DCE and VC to soluble products and CO$_2$ in the presence of fractured sandstone. This objective will be addressed in Chapter 2.

3) To determine if pyrite in the sandstone is responsible for catalyzing the abiotic transformation of cis-DCE to soluble products and CO$_2$, and if so, at what rate. This objective will be addressed in Chapter 3. This objective will be addressed in Chapter 3.
4) To determine if autoclaving alters the mineral composition of sandstone as well as its reactivity with respect to abiotic transformation of cis-DCE. This objective will be addressed in Chapter 3.

5) To measure a surface-area-normalized first order rate of abiotic transformation of cis-DCE. This objective will be addressed in Chapter 3.

6) To identify the microbe responsible for TCE reduction to cis-DCE at the industrial site in southern California and compare this microbe to others that are known to mediate this reaction via chlororespiration. This objective will be addressed in Chapter 4.

Chapters 2, 3 and 4 are “stand alone,” i.e., each presents an introduction, materials and methods, results and discussion section that fully address one or several of the specific objectives. The final chapter provides a comprehensive list of conclusions for this dissertation.

1.4 References


(39) Pierce, A. A.; Isotopic and Hydrogeochemical Investigation of Major Ion Origin and Trichloroethene Degradation in Fractured Sandstone. MS Thesis, Earth Sciences, University of Waterloo, **2005**.

BIOTIC and ABIOTIC ANAEROBIC TRANSFORMATIONS of
TRICHLOROETHENE and cis-1,2-DICHLOROETHENE in FRACTURED
SANDSTONE

2.0 Abstract

A fractured sandstone aquifer at an industrial site in southern California is contaminated with trichloroethene (TCE) and cis-1,2-dichloroethene (DCE) to depths in excess of 244 m. Field monitoring data suggest that TCE is undergoing reduction to cis-DCE and that additional attenuation is occurring. However, vinyl chloride (VC) and ethene have not been detected in significant amounts, so that if transformation is occurring, a process other than reductive dechlorination must be responsible. The objective of this study was to evaluate the occurrence of biotic and abiotic transformation processes at this site for TCE, cis-DCE and VC. Anaerobic microcosms were constructed with site groundwater and sandstone core samples. $^{14}$C-labeled compounds were used to detect transformation products (e.g., CO$_2$ and soluble products) that are not readily identifiable by headspace analysis. The microcosms confirmed the occurrence of biotic reduction of TCE to cis-DCE, driven by electron donor in the groundwater and/or sandstone. VC and ethene were not detected. Following incubation periods up to 22 months, the distribution of $^{14}$C indicated statistically significant transformation of $[^{14}\text{C}]$TCE and $[^{14}\text{C}]$cis-DCE in live microcosms, to as high as 10% $^{14}$CO$_2$ from TCE and 20% $^{14}$CO$_2$ from cis-DCE. In autoclaved microcosms, significant transformation of $[^{14}\text{C}]$TCE and $[^{14}\text{C}]$cis-DCE also occurred; although some $^{14}$CO$_2$ accumulated, the predominant $^{14}$C product was soluble and could not be stripped by N$_2$ from an acidic
solution (referred to as non-strippable residue, or NSR). Characterization of the NSR by high performance liquid and ion chromatography identified glycolate, acetate and formate as significant components. These results suggest that a combination of abiotic and biotic transformation processes is responsible for attenuation of TCE and cis-DCE in the fractured sandstone aquifer. Tracking the distribution of $^{14}\text{C}$ during the microcosm study was essential for observing these phenomena.

2.1 Introduction

Most assessments of natural attenuation of chlorinated ethenes focus on biologically catalyzed hydrogenolysis. This process is widely distributed and can be documented by monitoring volatile daughter products that are unique to degradation of the parent compounds ($^1$). However, there is mounting evidence that other processes contribute to natural attenuation, including anaerobic oxidation ($^2$-4) and abiotic reductive transformations ($^5$-9). These processes are considerably more difficult to document in situ if the products formed are not uniquely identifiable volatile compounds (i.e., less chlorinated ethenes and ethane). Transformation products such as CO$_2$ and chloride cannot typically be attributed solely to chlorinated ethene degradation.

Several recent studies have demonstrated the potential for abiotic transformation of chlorinated ethenes in the presence of soil minerals. Lee and Batchelor ($^6$-$^8$) observed surface-catalyzed abiotic reduction of chlorinated ethenes by the iron-bearing minerals pyrite, magnetite and green rust. Abiotic reduction of tetrachloroethene, trichloroethene (TCE), and 1,1-dichloroethene (DCE) has been demonstrated in aqueous solutions of iron sulfides ($^5,$10). Acetylene is a key transformation product during abiotic reduction,
indicating the prevalence of $\beta$-elimination as the dechlorination pathway (5-8,10,11). Abiotic transformations of chlorinated organics may, therefore, be significant at sites with iron-rich minerals, including iron sulfide, pyrite, fougereite, magnetite and Fe(II)-containing phyllosilicates. Ferrey et al. (9) attributed natural attenuation of $cis$- and 1,1-DCE to abiotic transformation processes in aquifer sediment containing magnetite at a contaminated site in Minnesota, based on equivalent removal rates from live and autoclaved microcosms. Kennedy et al. (12) attributed attenuation of TCE (commingled with fuel hydrocarbons) at Altus Air Force Base, Oklahoma to FeS-mediated abiotic degradation, in an aquifer with naturally high concentrations of sulfate and Fe(III) minerals. Volatile daughter products have been monitored in many of these studies (5-10), although none examined the potential for biotic and abiotic transformation of chlorinated ethenes to soluble products, as well as CO$_2$.

At an industrial site in southern California, thousands of kilograms of TCE were discharged to the surface for several decades following its use in degreasing operations. Some of the TCE percolated into the underlying sandstone and has been found in groundwater as deep as 244 m below grade. Field data indicate that most of the TCE has diffused into the rock matrix and some has undergone reductive dechlorination to $cis$-DCE. Only minor amounts of vinyl chloride (VC) and ethene have been found at the site, suggesting that biological dechlorination is incomplete. Nevertheless, the TCE and $cis$-DCE plumes do not appear to have traveled as far as would be expected (13). This raises the prospect that, in addition to attenuation by diffusion, TCE and $cis$-DCE are undergoing some type of biotic or abiotic transformation other than by reductive
dechlorination. The site contains minerals typical of what is found in sandstone for this region (i.e., iron sulfides, pyrite, fougerite, magnetite, biotite, vermiculite, and quartz) and the groundwater is predominantly anaerobic (13). This suggests that geochemical conditions may be conducive for transformation of chlorinated ethenes by abiotic as well as biotic processes.

The objective of this study was to evaluate the occurrence of biotic and abiotic transformation processes at this site for TCE, cis-DCE and VC. Site groundwater and sandstone core samples taken from four depths were used to construct microcosms. In addition to monitoring for volatile daughter products, use of $^{14}$C-labeled compounds made it possible to evaluate formation of $^{14}$CO$_2$ as well as soluble products that may not be easily detected in field samples. Biotic reductive dechlorination of TCE in the microcosms confirmed field observations of cis-DCE accumulation. In addition, significant amounts of $^{14}$C-labeled soluble products (including acetate, glycolate and formate) and CO$_2$ were detected in the microcosms. This appears to be the first report of soluble product formation from abiotic transformation of chlorinated ethenes in the presence of sandstone.

2.2 Materials and Methods

2.2.1 Site Geology and Sample Collection

The site is underlain with material referred to as the Chatsworth formation, consisting of interbedded sandstone and shale that was deposited by marine turbidites that were uplifted during the Upper Cretaceous (13). The core samples used in this study consist mainly of coarse grained sandstone, collected at depths of 161, 173, 222 and 265
These depths were selected to cover a range of sandstone composition (from typical to biotite-rich). Samples were collected with an air rotary drilling rig (Ingersoll-Rand TH-60) equipped to drill and retrieve PQ-size core (8.48-cm diameter). Water was used from hydrants on site, when necessary, to cool the drill bit. Cores were collected in 1.52-m lengths. All drilling equipment that came into contact with the rock core was decontaminated at the end of each core run, by washing and scrubbing with laboratory grade, non-phosphate detergent and rinsing with deionized water. Core catchers were cleaned between core runs using soapy water and a drill water rinse.

Cores were shipped to the University of Waterloo where they were crushed using a schedule 304 stainless steel cylinder and piston. Parts that came in contact with the rock were decontaminated between samples by washing with a phosphate-free detergent; rinsing sequentially with tap water, methanol, and distilled water; and drying with clean paper towels. Crushed rock was shipped to Clemson University and stored in an anaerobic chamber until the microcosms were prepared. Groundwater was obtained from a well close to the core hole and stored at 4°C prior to preparing the microcosms. At the time of microcosm preparation, the groundwater contained approximately 78 µg/L of TCE; cis-DCE and VC were below detection.

2.2.2 Chemicals

Neat [14C]TCE and [14C]cis-DCE were purchased from Sigma-Aldrich (5.4 mCi/mmol) and Moravek Biochemicals (1.1 mCi/mmol), respectively. [14C]VC was purchased dissolved in toluene from Perkin Elmer Life Sciences (1.4 mCi/mmol).
Formate dehydrogenase (8.8 units/mg protein) and β-NAD (reduced form, disodium salt) were obtained from Sigma Aldrich. All other chemicals used were reagent grade.

2.2.3 Microcosms

Three types were prepared: 1) live, designed to simulate in situ conditions; 2) autoclaved controls (AC), to determine the extent of abiotic activity; and 3) water controls (WC), to determine the extent of diffusive losses through the septa. The live treatments consisted of 48 bottles with $[^{14}\text{C}]\text{TCE}$ added (12 bottles with rock from each of the four depths tested: 161, 173, 222 and 265 m), 48 with $[^{14}\text{C}]\text{cis-DCE}$ added, and 48 with $[^{14}\text{C}]\text{VC}$ added (total of 144 live bottles). The same design was repeated for the AC treatments (i.e., total of 144 AC bottles). The WC treatments consisted of 12 bottles with $[^{14}\text{C}]\text{TCE}$ added, 12 with $[^{14}\text{C}]\text{cis-DCE}$ added, and 12 with $[^{14}\text{C}]\text{VC}$ added, yielding a grand total of 324 bottles. From each set of 12 bottles (e.g., live microcosms with rock from 161 m and $[^{14}\text{C}]\text{TCE}$ added), triplicates were analyzed for the distribution of $^{14}\text{C}$ at four time steps, corresponding to increasing incubation times.

The microcosms consisted of 160 mL serum bottles capped with Teflon-faced red rubber septa (for TCE and cis-DCE) or slotted gray butyl rubber septa (for VC). The live and AC bottles were constructed in an anaerobic chamber (approximately 99% $\text{N}_2$ and 1% $\text{H}_2$) with groundwater (50 mL) plus crushed rock (20 g). ACs were autoclaved (121°C for 1 h) on three consecutive days prior to adding the $^{14}\text{C}$ compounds. WCs were prepared in the anaerobic chamber with 50 mL of autoclaved distilled-deionized water.

$[^{14}\text{C}]\text{TCE}$ and $[^{14}\text{C}]\text{cis-DCE}$ were added to the microcosms using stock solutions (0.15-0.20 mL) of the compounds dissolved in distilled-deionized water. To avoid
adding toluene to the \[^{14}\text{C}]\text{VC} \text{ microcosms}, the VC/toluene solution (3 \, \mu\text{L}) was injected onto a gas chromatograph (GC) containing a stainless steel column (3.175-mm x 2.44-m) packed with 1\% SP-1000 on 60/80 Carbopak B (Supelco). N\_2 was used as the carrier gas (25-30 \, \text{mL/min}). The outlet of the column was connected to stainless steel tubing (1.59 mm) that exited the GC oven and terminated with a needle, which was inserted into the microcosm headspace during the interval when VC eluted (separated from toluene). The total \(^{14}\text{C}\) added was 0.50-0.77 \, \mu\text{Ci per bottle}.

The average initial amounts of TCE in the live, AC and WC bottles with \[^{14}\text{C}]\text{TCE} added were 0.163±0.036, 0.128±0.015 and 0.0786±0.0006 \, \mu\text{mol per bottle}, respectively. Taking into account the volume of liquid (50 mL) and headspace (99 mL, accounting for 11 mL occupied by the rock) and assuming equilibrium, the average initial aqueous phase concentrations of TCE were 253, 200, and 122 \, \mu\text{g/L}, respectively. The higher TCE levels in the live and AC treatments were due to TCE in the groundwater, some of which was lost during autoclaving. \textit{cis}-DCE and VC were not present.

In the live, AC and WC treatments with \[^{14}\text{C}]\text{cis-DCE} added, the average initial amount of \textit{cis}-DCE present was 0.407±0.081 \, \mu\text{mol per bottle}, resulting in an aqueous phase concentration of 618 \, \mu\text{g/L}. TCE (unlabeled) from the groundwater was also present in the live treatment (0.100±0.039 per bottle), with less in the AC treatment (0.057±0.017 per bottle). No TCE was present in the WC treatment. None of the treatments initially contained VC.

In the live, AC and WC treatments with \[^{14}\text{C}]\text{VC} added, the average initial amount of VC present was 0.245±0.017 \, \mu\text{mol per bottle}, resulting in an aqueous phase
concentration of 102 µg/L. The same concentrations of TCE initially present in the live and AC treatments with $[^{14}\text{C}]\text{cis-DCE}$ were present in these bottles. Initial concentrations of each compound in the microcosms were within the range reported for groundwater at the site (13).

Microcosms were incubated in an inverted position (liquid in contact with the septa), in the dark, at room temperature (22-24°C). The live and AC bottles were stored in an anaerobic chamber, to prevent diffusion of oxygen into the bottles. WC bottles were stored outside the chamber (due to space limitations).

2.2.4 Analytical Methods and $^{14}\text{C}$ Distribution

The total amount of volatile compounds present in the microcosms was monitored by GC analysis of headspace samples (0.5 mL) using a column packed with 1% SP-1000 on 60/80 Carbopak B (14). Detection limits for TCE, cis-DCE, trans-DCE, and VC were 2.9, 5.2, 2.2, and 0.48 µg/L, respectively. As needed, better resolution of cis- and trans-DCE was accomplished using a column packed with 10% SP-1000 on 80/100 Supelcoport (14). Sulfate was measured by ion chromatography (IC) on an AS9-HC anion exchange column (Dionex, 4 mm x 250 mm; 9 mM NaHCO$_3$ eluant, 1 mL/min). Dissolved organic carbon was measured on a Shimadzu total organic carbon analyzer (15).

The total amount of $^{14}\text{C}$ in the microcosms was quantified by counting samples of the headspace and liquid in liquid scintillation cocktail. Distribution of the $^{14}\text{C}$-labeled compounds was determined by a combination of headspace and aqueous phase analyses (14) at four time steps: 2-3 months (step 1), 7-8 months (step 2), 11-12 months (step 3)
and 15-16 months (step 4). $^{14}$C-labeled volatile compounds were analyzed with a GC/combustion technique (Appendix A). $^{14}$CO$_2$ and $^{14}$C-labeled nonvolatile compounds were measured after analysis of the $^{14}$C-labeled volatile compounds was completed (14). Samples of the liquid phase (10 mL) were transferred to a test tube that was connected to a second test tube containing NaOH (0.5 M). Nitrogen gas was sparged into the first tube and over into the second. The pH in the first tube was then lowered with HCl, to facilitate stripping of CO$_2$, which was trapped in the second tube. The term given to the liquid remaining in the first tube after acidic sparging is nonstrippable residue (NSR). The percentage of NSR associated with particulates was determined by counting the level of $^{14}$C activity remaining after centrifugation (10,000g for 10 min; Sorvall Evolution RC Centrifuge) and comparing this to the total $^{14}$C-NSR activity.

The percent distribution of $^{14}$C in a serum bottle for each compound or NSR (C) was:

$$\% C = \frac{x}{T_o} \times 100 \quad (1)$$

where $x = ^{14}$C per bottle for compound $x$; and $T_o$ = the total $^{14}$C added per bottle at time zero. The amount of $^{14}$C lost to diffusion and adsorption during incubation was:

$$\% \text{Loss} = \frac{(T_o - T_f)}{T_o} \times 100 \quad (2)$$

where $T_f$ is the total $^{14}$C remaining in a bottle at the final sampling time (excluding $^{14}$C that sorbed to the sandstone). The amount of $^{14}$C in the “unaccounted for” (UAF) category was:

$$\% \text{UAF} = \frac{(T_f - \Sigma C)}{T_o} \times 100 \quad (3)$$
where $\Sigma C =$ sum of the $^{14}$C associated with all of the identifiable compounds or categories (CO$_2$, NSR, TCE, cis-DCE, and VC).

2.2.5 $^{14}$C-NSR characterization

Significant amounts of $^{14}$C-NSR were recovered in a number of the AC microcosms. Initial characterization of the NSR was performed by separation on a Waters high performance liquid chromatograph (HPLC) equipped with an Aminex HPX-87H column (300 mm x 7.6 mm), $\text{H}_2\text{SO}_4$ eluant (0.1 N, 0.6 mL/min), and a UV/Vis detector (model 490E) (14). As eluant passed through the detector, fractions were collected in liquid scintillation cocktail. Several fractions had high levels of $^{14}$C activity but the total concentration of compounds present was too low for detection by UV/Vis (210 nm). To facilitate identification of these fractions, the NSR was concentrated by lyophilization (see below). The NSR concentrate was analyzed by HPLC in the same manner described above. Several peaks became detectable by UV/Vis and coincided with the fractions that contained the $^{14}$C activity. Preliminary identification of several of these peaks was made by comparing their retention times to authentic material.

The identity of compounds in NSR was evaluated further using the same procedure but with a Dionex IC equipped with an IonPac AS15 anion exchange column. A mixed eluant was used (10% 0.1 M NaOH and 90% distilled-deionized water, 1.6 mL/min). The retention time of peaks on the IC that contained $^{14}$C activity was also matched to authentic material. The presence of formate in lyophilized NSR was confirmed using a formate dehydrogenase assay (16) (Appendix A).
Lyophilization was used to concentrate samples of NSR to facilitate detection of the $^{14}$C-labeled compounds that were present. Liquid samples (25 mL) from four AC or four WC microcosms were combined in a plastic jar (Nalgene) with a screw cap containing two holes (2 mm). The sample was acidified (pH 4.4) and sparged to remove CO$_2$. The pH was then raised to 10 and the sample was frozen, placed in a wide mouth filter seal flask, attached to a -80°C freeze dryer (VirTis Benchtop 6K Freeze drier model NEWBT6K-ES-ElV2), and a vacuum was applied (approximately 54 mTorr for 60 h) until lyophilization was complete. The jar was removed, distilled-deionized water was added (2 mL) and the concentrate was filtered (0.45 µm). The efficiency of lyophilization was evaluated based on the ratio of the total $^{14}$C recovered in the concentrate and the total $^{14}$C activity prior to freeze drying.

2.3 Results

2.3.1 TCE

Within 60 days, complete reduction of the TCE to DCE occurred in three of the 12 live microcosms for the 161 m depth and six of the 12 for the 265 m depth. Average results for three live microcosms with rock from 265 m are shown in Figure 2.1. The molar ratio of cis- to trans-DCE was 25:1. After 150 days, conversion of TCE to DCE was complete in four of the 12 bottles for the 161 m depth, one of the 12 bottles for the 222 m depth, and seven of the 12 bottles for the 265 m depth. No other live bottles underwent complete reduction of TCE through day 450. In bottles that exhibited reduction of TCE to cis-DCE there was no further dechlorination to VC. Reduction of TCE to cis-DCE was not a significant transformation process in the AC or WC bottles.
Figure 2.1. GC headspace monitoring results for live microcosms prepared with sandstone from 265 m that received $[^{14}\text{C}]$TCE and exhibited significant reductive dechlorination to cis-DCE. Error bars are standard deviations for triplicate microcosms. Larger error bars appear on day 30 because one of the three microcosms completed dechlorination of TCE but the other two microcosms did not.
To determine the electron donor capacity for reductive dechlorination, TCE was added repeatedly to three live bottles (266 m) that had dechlorinated TCE to cis-DCE within the first 60 days (Figure 2.1), after analyzing for the distribution of $^{14}$C. The average total amount of TCE reduced to DCE over 385 days of incubation was 78 µmol per bottle, requiring 156 micro-electron equivalents (µeeq) of electron donor. Electron donor demand was also exerted for reduction of 195 mg/L of sulfate in the groundwater, consuming up to 810 µeeq per bottle, for a total demand of 966 µeeq. There was no detectable nitrate in the groundwater. Hydrogen from the anaerobic chamber in which the microcosms were prepared (approximately 1%) could have provided up to 80 µeeq of electron donor (based on 60 mL headspace), so the balance (880 µeeq) apparently came from the groundwater (containing 6.2 mg/L of dissolved organic carbon) and/or sediment (containing 0.1% organic matter; Appendix A).

On the basis of (Appendix A), decreases in TCE also occurred in microcosms without accumulation of cis-DCE, although the magnitude of decrease was considerably less. In these microcosms, the highest zero-order rate of decrease in TCE for bottles incubated 450 days was 0.41 µM/yr (Table 2.1). There was no significant difference in rates of TCE loss between the live and AC treatments except for the 265 m depth, in which loss of TCE from the ACs was not statistically significant. The rate of loss of TCE from the WCs was also not statistically significant (p>0.05). The distribution of $^{14}$C in live bottles to which $[^{14}$C]TCE was added confirmed that cis-DCE was the main volatile product when complete reduction occurred (Figure 2.2a). Three of 12 replicates were analyzed at each time step.
Table 2.1. Zero-Order Rates of Transformation Based on GC Monitoring of Microcosms Incubated at Least 450 Days<sup>a</sup>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TCE (µM/yr)</th>
<th>cis-DCE (µM/yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.72±0.36</td>
</tr>
<tr>
<td>161 m, Live</td>
<td>0.41±0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.95±0.62</td>
</tr>
<tr>
<td>161 m, AC</td>
<td>0.27±0.26</td>
<td>0.95±0.72</td>
</tr>
<tr>
<td>173 m, Live</td>
<td>0.28±0.19</td>
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</tr>
<tr>
<td>173 m, AC</td>
<td>0.31±0.18</td>
<td>1.7±1.0</td>
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<tr>
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<td>0.94±0.83</td>
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<tr>
<td>222 m, AC</td>
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<td>0</td>
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<tr>
<td>265 m, Live</td>
<td>0.41±0.15</td>
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</tr>
<tr>
<td>265 m, AC</td>
<td>0</td>
<td>1.5±0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> GC data used to determine these rates are presented in Appendix A.
<sup>b</sup> Indicates the rate was not statistically different from zero (α=0.05).
<sup>c</sup> ± the standard error of the slope of the regression line used to determine the rate.
Figure 2.2. Distribution of $^{14}$C compounds in microcosms with $[^{14}\text{C}]$TCE added; (a) volatiles in live treatments, (b) volatiles in ACs, (c) CO$_2$ + NSR in live treatments, and (d) CO$_2$ + NSR in ACs (d); WC results are shown in each panel for comparison. The numbers below the bars indicate the four time steps when microcosms were analyzed for $^{14}$C distribution (2-3, 7-8, 11-12, and 15-16 months). Error bars represent the standard deviation for the sum based on triplicate serum bottles. In (c) and (d), asterisk(s) above a bar indicate that the sum of $^{14}$CO$_2$ + $[^{14}\text{C}]$NSR is statistically greater than in the WCs at the 10% (*) or 5% (**) confidence level.
Microcosms that exhibited significant levels of reductive dechlorination were chosen for analysis at the earlier time steps. This explains why cis-DCE was a predominant product for the earlier time steps, while TCE was still predominant at later times. $[^{14}\text{C}]\text{TCE}$ was the predominant volatile compound in the ACs (Figure 2.2b), followed by smaller amounts of the UAF fraction. $^{14}\text{C}$ losses averaged 31.8±17.1% in the live bottles, 16.9±14.5% in the ACs, and 17.8±16.7% in the WCs, and increased over time.

The nonvolatile transformation products, $^{14}\text{CO}_2$ and $[^{14}\text{CO}_2]\text{NSR}$, were generally significantly higher in the live and AC microcosms than in the WCs (based on a Student’s $t$-test), with $^{14}\text{CO}_2$ predominating in the live bottles (up to 9.5%, Figure 2.2c) and a mixture of $[^{14}\text{C}]\text{NSR}$ (up to 13%) + $^{14}\text{CO}_2$ (up to 12%) in the ACs (Figure 2.2d). The sum of $^{14}\text{CO}_2 +[^{14}\text{C}]\text{NSR}$ was generally higher in the ACs than in the live bottles. None of the treatments exhibited a statistically significant increase in $^{14}\text{CO}_2 +[^{14}\text{C}]\text{NSR}$ over time (based on the slope of the trend line for the four time steps, $\alpha=0.05$). The presence of NSR in the WCs indicated that some of this $^{14}\text{C}$ activity was present in the stock solution used to spike the microcosms.

2.3.2 cis-DCE

Based on GC data (Appendix A), there was a statistically significant decrease in the total amount of cis-DCE over time in most of the treatments. Occasionally a minor amount of VC appeared in the live treatments, although it was consistently well below the amount of DCE lost. Average zero-order rates of decrease in cis-DCE were determined for all of the bottles that were incubated for 627 days (Table 2.1). In two of the treatments (live for 173 m and AC for 222 m) the rate of decrease was not significant.
In all of the others, the average rates of loss were significant and were greater in the live and AC treatments than in the WCs. However, due to scatter in the headspace monitoring results for cis-DCE, only one of the treatments (AC for 173 m) had a rate that was statistically greater than the average rate for the WCs.

$[^{14}C]cis$-DCE was the largest category of $^{14}$C remaining in all of the treatments at the time of analysis, followed by an average of 5.0% UAF volatiles (Figure 2.3a and 2.3b). $^{14}$C losses averaged 23.5±6.9% in the live bottles, 22.2±7.4% in the ACs, and 16.9±6.7% in the WCs, and tended to increase over time. Consistent with the headspace monitoring results, only trace amounts of $[^{14}C]$VC were detected in the live treatments (Figure 2.3a). In a number of the live treatments, a significant amount of $^{14}$C activity in the headspace samples eluted off the Carbopack column in the first 2.5 min. For these bottles, a second headspace sample was analyzed on a Carboxieve SII column in order to achieve better separation. The only significant $^{14}$C activity eluting from the Carboxieve column was CO$_2$; no detectable activity was associated with methane, ethane, ethene, or acetylene. The Carboxieve SII results were consistent with significant levels of $^{14}$CO$_2$ in the aqueous phase of most of the live bottles (see below).

The nonvolatile transformation products from $[^{14}C]$cis-DCE, $^{14}$CO$_2$ and $[^{14}C]$NSR, were consistently higher than in the WCs (based on a Student’s $t$-test), with $^{14}$CO$_2$ predominating in the live bottles (up to 19%, Figure 2.3c) and $[^{14}C]$NSR (up to 22%) in the ACs (Figure 2.3d). The sum of $^{14}$CO$_2$ + $[^{14}C]$NSR was generally higher in the ACs than in the live bottles. None of the treatments exhibited a statistically
Figure 2.3. Distribution of $^{14}$C compounds in microcosms with $[^{14}$C]$cis$-DCE added; (a) volatiles in live treatments, (b) volatiles in ACs, (c) CO$_2$ + NSR in live treatments, and (d) CO$_2$ + NSR in ACs (d); WC results are shown in each panel for comparison. The numbers below the bars indicate the four time steps when microcosms were analyzed for $^{14}$C distribution (7-8, 11-12, 15-16, and 21-22 months). Error bars represent the standard deviation for the sum based on triplicate serum bottles. In (c) and (d), asterisk(s) above a bar indicate that the sum of $^{14}$CO$_2$ + $[^{14}$C]NSR is statistically greater than in the WCs at the 10% (*) or 5% (**) confidence level.
significant increase in \(^{14}\text{CO}_2 + [^{14}\text{C}]\text{NSR}\) over time (based on the slope of the trend line for the four time steps, \(\alpha=0.05\)).

### 2.3.3 VC

Based on GC monitoring of headspace samples, losses of VC from all of the treatments were minor. There was no evidence of ethene or ethane accumulation, indicating that reductive dechlorination was not a significant fate process for VC (data not shown). The majority of the \([^{14}\text{C}]\text{VC}\) added was recovered in the live and AC treatments as \([^{14}\text{C}]\text{VC}\), ranging from 77% to 91% across all depths and time steps. Consistent with the GC results, 14\(^{\text{C}}\)-labeled methane, ethane, ethene or acetylene were not detected. An average of 0.35% of the total \([^{14}\text{C}]\text{VC}\) added consisted of UAF volatiles. 14\(^{\text{C}}\) losses averaged 12.1±5.3% in the live bottles, 14.3±5.5% in the ACs, and 17.3±4.5% in the WCs.

\(^{14}\text{CO}_2\) and \([^{14}\text{C}]\text{NSR}\) were generally below 2% of the \([^{14}\text{C}]\text{VC}\) added to the live microcosms. Although this was lower compared to the \([^{14}\text{C}]\text{TCE}\) and \([^{14}\text{C}]\text{cis-DCE}\) results, the accumulation was statistically greater than the WCs for several of the live treatments (161 m depth for step #3, 173 m for steps #2 and #4, and at 265 m for step #2). There was virtually no \(^{14}\text{CO}_2\) or \([^{14}\text{C}]\text{NSR}\) in the WCs. Accumulation of \([^{14}\text{C}]\text{NSR}\) was notably higher in the AC treatments (up to 12% in the time step #4 microcosms for the 222 m depth) in comparison to the live ones, with only traces of \(^{14}\text{CO}_2\) recovered. For all depths and time steps, the amount of \([^{14}\text{C}]\text{NSR}\) in the ACs was statistically higher than in the corresponding WCs (Appendix A, Figure A.3).
2.3.4 NSR Characterization

NSR was a significant percentage of the $^{14}$C recovered, especially in several of the AC microcosms that received [{}^{14}$C]TCE (Figure 2.2d) and [{}^{14}$C]cis-DCE (Figure 2.3d). All of the [{}^{14}$C]NSR was soluble (based on separation by centrifugation). When the NSR was passed through an organic acids HPLC column, most of the $^{14}$C activity present was recovered in four collection intervals (approximately 1.2 min each). However, the concentration of compounds in the NSR was not sufficient to permit detection by UV/Vis. To facilitate detection, samples of NSR from several AC bottles were combined and concentrated approximately 50-fold by lyophilizing; the same was done with samples from WC bottles. An average of 68% of the $^{14}$C in the NSR was recovered in the AC concentrate after lyophilizing; considerably lower recoveries were obtained with the WCs (Table 2.2). Aliquots of the concentrate were evaluated on the organic acids HPLC column. The sum of $^{14}$C activity in all of the HPLC fractions from the lyophilized AC samples accounted for 90-97% of the $^{14}$C injected onto the HPLC column, compared to an average of 58% for the WCs (Table 2.2).

A number of peaks appeared in UV/Vis chromatograms for the concentrated NSR. HPLC fractions corresponding to four of the peaks contained significant amounts of $^{14}$C activity. Matches in retention times to the $^{14}$C peaks in the NSR concentrate were obtained with neat samples of glycolate, formate, and acetate (Appendix A). Other compounds were also tested (oxalate, trichloroacetate, glyoxal, glycolaldehyde, and chloroacetate) but their retention times did not match up with the peaks containing $^{14}$C. The highest levels of $^{14}$C activity recovered in the lyophilized AC samples were 46% for
Table 2.2. Efficiency of NSR Lyophilization and Distribution of $^{14}$C in Lyophilized Samples from Autoclaved Control and Water Control Microcosms with $[^{14}]$cis-DCE and $[^{14}]$TCE Added as Determined by HPLC Separation

<table>
<thead>
<tr>
<th>Sample</th>
<th>NSR before lyophilizing (% of total $^{14}$C added)</th>
<th>% of $^{14}$C-NSR remaining after lyophilizing</th>
<th>% recovery of lyophilized $^{14}$C-NSR in HPLC fractions</th>
<th>Unretained</th>
<th>Glycolate</th>
<th>Formate</th>
<th>Acetate</th>
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<th>Sample</th>
<th>NSR before lyophilizing (% of total $^{14}$C added)</th>
<th>% of $^{14}$C-NSR remaining after lyophilizing</th>
<th>% recovery of lyophilized $^{14}$C-NSR in HPLC fractions</th>
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<th>% of $^{14}$C-NSR remaining after lyophilizing</th>
<th>% recovery of lyophilized $^{14}$C-NSR in HPLC fractions</th>
<th>Unretained</th>
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<th>Sample</th>
<th>NSR before lyophilizing (% of total $^{14}$C added)</th>
<th>% of $^{14}$C-NSR remaining after lyophilizing</th>
<th>% recovery of lyophilized $^{14}$C-NSR in HPLC fractions</th>
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<th>Glycolate</th>
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\(\text{a}\) Each sample was a composite of 25 mL of liquid from four autoclaved control or water control microcosms.  
\(\text{b}\) Amount of $[^{14}]$NSR as a percentage of the total $[^{14}]$TCE or $[^{14}]$cis-DCE added.  
\(\text{c}\) (total $^{14}$C remaining after lyophilizing)/(total $^{14}$C present prior to lyophilization).  
\(\text{d}\) $\sum$ of $^{14}$C in all HPLC fractions)/(total $^{14}$C of the lyophilized NSR injected onto the HPLC).  
\(\text{e}\) $^{14}$C activity not measured prior to lyophilizing.  
\(\text{f}\) Identification confirmed using a formate dehydrogenase assay (see text).  
\(\text{g}\) $^{14}$C activity not measured prior to lyophilizing.
glycolate, 25% for formate, and 41% for acetate (Table 2.2). The $^{14}\text{C}$ activity in the AC treatments attributable to glycolate + formate + acetate was greater than the total amount of $[^{14}\text{C}]\text{NSR}$ in the WC samples (data not shown). In the ACs and WCs, 30-62% of the $^{14}\text{C}$ in NSR concentrate was unretained on the organic acids column, i.e., it emerged at the same time as the injection peak (Table 2.2).

In order to confirm the presence of glycolate, formate and acetate in the NSR, the lyophilized samples were separated by IC using a column with different characteristics in comparison to the organic acids column. Similar results were obtained (Appendix A), i.e., the IC peaks that contained $^{14}\text{C}$ activity matched up to the retention times for glycolate, formate and acetate. There were two notable differences between the separation results for the IC and HPLC. First, there was no $^{14}\text{C}$ activity associated with the unretained fraction from the IC column; and second, there was a lower percent recovery of $^{14}\text{C}$ in the fractions collected off of the IC column (averaging 53.4% for the AC samples). This suggests that the unretained $[^{14}\text{C}]\text{NSR}$ collected off of the organic acids column did not elute from the IC column, at least for the run time used (30 min).

The presence of formate in NSR concentrate from the AC samples was further confirmed using formate dehydrogenase. NADH accumulated due to reduction of NAD$^+$, coupled to the oxidation of formate to CO$_2$. The same increase occurred with a formate standard but not with NSR concentrate from WC samples or reagent blanks (no formate added). The presence of $^{14}\text{CO}_2$ in the assay vials with $[^{14}\text{C}]\text{NSR}$ from AC samples provided further confirmation that $[^{14}\text{C}]\text{formate}$ was present in the NSR concentrate (Appendix A).
2.4 Discussion

The results of this study demonstrate the occurrence of biotic and abiotic transformations in sandstone and groundwater samples from the southern California industrial site where TCE was released to the subsurface several decades ago. Reductive dechlorination of TCE to cis-DCE in many of the live microcosms is consistent with field data (13). TCE reduction to DCE in the microcosms was a biotic process, based on the predominance of cis- over trans-DCE as the dechlorination product (2), the lack of reductive dechlorination in AC treatments, and the lag period that preceded a rapid onset of reduction in several of the microcosms. A limited amount of electron donor is available in the groundwater and/or sandstone for TCE reduction. It is not yet clear why some microcosms within a treatment exhibited significant reduction of TCE to cis-DCE, while others did not, since the crushed sandstone from each depth was thoroughly mixed prior to distributing it to the bottles. The distribution of dechlorinating microbes may not have been uniform within the sandstone, in spite of mixing. Exposure of the rock cores to air during sample collection and crushing may have adversely affected the recovery of anaerobic microbial activity, as well as abiotic transformation capacity. The lack of VC and ethene accumulation suggests the absence of the Dehalococcoides population needed for these dechlorination steps (17,18).

Evidence for abiotic transformation of TCE and cis-DCE is based on statistically significant accumulation of [14C]NSR and 14CO2 in the AC microcosms at all depths, in comparison to the WC bottles (Figure 2.2d and 2.3d). The lack of an increase in NSR and CO2 formation over time indicates that these transformations were complete by the
time the first set of $^{14}$C analyses were performed (2-3 months for TCE, 7-8 months for cis-DCE), and that the transformation capacity of the sandstone was exhausted. The ratio of sandstone to groundwater used in this study (0.4 g/g) was intermediate in comparison to other studies reporting abiotic transformations of TCE in groundwater ($^8,^9,^{11}$), which likely affects the rate and extent of abiotic transformation. Based on elemental analysis (Table A-1) and data from drillers’ logs ($^13$), rock from 161 m was biotite-rich, while rock from the other depths was typical sandstone. Nevertheless, the live and AC bottles from 161 m exhibited similar amounts of TCE and cis-DCE transformation to CO$_2$ + NSR, suggesting that biotite is not a significant factor. Crushing the sandstone increased its surface area and most likely increased the rate of reaction, but to what extent is not yet known. Autoclaving to achieve abiotic conditions may also affect the reactivity of the sandstone. The presence of acetylene at the field site ($^13$) also confirms the occurrence of abiotic processes as demonstrated by the microcosm results.

The presence of $[^{14}]$NSR in the $[^{14}]$TCE and $[^{14}]$cis-DCE WCs suggests some was present in the stock solutions. Prior to preparing the microcosms, the radiochemical purity of the volatile component of the stock solutions was verified, although the presence of NSR in the stock solutions was not evaluated. $[^{14}]$VC was added to the microcosms after chromatographic separation and the corresponding WC microcosms had lower $[^{14}]$NSR levels. In future studies we recommend delivering labeled compounds in the same manner as VC, or at least confirming in advance the absence of NSR in aqueous stock solutions.
Figure 2.4 summarizes the types of transformations observed in this study. Biotic reduction of TCE to cis-DCE was most prominent in microcosms prepared with sandstone from 265 m, but also occurred with samples from 161 m and 222 m. Acetylene was not detected as a significant product, as has been reported previously (5-8,10,11). In live microcosms that received [$^{14}$C]TCE but did not exhibit significant hydrogenolysis, NSR and CO$_2$ accumulated. Microcosms that received [$^{14}$C]cis-DCE also accumulated statistically significant amounts of NSR and CO$_2$. NSR formation from TCE and cis-DCE is presumed to be abiotic, since NSR was more prevalent in AC microcosms versus live treatments. Compound specific stable carbon isotope analysis of field samples indicate that cis-DCE has not been affected significantly by biodegradation (13). It is not yet known if CO$_2$ formation occurs via NSR, directly from TCE and cis-DCE, or both.

Glycolate, formate and acetate were identified as components of NSR from the AC microcosms. The thermodynamics for transformation of TCE and cis-DCE to these organic acids are quite favorable (Appendix A). Although the transformation mechanism is not yet known, the fractured sandstone contains as much as 1.9% iron (Appendix A), which is often associated with abiotic transformations. Formation of glycolate and formate from TCE was reported in soil subjected to thermal remediation and was considerably enhanced by the presence of goethite (19). The crushed rock used in this study does not contain goethite; the minerals present are typical of what is found in
Figure 2.4. Transformations observed in this study and potential pathways based on $^{14}$C results; A = abiotic process; B = biotic process, A/B = abiotic and/or biotic process, [H] = H$^+ + e^−$. 
sandstone in parts of southern California (i.e., iron sulfides, pyrite, fougerite, magnetite, biotite, vermiculite, and quartz). The role of pyrite and fougerite (green rust) in abiotic reductive transformation of TCE to acetylene has been reported, although prior studies did not attempt to identify possible soluble products (5-8,10,11,20).

Hydrogenolysis is likely to remain the focus of natural and enhanced remediation of chlorinated ethenes. Nevertheless, evidence is mounting that alternative pathways exist for natural attenuation, with some involving abiotic processes. The results of this study demonstrate the importance of looking not only for volatile transformation products but soluble ones as well. However, this presents a significant challenge for documenting natural attenuation, since the tools available to demonstrate activity remain limited to microcosm studies with \(^{14}\)C labeled compounds. As additional information is obtained on the mechanisms of transformation, it should become possible to better predict the in situ conditions under which activity may be expected to occur.

2.5 References


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(13) Pierce, A. A.; Isotopic and Hydrogeochemical Investigation of Major Ion Origin and Trichloroethene Degradation in Fractured Sandstone. MS Thesis, Earth Sciences, University of Waterloo, **2005**.


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ANAEROBIC ABIOTIC TRANSFORMATION of cis-1,2-DICHLOROETHENE 
CATALYZED by MINERALS in FRACTURED SANDSTONE

3.0 Abstract

A fractured sandstone aquifer at an industrial site in southern California is contaminated with trichloroethene (TCE) to depths in excess of 244 m. Field monitoring data suggest that TCE is undergoing reduction to cis-1,2-dichloroethene (DCE) and that additional attenuation is occurring. However, vinyl chloride (VC) and ethene have not been detected in significant amounts, so that if transformation is occurring, a process other than reductive dechlorination must be involved. A previous microcosm study using $^{14}$C labeled compounds demonstrated the occurrence of biotic and abiotic transformations of TCE, cis-DCE and VC at this site. In live microcosms, TCE was reductively dechlorinated to cis-DCE with only minor amounts of further dechlorination, confirming field observations of cis-DCE accumulation. In autoclaved microcosms, significant transformation of $[^{14}\text{C}]$TCE and $[^{14}\text{C}]$cis-DCE to soluble products (including glycolate, formate and acetate) and $^{14}\text{CO}_2$ also occurred. The objectives of this study were to determine if pyrite in the sandstone is responsible for catalyzing the abiotic transformation of cis-DCE to soluble products and CO$_2$; to quantify the rate of cis-DCE transformation; and to evaluate the effect of autoclaving as a method of sterilization for measuring abiotic transformation of cis-DCE in fractured sandstone. Microcosms (2 L glass bottles equipped with mini-nert valves) were prepared with groundwater and approximately 30 mg/L cis-DCE (including some $^{14}$C-labeled) plus pyrite, pyrite-rich sandstone, and typical sandstone that was crushed and autoclaved. During 120 days of
incubation, the highest level of cis-DCE transformation occurred with typical sandstone (11-14\% $^{14}$CO$_2$, 1-3\% $^{14}$C-soluble products), followed by pyrite-rich sandstone (2-4\% $^{14}$CO$_2$, 1\% $^{14}$C-soluble products). Transformation in the treatment with pyrite was only slightly higher than groundwater-only controls, indicating that pyrite was not the principal mineral involved. A second experiment was performed using only the typical sandstone, with three treatments (live, autoclaved, and sterilized with propylene oxide). Surface-normalized transformation rates for cis-DCE were higher for the live treatment (1.31E-05 L m\(^{-2}\) d\(^{-1}\)) versus the autoclaved treatment (1.08E-05 L m\(^{-2}\) d\(^{-1}\)), although the extent of transformation in the autoclaved sandstone was higher initially the ultimate extent of transformation was similar in both treatments (approximately 7\% of the $[^{14}$C]cis-DCE added converted to $^{14}$CO$_2$ + $^{14}$C-soluble products). The presence of glycolate, formate and acetate as components of the soluble products was confirmed. Small amounts of VC, ethene, ethane and acetylene were detected in the live treatment, while only acetylene was detected in the autoclaved treatment. Use of propylene oxide to sterilize the sandstone resulted in substantial inhibition of cis-DCE transformation. XPS analysis of the sandstone indicated that autoclaving increased the availability of magnetite and goethite. In the non-autoclaved sandstone (i.e., live treatment), ferrous (FeO) and ferric oxides (Fe$_2$O$_3$) predominated, suggesting that these minerals may be central to cis-DCE transformation under field conditions. The results of this study provide further insight into the co-occurrence of abiotic and biotic transformation process for chlorinated ethenes in fractured sandstone.
3.1 Introduction

At an industrial site in southern California underlain by fractured sandstone, thousands of kilograms of trichloroethene (TCE) were reportedly discharged to the surface for several decades following its use in degreasing operations. Field data indicate that a portion of the TCE has diffused into the rock matrix and some has undergone reductive dechlorination to cis-1,2-dichloroethene (DCE) to depths in excess of 244 m. Only minor amounts of vinyl chloride (VC) and ethene have been found at the site, suggesting that biological dechlorination is incomplete. At several TCE contaminated sites there is evidence to suggest that TCE is reductively dechlorinated to cis-DCE, with little or no further reduction to VC and ethene (I,2), as seen at this site. Natural attenuation, by abiotic transformation of cis-DCE, may be an explanation for the removal of cis-DCE at these sites.

A previous microcosm study performed with crushed rock and groundwater from this site provided evidence that cis-DCE is transformed abiotically (I). In that study, live microcosms confirmed field evidence for reductive dechlorination of TCE to cis-DCE, with only minor amounts of VC appearing. In both the live and autoclaved microcosms spiked with [14C]TCE and [14C]cis-DCE there was transformation to carbon dioxide and a soluble product, hereafter referred to as non strippable residue (NSR), based on compounds that remained after sparging with nitrogen under acidic conditions. Glycolate, formate and acetate were identified as three components of the NSR. The extent of transformation to CO₂ was consistently greater in the live microcosms and the extent of transformation to NSR was greater in the autoclaved microcosms. This suggests
that the transformation to NSR was abiotic and may then be followed by biotic or abiotic transformation of the NSR to CO₂. However, this does not rule out direct transformation of TCE and cis-DCE to CO₂, either by a biotic or abiotic process.

The study by Darlington et al. (1) appears to be the first to provide evidence for anaerobic abiotic transformation of TCE and cis-DCE to soluble products, specifically glycolate, formate and acetate. Costanza et al. (3) also observed formation of glycolate, formate and carbon dioxide, although the parent compound was tetrachloroethene and transformation was induced by thermal remediation under aerobic conditions. In the study by Darlington et al. (1) the autoclaved microcosms showed greater transformation to NSR than the live microcosms.

Other than the study by Darlington et al. (1), previous laboratory studies that investigated abiotic transformation of cis-DCE only measured volatile transformation products (2,4-9). Evidence provided by these studies suggests that zero valent iron and zinc can serve as catalysts for the abiotic transformation of chlorinated ethenes to acetylene and ethene (4,5). Zero valent iron, however, does not occur naturally and can only be used for enhanced remediation. Naturally occurring minerals like pyrite, magnetite and green rust were found to catalyze the transformation of TCE and cis-DCE to as much as 71% acetylene and minor amounts of VC (6,7). In these abiotic studies the mass balances did not account for all the TCE or cis-DCE initially added to the microcosms.

Autoclaving is widely used as a method to sterilize soil. Although speculation has existed in the environmental engineering community as to the efficiency of sterilization
by autoclaving, it is still a widely used method to inactivate microorganisms in soil. In the study by Darlington et al. (1), the extent of abiotic transformation in autoclaved microcosms was greater than in live microcosms. This suggests that autoclaving the sandstone may somehow alter its reactivity, thereby enhancing abiotic transformation. Several studies have compared the characteristics of live sediment to autoclaved sediment. Extractable manganese and pH levels increased in soils that were autoclaved (10-12). Extractable iron levels, however, decreased in soils that were autoclaved (10-12). Ammonia and nitrate nitrogen increased in autoclaved sandy loam soils (10).

Previous studies provide evidence for abiotic transformation of chlorinated ethenes (PCE, TCE, cis-DCE and VC) catalyzed by naturally occurring minerals (7,13,14). Since portions of the sandstone at the southern California site that is the subject of this research is high in pyrite, one of the main objectives of this study was to determine if pyrite is responsible for catalyzing the abiotic transformation of cis-DCE to NSR and CO₂, or if some other mineral is involved. Also, since the extent of transformation was greater in autoclaved versus live microcosms (1), another objective of this study was to compare the rate and extent of cis-DCE transformation with an alternative method of sterilization, i.e., use of propylene oxide. In the previous microcosm study, the low initial concentrations made it difficult to determine the rates of transformation. Therefore, another objective of this study was to measure first order rates for cis-DCE transformation, by using higher initial concentrations.

Although abiotic transformation occurred with TCE and cis-DCE in the prior study (1), the current study focuses on cis-DCE. With TCE, biotic reductive
dechlorination is a significant pathway, whereas with cis-DCE abiotic transformation plays a predominant role. Also, the extent of transformation to soluble products was greater for cis-DCE than for TCE. In this study, experiments were conducted in bottles that were large enough to permit repeated sampling of the aqueous phase for analysis of $[^{14}\text{C}]\text{NSR}$ and $^{14}\text{CO}_2$.

3.2 Materials & Methods

3.2.1 Sample Collection, Processing, and Storage

Cores identified as pyrite rich and typical sandstone by drillers’ logs were collected from depths of 125-170 m and 90-230 m at the site respectively. Samples were collected with an air rotary drilling rig (Ingersoll-Rand TH-60) equipped to drill and retrieve PQ-size core (8.48-cm diameter). Water was used from hydrants on site, when necessary, to cool the drill bit. All drilling equipment that came into contact with the rock core was decontaminated at the end of each core run, by washing and scrubbing with laboratory grade, non-phosphate detergent and rinsing with deionized water. Core catchers were cleaned between core runs using soapy water and a drill water rinse.

The samples were crushed at the University of Waterloo using a schedule 304 stainless steel cylinder and piston, shipped to Clemson University where they were crushed further with a mortar (10 cm high, 16.5 cm outer diameter, 14 cm inner diameter) and pestle (21.5 cm long). The crushed sandstone was successively passed through a 0.25 mm and a 0.075 mm sieve by shaking (approximately 30 min) on a mechanized sieving apparatus (Ro-Top Testing Sieve Shaker, Model B, Tyler Combustion Engineering Inc.). Three fractions were obtained: <0.075 mm; 0.075-0.25 mm; and >0.25 mm. The crushed
rock was stored in an anaerobic chamber for about 150 days until the microcosms were prepared. Additional cores identified as typical sandstone were collected from the same core hole and shipped to Clemson University where they were crushed using a manual hydraulic press (20 Ton Bottle Jack, model #20 D). The smaller pieces of core were then crushed with a Brinkmann Table top ZM-1 grinding mill, model #027160000, and passed through a 0.85 mm sieve. The crushed rock that passed through the 0.85 mm sieve was stored in an anaerobic chamber and used for additional tests. The crushed typical sandstone was stored in an anaerobic chamber for about 60 days before the experiment was set up.

Groundwater was obtained from a well close to the core hole. Upon receipt, the presence of volatiles in the groundwater was measured by equilibrating a 50 mL sample in a 160 mL serum bottle, followed by analysis of a 0.5 mL headspace sample on a gas chromatograph (GC) using a flame ionization detector (FID). The TCE concentration in the groundwater before setting up the experiments was 1.3 µM. The groundwater was stored briefly at 4°C prior to preparing the experiments. The groundwater was sparged, as described below, before being used to set up the experiments so that the only volatiles present at time zero was the added cis-DCE.

3.2.2 Chemicals

Neat \(^{[14]C}\)cis-DCE dissolved in toluene was purchased from Moravek Biochemicals (4 mCi/mmol). Research grade pyrite mineral (Zacatecas, Mexico) was obtained from Ward’s Natural Science (Rochester, NY), crushed with a mortar and pestle, and sieved (see below). The fraction in the size range of 0.075-0.25 mm was acid
washed and freeze dried, as previously described (7). Propylene oxide (98%) was purchased from VWR. VC (99.5%) was obtained from Fluka. Ethene (99.9%) and acetylene (99.6%) were obtained from Matheson Tri Gas, Inc. (Irving, TX). Ethane (99.5%) was obtained from National Welders (Charlotte, NC). All other chemicals used were reagent grade.

3.2.3 Experimental Set-up

Two experiments were conducted. The purpose of experiment I was to determine if pyrite was catalyzing the abiotic transformation of cis-DCE to NSR and CO₂. The purpose of experiment II was to compare the effect of autoclaving and sterilization of the sandstone by use of propylene oxide on the rate and extent of abiotic transformation of cis-DCE.

Experiment I consisted of five treatments: 1) groundwater + typical sandstone; 2) groundwater + pyrite-rich sandstone; 3) buffered water (10 mM NaHCO₃) (7) + pure pyrite; 4) groundwater alone; and 5) buffered water alone. All of the treatments except pure pyrite were sterilized by autoclaving. Pure pyrite was sterilized by washing twice with a 1 M HCl acid solution then rinsing with DDI water until the pH was above 6.7. The pyrite was then freeze dried before sieving and storing in the anaerobic glove box (7). This method of preparation was used to remove oxidized iron oxides from the surface of the pyrite. All treatments were prepared in duplicate.

Experiment II consisted of four treatments: 1) groundwater + typical sandstone, intended to simulate live (i.e., in situ) conditions; 2) groundwater + typical sandstone that was autoclaved (see below); 3) groundwater + typical sandstone that was sterilized using
propylene oxide; and 4) groundwater that was autoclaved (60 min). All treatments were prepared in triplicate. Any volatiles present in the groundwater were removed by sparging with N\textsubscript{2}/CO\textsubscript{2} (70\%:30\% v/v) for 5 minutes, then a headspace sample was analyzed. If there were volatiles present, the GW was sparged for another minute. The pH of the groundwater was 7.0 before sparging and 6.3 after sparging.

Both experiments were conducted in bottles (total volume = 2300 mL) with a side arm attached. These bottles were custom-fabricated by Glass Warehouse (Millville, NJ) by attaching a 5-7 cm long glass tube to the side of a glass bottle (Wheaton #219921) approximately 5 cm from the cap (Appendix B, Figure B.1). The end of the added tube was threaded to allow a mininert valve (13 mm, 425 thread size; VICI, Inc.) to be added. The main bottle opening was large enough (45 mm) to permit addition of the amount of crushed sediment and groundwater necessary for this experiment and was then sealed with an unpunctured Teflon-lined cap (Wheaton, #240256).

The treatments with sandstone added contained 1.2 g crushed rock per g groundwater (i.e., 1400 g rock + 1200 mL groundwater per bottle). For experiment I, crushed rock in the size range 0.075-0.25 mm was used. However, the treatment with pyrite was prepared with a different ratio (0.083 g pyrite per g buffered water, i.e., 100 g pyrite + 1200 mL buffered water per bottle), in order to more closely resemble the ratio used by Lee and Batchelor (7).

Preliminary tests conducted with varying sizes of sandstone indicated that the rate and extent of cis-DCE transformation was not affected by particle size (Appendix B,
Figure B.2). Consequently, for experiment II, crushed rock that passed through a 0.85 mm sieve was used.

All treatments were prepared in an anaerobic chamber containing an atmosphere of approximately 98.5% N\textsubscript{2} and 1.5% H\textsubscript{2}. Treatments with sandstone were autoclaved at 121°C for 1 h on three consecutive days. Groundwater and buffered water controls were sterilized by autoclaving once for 1 h at 121°C. During autoclaving the side arm tubes on the bottles were sealed with Teflon-lined caps, to prevent damage to the mininert valves. After cooling, the caps were replaced with mininert valves (inside the anaerobic chamber). The valves were separately sterilized by soaking them with ethanol, which was then allowed to evaporate.

One of the treatments for experiment II involved sterilization of the sandstone using propylene oxide. This was accomplished by adding 700 mL of propylene oxide to 1400 g of crushed rock and sealing the bottles with Teflon-lined caps. After 96 h of contact, the larger cap was removed and as much propylene oxide as possible was decanted without discharging any of the rock. The remaining propylene oxide was removed by placing the bottles in a 35°C incubator and applying a vacuum. The absence of residual propylene oxide was confirmed by GC analysis of headspace samples. The groundwater added to these bottles was sterilized by filtration (0.2 µm, Pall Corporation).

Neat \textit{cis}-DCE and \textsuperscript{[\textit{14}C]}\textit{cis}-DCE was added to all bottles. Neat \textit{cis}-DCE (42 µL) was added to obtain an initial aqueous concentration of approximately 40 mg/L. This value is within the range of concentrations for pore water reported at the site (15). \textsuperscript{[\textit{14}C]}\textit{cis}-DCE was separated from the toluene on a GC, as previously described for...
Approximately 20 µL of the \[^{14}\text{C}]\text{cis-DCE}\) solution was injected onto the GC. An isothermal program was used (200°C) with \(\text{N}_2\) as the carrier gas (approximately 30 mL/min). During the interval when \[^{14}\text{C}]\text{cis-DCE}\) eluted from the column (1.1 to 2.7 min), the flow was injected into the microcosm via the mininert valve. The next addition of \[^{14}\text{C}]\text{cis-DCE}\) was made approximately 1 h later, to allow sufficient time for all of the toluene to exit the column. After the \(^{14}\text{C}\)-labeled and unlabeled \(\text{cis-DCE}\) were added, the bottles were placed in plastic containers, supported with packaging to prevent breakage, and incubated on a rotary tumbler (20 rpm).

3.2.4 Analytical Procedures

a) Crushed Rock

Elemental analysis of the crushed sandstone used in the autoclaved, propylene oxide sterilized, and live treatments for experiment II were performed by the Agricultural Service Laboratory at Clemson University. The parameters (and methods used) included extractable nitrogen (Kjeldahl digestion); phosphorus, potassium, calcium, magnesium (dry ash method); sulfur, zinc, copper, manganese, iron, sodium (wet ash procedure); and organic matter (loss on ignition) (16,17).

X-ray photoelectron spectroscopy (XPS; Kratos Axis 165) analysis was performed on live, propylene oxide treated, and autoclaved crushed sandstone that was less than 850 µm in size. Approximately 2 mg of sample was placed on a sample disc, inserted into the electron microscope, and a vacuum applied. Monoaluminum survey scan and high resolution scan were performed to determine binding energies. Three areas (30
µm each) of each sample were analyzed. Surface area and pore diameter measurements were conducted as described by Dastgheib et al. (18).

b) Chlorinated Ethenes and Transformation Products

The amount of cis-DCE remaining in the bottles was monitored by GC analysis of headspace samples (0.5 mL), as previously described (1). A Hewlett Packard 5890 Series II GC was used, equipped with an FID and a 2.44-m by 3.175-mm column packed with 1% SP-1000 on 60/80 Carbopak B (Supelco). Methane, ethene, ethane and acetylene were measured on a Hewlett Packard 5890 Series II GC, equipped with an FID detector and a Carbosieve SII column. Since liquid samples were removed from the bottles to measure \(^{14}\text{CO}_2 + [^{14}\text{C}]\text{NSR}\), the ratio of liquid to headspace changed at each sampling event, which necessitated an adjustment to the GC response factors used to determine the amount of volatile compounds present. A correlation was developed between the GC response factors and the ratio of headspace to liquid volume, thereby permitting the use of a new response factor after each liquid sampling event. The correlation is presented in Appendix B, Figure B.3.

c) Analysis of \(^{14}\text{C}\)

The initial amount of \(^{14}\text{C}\) activity present in all of the bottles was determined by counting a headspace sample (0.5 mL) and a liquid sample (0.2 mL) in liquid scintillation cocktail, as previously described (1). For experiment I, analysis of the \(^{14}\text{C}\) distribution was performed at four time steps (30, 60, 90 and 120 days). The bottles were removed from the tumbler and headspace samples were taken (via the Mininert valve) to measure the total VOCs present (by GC/FID) and the distribution of \(^{14}\text{C}\) (using a combustion
method, as previously described (1)). The bottles were then transferred to the anaerobic chamber and allowed to settle for approximately 30 min. The caps were removed, 100 mL of liquid was quickly poured off, and the bottles were resealed and returned to the tumbler. NSR analysis was performed with a 10 mL sample and the remaining liquid (90 mL) was lyophilized, as previously described (1). Analysis of the $^{14}$C distribution for experiment II was similar, except that smaller liquid samples were removed (50 mL) to allow for a greater number of sampling events.

A number of lyophilized samples contained sufficient concentrations of $^{14}$C ($>0.0023 \ \mu$Ci/L, or when the amount of $[^{14}$C]NSR exceeded 1% of the $^{14}$C initially added) to warrant further evaluation by fractionation on a high performance liquid chromatograph (HPLC) equipped with an organic acids column, as previously described (1). At the completion of the incubation period for experiment I (120 days), a larger sample size was removed (500 mL) from the bottles and lyophilized, to provide a higher concentration of soluble $^{14}$C products for analysis by HPLC.

As indicated above, the large volume of liquid removed at each sampling event for analysis of $^{14}$CO$_2$ and $[^{14}$C]NSR necessitated opening the bottles and pouring off liquid samples. This procedure resulted in losses of cis-DCE and its daughter products, including acetylene, ethene and ethane. A conservative estimate of the amounts removed was made by assuming that the mass loss per sampling event occurred only via the liquid, i.e., none via the gas phase. Based on four sampling events for experiment I, the calculated loss was 27% of the cis-DCE added (Appendix B, Table B.1). Although experiment II had a larger number of sampling events, estimated loss of cis-DCE was
smaller (22%), since the volume of liquid removed per sampling event was one-half the amount removed in experiment I (i.e., 50 mL versus 100 mL).

3.2.5 Mass Losses of Volatiles During Sampling

Since it was necessary to open the bottles to remove large volumes of water at each sampling event, it was important to quantify the amount of VOCs removed during the events. This was accomplished by calculation (assuming losses only from the aqueous phase) and experimentation, based on the number of sampling events for experiment II. Groundwater and neat cis-DCE were added to duplicate bottles, at the same levels added for experiment II. VC, acetylene, ethene and ethane were also added, at levels comparable to the highest amounts detected during experiment II (see Results, section 3.3.2). After reaching equilibrium between the liquid and headspace, the amount of volatiles present was measured by GC-FID analysis of headspace samples. Liquid samples (50 mL) were then removed using the same procedure as for experiment II, the bottles were recapped, and the liquid and aqueous phases were allowed to reach equilibrium again. Then the amount of volatiles remaining in the bottles was measured and the process was repeated. After 6 sampling events, the measured losses were 13% for cis-DCE, 26% for VC, 25% for acetylene, 60% for ethene and 61% for ethane (Appendix B, Table B.1). For cis-DCE, the measured losses were less than or equal to the calculated losses. This confirmed that relatively small amounts of cis-DCE were lost during sampling via diffusion from the headspace. This is not surprising, since the bottles were opened for very short periods of time (e.g., 15 s) and there was no agitation of the headspace during sampling. For the more volatile gases (based on much higher
Henry’s law constants), however, the measured amounts of mass loss per sampling event were much higher than calculated losses based only on mass loss via the liquid removed, since most of the mass of these compounds resided in the headspace and diffusive losses were significant even though the bottles were opened for only a short time per event.

3.3 Results

3.3.1 Experiment I

Although the GC–FID method used to analyze the headspace of the bottles was able to test for cis-DCE, VC, acetylene, ethene and ethane, the only volatile product that appeared during the 120 day incubation period for the autoclaved typical and pyrite rich sandstone treatments and the autoclaved pure pyrite treatment was cis-DCE. This was confirmed by analysis of the $^{14}\text{C}$ compounds in the headspace. The percentage of $[^{14}\text{C}]\text{cis-DCE}$ remaining after 30 days was 96% in the treatment with pyrite rich sandstone, 83% in the treatment with typical sandstone, 95% in the pure pyrite treatment, 99% in the buffered water control, and 98% in the groundwater control (Figure 3.1a). The percentage of $[^{14}\text{C}]\text{cis-DCE}$ remaining at the end of the incubation period (120 days) was 70% in the treatment with pyrite rich sandstone, 46% in the treatment with typical sandstone, 82% in the pure pyrite treatment, 83% in the buffered water control, and 65% in the groundwater control. The estimated amount of cis-DCE remaining, based on mass loss occurring only via the liquid, was 78% (Appendix B, Table B.1). Lower measured amounts in the treatments with typical sandstone are consistent with the higher amounts of CO$_2$ and NSR accumulation for these treatments (Figure 3.1b).
Figure 3.1. Distribution of $^{14}$C compounds obtained by liquid analysis in experiment I; a) volatile compounds; and b) NSR and CO$_2$. Numbers below the bars indicate the four time steps when the bottles were analyzed for $^{14}$C products (step 1 = 30 d; step 2 = 60 d; step 3 = 90 d; and step 4 = 120 d). Error bars represent the standard deviation for the sum based on duplicate bottles. SS = sandstone; BW = buffered water.
After 60 days of incubation the transformation of $[^{14}\text{C}]\text{cis}$-DCE reached a maximum of 2.8% $^{14}\text{CO}_2$ and 0.9% $[^{14}\text{C}]$NSR in the pyrite rich sandstone treatment, 14% $^{14}\text{CO}_2$ and 1.6% $[^{14}\text{C}]$NSR in the typical sandstone treatment, and 0.27% $^{14}\text{CO}_2$ and 1.25% $[^{14}\text{C}]$NSR in the pure pyrite treatment (Figure 3.1b). Transformation of $[^{14}\text{C}]\text{cis}$-DCE to $^{14}\text{CO}_2$ and $[^{14}\text{C}]$NSR was complete after 30 days of incubation, based on the lack of a statistically significant increase during the subsequent sampling events. The maximum amount of $^{14}\text{CO}_2$ and $[^{14}\text{C}]$NSR in the groundwater control was 0.80% and 0.26%, respectively. Similarly minor amounts accumulated in the buffered water controls.

The composition of lyophilized NSR was determined in samples from the pyrite rich sandstone and typical sandstone treatments, after 60 and 120 days of incubation. An insufficient amount of $[^{14}\text{C}]$NSR accumulated in the pure pyrite treatment and the groundwater and buffered water controls to permit further analysis. Table 3.1 shows the distribution of compounds present in the NSR at both time steps. In samples from the pyrite rich sandstone treatment, the average NSR remaining after lyophilizing was 83%. HPLC fractioning of these samples revealed 9.1% acetate, 5.8% glycolate, 5.7% formate, 57% in the unretained fraction, and 23% in other fractions (Table 3.1). After 120 days of incubation, there was a notable decrease in $^{14}\text{C}$ recovered after lyophilization, although the distribution of $[^{14}\text{C}]$NSR did not change substantially: 15% acetate, 4.5% glycolate, 2.8% formate, 60% in the unretained fraction, and 19% in other fractions.

In 60 day samples from the typical sandstone treatment, the average NSR remaining after lyophilizing was 58%. HPLC fractioning of these samples revealed only
Table 3.1. Distribution of $^{14}$C labeled compounds present in the NSR after 60 and 120 days of incubation in Experiment 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Recovery of $^{14}$C-NSR in HPLC fractions $^a$</th>
<th>% of $^{14}$C-NSR remaining after lyophilizing $^b$</th>
<th>% of $^{14}$C-NSR remaining after lyophilizing $^c$</th>
<th>% of lyophilized NSR in HPLC fractions $^d$</th>
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Pyrite Rich Sandstone at 120 days

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<th>% of $^{14}$C-NSR remaining after lyophilizing $^b$</th>
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Typical Sandstone at 60 days

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<th>% of $^{14}$C-NSR remaining after lyophilizing $^b$</th>
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Typical Sandstone at 120 days

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<th>% of $^{14}$C-NSR remaining after lyophilizing $^b$</th>
<th>% of $^{14}$C-NSR remaining after lyophilizing $^c$</th>
<th>% of lyophilized NSR in HPLC fractions $^d$</th>
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<td>3.0</td>
</tr>
</tbody>
</table>

$^a$ Each sample was a 100 mL.

$^b$ Amount of $[^{14}]$CNSR as a percentage of the total $[^{14}]$Ccis-DCE added.

$^c$ (total $^{14}$C remaining after lyophilizing)/(total $^{14}$C present prior to lyophilization).

$^d$ $\Sigma$(14C in all HPLC fractions)/(total 14C of the lyophilized NSR injected onto the HPLC).

$^e$ $[^{14}]$C in the fraction indicated)/(total $^{14}$C of the lyophilized NSR injected onto the HPLC).

$^f$ Only one sample is shown because the other bottle broke.
3.2-4.2% associated with acetate, glycolate, and formate; most of the $^{14}$C activity was associated with the unretained fraction (Table 3.1). After 120 days of incubation, the distribution in the one remaining bottle (the other was broken) was similar.

The $^{14}$C results from experiment I (Figure 3.1) clearly indicate that the highest amount of cis-DCE transformation occurred with typical sandstone. Consequently, only typical sandstone was used for experiment II. The lack of an increase in $^{14}$CO$_2$ + [$^{14}$C]NSR over time for the typical sandstone treatment indicated that samples needed to be taken more frequently to establish a rate of transformation. A set of preliminary experiments were conducted to identify an appropriate sampling rate (Appendix B, Figure B.2). As part of these preliminary experiments, the effect of rock size on the rate and extent of cis-DCE transformation was examined. The size range used for experiment I (0.075-0.25 mm) was similar to previous studies. However, this range is not necessarily representative of the sandstone. The preliminary experiments revealed that typical sandstone smaller than 0.85 mm gave similar results (Appendix B, Figure B.2), so this range was used for experiment II.

3.3.2 Experiment II

GC-FID headspace monitoring results are shown in Figure 3.2. Decreases in cis-DCE for the live, autoclaved and propylene oxide treatments were considerably greater than in the groundwater control. VC, ethene and ethane accumulated in the live and propylene oxide treatments, while none formed in the autoclaved treatments. Acetylene accumulated in all of the treatments, and was the only volatile product detected in the autoclaved treatment. Nevertheless, the magnitude of volatile daughter product accumulation was
Figure 3.2. Volatile compounds formed from cis-DCE in the treatments tested in experiment II. Compounds were determined by GC-FID analysis. Error bars represent the standard deviation based on triplicate bottles.
small; compared to the initial concentration of cis-DCE (approximately 500 μM), the highest accumulation of a volatile product was ethene in the live treatment, reaching a maximum of 20 μM. Most of the volatiles reached a maximum early in the incubation period, followed by a decrease. Some of the decrease was undoubtedly due to losses during removal of liquid samples (Appendix B, Table B.1). Additional decreases may have involved transformation to other products.

During experiment II, $^{14}$CO$_2$ + $[^{14}\text{C}]$NSR, as well as the distribution of $^{14}$C in NSR, were analyzed at more frequent time intervals. At the first time step (0.5 days), 2.9% $^{14}$CO$_2$ + $[^{14}\text{C}]$NSR was formed in the autoclaved treatment, of which 2.6% was $[^{14}\text{C}]$NSR (Figure 3.3a). NSR continued to increase until day 2, when the $[^{14}\text{C}]$NSR + $^{14}$CO$_2$ was 4.1%. After day 5 the amount of $[^{14}\text{C}]$NSR decreased gradually while the percentage of $^{14}$CO$_2$ increased to a maximum at day 90. The treatment with live sandstone followed a different trend. $[^{14}\text{C}]$NSR increased through day 90, then declined. The maximum $[^{14}\text{C}]$NSR formed in the live treatment was 3.1% (Figure 3.3b), compared to 4.1% in the autoclaved treatment (Figure 3.3b). The highest level of $[^{14}\text{C}]$NSR reached in the propylene oxide treatment was only 0.51% after 52 days of incubation and 0.21% CO$_2$ after 4 days of incubation (Figure 3.3e). Insignificant amounts of $[^{14}\text{C}]$NSR and $^{14}$CO$_2$ formed in the groundwater control treatment (Figure 3.3f).

Accumulation of $[^{14}\text{C}]$NSR in the autoclaved and live treatments was sufficiently high during several sampling steps to permit fractionation of lyophilized NSR by HPLC. In samples from the autoclaved sandstone treatment, glycolate was predominant through day 24, followed by a decrease in glycolate and an increase in acetate and the unretained
Figure 3.3. Experiment II distribution of $^{14}$C compounds; a) CO$_2$ + NSR in autoclaved typical sandstone and in b) live typical sandstone; c) HPLC fractionation of lyophilized NSR from the autoclaved and d) live bottles; e) CO$_2$ + NSR in propylene oxide treated typical sandstone and in f) the groundwater (GW) controls. Error bars represent the standard deviation for total amounts based on triplicate analyses. UR = unretained.
fraction (Figure 3.3c). In samples from the live treatment, glycolate, formate and acetate increased from day 24 to 90 and acetate decreased at day 120 as the formate increased.

The distribution of \(^{14}\text{C}\)-labeled volatile compounds was measured for samples taken on day 52 (Table 3.2). The \(^{14}\text{C}\) results supported the GC-FID results, which revealed a greater level of \textit{cis}-DCE transformation to VC, acetylene, ethene and ethane in the live treatment, followed by the propylene oxide treatment. At day 52, 1.4\% of the \(^{14}\text{C}\)\textit{cis}-DCE added was recovered as \(^{14}\text{C}\)-labeled ethane. The only notable \(^{14}\text{C}\) volatile product recovered from the autoclaved treatment was acetylene. The groundwater controls did not have any \(^{14}\text{C}\)-labeled ethene, ethane, or acetylene.

\textit{3.3.3 Sediment Analysis}

Autoclaved typical sandstone had the highest surface area and more than double the pore diameter of the live sandstone (Table 3.3). The autoclaved sandstone also had one order of magnitude more pore volume than the live sandstone and propylene oxide treated sandstone (Table 3.3). pH and extractable elemental composition were determined before and after sterilizing the typical sandstone by autoclaving and exposure to propylene oxide. The live sandstone had a pH of 7.7, which increased by 0.25 units after autoclaving and 0.45 units after exposure to propylene oxide (Table 3.4). Extractable Fe, Mg, K and Zn decreased after autoclaving, while no substantial decreases occurred in the propylene oxide treatments. Extractable sulfur and phosphorous more than doubled in the autoclaved treatment. A smaller increase in the S content was seen in the propylene oxide treatment. There were no significant differences in Na and Ca among the three treatments.
Table 3.2. Distribution of volatile $^{14}$C labeled compounds formed in experiment II after 52 days of incubation.

<table>
<thead>
<tr>
<th></th>
<th>Autoclaved</th>
<th>Live</th>
<th>Oxide</th>
<th>GW control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>amount of each compound as a percent of $[^{14}]C$ cis-DCE added</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethene</td>
<td>0</td>
<td>1.8±0.23</td>
<td>0.43±0.15</td>
<td>0</td>
</tr>
<tr>
<td>acetylene</td>
<td>0.031±0.00a</td>
<td>0.82±0.05</td>
<td>0.29±0.08</td>
<td>0</td>
</tr>
<tr>
<td>ethane</td>
<td>0.021±0.00</td>
<td>1.4±0.54</td>
<td>0.32±0.13</td>
<td>0</td>
</tr>
<tr>
<td>VC</td>
<td>0.19±0.08</td>
<td>0.68±0.57</td>
<td>0.16±0.21</td>
<td>0.14±0.04</td>
</tr>
<tr>
<td>cis-DCE</td>
<td>46±6.1</td>
<td>42±5.0</td>
<td>35±2.0</td>
<td>40±5.0</td>
</tr>
</tbody>
</table>

* Standard deviation of triplicate analyses.
Table 3.3. Rock properties and kinetic parameters for experiment II.

<table>
<thead>
<tr>
<th></th>
<th>First Order Rate of cis-DCE Transformation (yr⁻¹)</th>
<th>Volume of Pores (cm³/kg)</th>
<th>Adsorption Pore Diameter (nm)</th>
<th>BET Surface Area (SA)(m²/g)</th>
<th>SA Normalized Rates of Transformation of cis-DCE (L/m²d)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live</td>
<td>6.5 ± 1.28ᵃ</td>
<td>0.40 ± 0.02</td>
<td>1.4 ± 0.9</td>
<td>1.1 ± 0.09</td>
<td>1.31E-05 ± 2.8E-06ᶜ</td>
</tr>
<tr>
<td>Autoclaved</td>
<td>5.3 ± 0.41</td>
<td>1.4 ± 0.09</td>
<td>3.9 ± 0.41</td>
<td>1.7 ± 0.13</td>
<td>1.08E-05 ± 1.1E-06</td>
</tr>
<tr>
<td>Propylene Oxide</td>
<td>4.7 ± 1.29</td>
<td>0.58 ± 0.01</td>
<td>8.5 ± 0.6</td>
<td>1.0 ± 0.01</td>
<td>1.00E-06 ± 3E-09</td>
</tr>
</tbody>
</table>

ᵃ Standard error at the 95% confidence level.
ᵇ Normalized according to the BET surface area.
ᶜ Based on propagation of error.
Table 3.4. Extractable elements present in treated and untreated sandstone$^a$

<table>
<thead>
<tr>
<th>Compound (ppm)</th>
<th>Live</th>
<th>Autoclaved</th>
<th>Propylene Oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>2.0 ± 0.5$^b$</td>
<td>8.7 ± 1.0</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>K</td>
<td>35 ± 2.0</td>
<td>30 ± 1.0</td>
<td>28 ± 3.0</td>
</tr>
<tr>
<td>Ca</td>
<td>5,594 ± 241</td>
<td>6,753 ± 1,379</td>
<td>6,125 ± 408</td>
</tr>
<tr>
<td>Mg</td>
<td>77 ± 2.0</td>
<td>44 ± 0.0</td>
<td>70 ± 11</td>
</tr>
<tr>
<td>Zn</td>
<td>1.2 ± 0.1</td>
<td>0.65 ± 0.3</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>Mn</td>
<td>24 ± 1.0</td>
<td>26 ± 4.0</td>
<td>24 ± 1.0</td>
</tr>
<tr>
<td>Na</td>
<td>20 ± 0.0</td>
<td>23 ± 0.0</td>
<td>17 ± 0.1</td>
</tr>
<tr>
<td>S</td>
<td>68 ± 1.0</td>
<td>120 ± 0.0</td>
<td>79 ± 0.0</td>
</tr>
<tr>
<td>Fe</td>
<td>182 ± 2.0</td>
<td>96 ± 5.0</td>
<td>188 ±12</td>
</tr>
<tr>
<td>Soil pH</td>
<td>7.7 ± 0</td>
<td>7.9 ± 0.1</td>
<td>8.1 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$ Values represent extractable amounts of each element, which differs from the amounts in Table A.1 which represent total amounts of each element present in the sediment.

$^b$ Range of duplicate analyses.
Results for XPS analysis of the surfaces of the live, autoclaved and propylene oxide treated sandstone are shown in Table 3.5. There was a decrease in silica and aluminum-based oxides in the autoclaved sandstone compared to the live. The propylene oxide treatment had similar amounts of albite as the live but also experienced a decrease in aluminum oxide. Iron oxides were also detected. Both hematite (Fe$_2$O$_3$) and Fe(II)oxide (FeO) were present in the live and autoclaved sandstone. However, more oxidized forms of iron also appeared in the autoclaved sandstone, i.e., magnetite (Fe$_3$O$_4$; 3.5%) and goethite (FeOOH; 0.55%). In the propylene oxide treated sandstone, FeO and FeOOH were the predominant forms of iron oxide. Overall, the total amount of oxygen-containing compounds varied for each of the treatments. The oxygen-containing compounds constituted 100% of the total oxygen in the live sandstone, 80% in the autoclaved treatment, and 93% in the propylene oxide treated sandstone (Table 3.5).

### 3.3.4 Rate of cis-DCE transformation

The rate of transformation of cis-DCE was determined from aqueous phase concentration data, obtained from GC-FID analysis of headspace samples during experiment II. These data were used rather than results from experiment I because headspace analyses were performed at more frequent time steps and more clearly revealed a trend in the decrease of cis-DCE, as well as product formation over time. The magnitude of cis-DCE losses due to sampling was small in comparison to transformation processes (Appendix B, Table B.1), as indicated by differences between the groundwater control and other treatments (Figure 3.2a). Pseudo first order rates of decrease in cis-DCE were determined by linear regression (Appendix B, Figure B.4). Rates were then
Table 3.5. Oxygen containing compounds present in live, autoclaved and propylene oxide treated sandstone determined by XPS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hematite (Fe₂O₃)</th>
<th>Ferrous Oxide (FeO)</th>
<th>Magnetite (Fe₃O₄)</th>
<th>Goethite Oxide (FeOOH)</th>
<th>Aluminum Oxide (Al₂O₃)</th>
<th>Calcium Oxide (CaO)</th>
<th>Calcium Carbonate (CaCO₃)</th>
<th>Albite (NaAlSi₅O₈)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live</td>
<td>1.8 ± 0.0</td>
<td>0.25 ± 0.0</td>
<td>0.0</td>
<td>20 ± 1.9</td>
<td>0.0</td>
<td>32 ± 2.3</td>
<td>45 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Autoclaved</td>
<td>0.16 ± 0.0</td>
<td>0.25 ± 0.0</td>
<td>3.6 ± 0.0</td>
<td>0.55 ± 0.0</td>
<td>12 ± 2.0</td>
<td>0.77 ± 0.0</td>
<td>28 ± 2.3</td>
<td>30 ± 2.3</td>
</tr>
<tr>
<td>Propylene Oxide</td>
<td>0.0</td>
<td>2.6 ± 0.0</td>
<td>0.0</td>
<td>2.2 ± 0.0</td>
<td>14 ± 1.9</td>
<td>0.68 ± 0.0</td>
<td>33 ± 2.4</td>
<td>40 ± 2.2</td>
</tr>
</tbody>
</table>

*Standard deviation of triplicate analyses*
normalized to surface area using the density calculated by the mass of sediment and liquid added to the bottle, along with the measured surface area. The surface area-normalized first order rate of transformation for cis-DCE was highest for the live sandstone (1.31E-05 L/m²d), followed by the autoclaved treatment (1.08E-05 L/m²d) and the propylene oxide treated sandstone (Table 3.3). The first order rates were all statistically greater than the rate of cis-DCE loss from the water controls (t-test of the slopes of the regression lines, p<0.025).

3.4 Discussion

This study confirmed the abiotic transformation of cis-DCE to glycolate, formate, acetate and other soluble products, as previously reported with typical sandstone from the same site in southern California (1). A greater amount of CO₂ formation in the live treatments was also consistent with the previous study. By using a higher initial concentration of cis-DCE in the current study, formation of low levels of VC, ethene, ethane and acetylene was observed. Based on these findings, a modified pathway for transformation of cis-DCE is shown in Figure 3.4. Formation of ethene may occur via reductive dechlorination through VC or β-elimination through acetylene.

In the microcosm study by Darlington et al. (1), both live and autoclaved sandstone from this site catalyzed the transformation of cis-DCE to CO₂ + NSR. However, a greater extent of transformation of cis-DCE to NSR occurred in the autoclaved microcosms and oxidation of cis-DCE to CO₂ predominated in the live microcosms. Although some NSR transferred from the cis-DCE stock solution into the
Figure 3.4. Abiotic transformations observed in this study. \([H] = H^+ + e^-\).
microcosms in the previous study, the amount of NSR + CO₂ present in the live and autoclaved microcosms was statistically greater than that present in the water controls.

In this study, [¹⁴C]cis-DCE was added to the bottles via the GC, ensuring that no NSR or any other compounds were added at the start of the experiment. Therefore, the appearance of NSR + CO₂ was solely a result of cis-DCE transformation. In the previous study (1) and experiment I of this study, no obvious trend was seen in the formation of [¹⁴C]NSR + ¹⁴CO₂ as a consequence of a lack of sampling data within the first 30 days of incubation. In experiment II, however, more frequent analysis of the transformation products provided evidence that organic acids were formed first, followed by oxidation to CO₂. Of the three organic acids identified, glycolate accumulated first, followed by formate and acetate. These products gradually decreased as the proportion of unretained [¹⁴C]NSR and ¹⁴CO₂ increased. The previous study (1) suggested that the sandstone has a finite capacity for transformation. In this study the maximum amount of cis-DCE transformed was 16% in experiment I and 10% in experiment II, at which point transformation leveled off. This confirmed the observation that the sandstone has a finite transformation capacity.

The rate of transformation of cis-DCE in the live, autoclaved and propylene oxide controls are higher than the abiotic rates reported by Ferrey et al. (2), which ranged from 0.31-2.3 yr⁻¹. In their study, the abiotic and biotic rates of cis-DCE transformation were similar which also occurred in this study. The slightly faster rate of transformation obtained for the live microcosms contradicts with the extent of transformation seen over time in the live treatment compared to the autoclaved treatment. The live treatments
however could have had a faster rate of *cis*-DCE transformation due to a combination of biotic and abiotic transformation processes. When considering which method should be used for sterilizing solids, it is important to consider the soil type and transformation pathways that may be present. One major difference between this study and that of Ferrey et al. (2) is the type of solids used. Ferrey et al. (2) collected unconsolidated sandy aquifer material from 50-56 m below the surface, compared to the typical sandstone used in this study, which was collected at depths of 165-265 m below the surface.

The results from experiment I demonstrated that pyrite is not the predominant catalyst for the abiotic transformation of *cis*-DCE to NSR + CO\(_2\). The extent of *cis*-DCE transformation was four times higher in the autoclaved treatment with typical sandstone in comparison to the treatment with pyrite-rich sandstone, and both were higher than the treatment with pure pyrite. Lee and Batchelor (7) demonstrated the abiotic transformation of *cis*-DCE in the presence of pyrite, with acetylene as the main product.

Because of its higher reactivity during experiment I, all treatments for experiment II were prepared with typical sandstone. Initially, the extent of \([^{14}\text{C}]\text{cis-DCE}\) transformation to \([^{14}\text{C}]\text{NSR} + ^{14}\text{CO}_2\) was highest in the treatment with autoclaved sandstone, suggesting that autoclaving somehow enhanced this activity. However, as the incubation period lengthened the transformation of \([^{14}\text{C}]\text{cis-DCE}\) to \([^{14}\text{C}]\text{NSR} + ^{14}\text{CO}_2\) in the treatment with live sandstone continued to increase and eventually reached the same extent. This differed from the previous study (1), in which the extent of *cis*-DCE transformation remained higher in the autoclaved microcosms through more than two years of incubation. Although typical sandstone was used in both studies, the cores used
were taken from different depths, which may explain the differences in behavior. Regardless, it is apparent that autoclaving significantly altered the rate of transformation and, at least in the previous study, also altered the extent.

First order rates of cis-DCE transformation normalized by surface area were determined using the GC-FID monitoring data. The fastest rate of transformation occurred in the treatment with live sandstone which could have been due to a combination of biotic and abiotic transformations. The cis-DCE data, however, showed a slower rate of $^{14}$C]NSR formation. For example, after 0.5 d of incubation in the autoclaved sandstone, $^{14}$C]NSR formation was more than twice that of the treatment with live sandstone and more than ten times that in the treatment with sandstone sterilized with propylene oxide. Autoclaving increased both the surface area and pore volume of the sandstone, exposing the cis-DCE to a greater number of reactive surfaces that coincided with an increased rate of reaction. Jenneman et al. (19) reported that autoclaving increased the porosity of Berea sandstone, which contains more silica and less carbonates than the sandstone used in this study. Compared to field soil and fractured rock, considerably higher first order rates of abiotic transformation of cis-DCE have been reported for pure minerals such as pyrite, magnetite and green rust, ranging from 216-378 yr$^{-1}$ (6,7).

The treatment with sandstone sterilized by propylene oxide had the lowest rate and extent of $^{14}$C]cis-DCE transformation to $^{14}$C]NSR + $^{14}$CO$_2$ and also had the lowest surface area. Wolf et al. (12) also reported decreased surface area for soils treated with propylene oxide. This may be due to residual carbon remaining after propylene oxide is
evaporated by vacuum (10). In this study, GC analysis of the headspace of bottles treated with propylene oxide indicated that none was detected. Nevertheless, residual organic carbon may have still been present, as a consequence of reaction of the propylene oxide with the rock (11,20). Dao et al. (20) observed that residual organic carbon and a change in pH after treatment of soils with propylene oxide decreased the absorption of aniline-HCl but had no effect on adsorption of diuron. In this study, treating the sandstone with propylene oxide may have reduced the sites available for adsorption and thereby decreased the rate and extent of cis-DCE transformation in comparison to treatments with live sandstone and autoclaved sandstone.

Analysis of extractable elements revealed significant differences among the compositions of typical sandstone in the live, autoclaved and propylene oxide treated rock. There was an overall decrease in the Al, Si and K content in the autoclaved and propylene oxide treated sandstone. This observation is consistent with a study by Croker et al. (21), which suggests that autoclaving sandstone causes dissolution of the clay that usually cements the silica grains. This change in cementation may explain the increase in pore diameter and pore volume seen in the autoclaved soils in this study. It is not yet known if the changes in extractable elements contributed to differences in the rates and extent of cis-DCE transformation among the various treatments with sandstone present.

Exposure of the sandstone to autoclaving and propylene oxide resulted in an increase in pH. Sterilization with propylene oxide has previously been found to increase the pH of soils by as much as 1.1 units (10-12). An increase occurs because propylene oxide esterifies the carboxyl groups in proteins. It reacts with labile H atoms in the
carboxy and hydroxyl groups on the organic and inorganic fractions of the soil matter, thereby increasing the pH (22). An average increase of 0.4 units, closer to what was obtained by this study, was reported by Bartlett and Zelazny (23), who investigated the effect of propylene oxide on the pH of 16 different soils. Their study revealed that soils with higher organic matter content showed larger pH increases when treated with propylene oxide. This is consistent with the lower pH change observed in this study, since the sandstone contains only 0.1% organic matter (1). Smaller pH changes were observed in the autoclaved sandstone (compared to the live and propylene oxide treatments), consistent with previous studies that have reported minor increases in pH as a result of autoclaving (10-12). This suggests that for reactions that are sensitive to changes in pH, such as abiotic transformations, propylene oxide is not a suitable method for sterilizing soils. This may explain why the smallest degree of $[^{14}\text{C}]\text{cis-DCE}$ transformation to $[^{14}\text{C}]\text{NSR} + ^{14}\text{CO}_2$ occurred in this study with propylene oxide treated sandstone. This increase in pH may also explain the decreases observed in Al and Fe, due to complexation and precipitation of their hydroxides (12) making the elements unextractable.

XPS analysis of the surface of the sandstone revealed the presence of iron oxides and the minerals magnetite and goethite. Magnetite was highest in the autoclaved sandstone, suggesting that magnetite may be responsible for the rapid transformation of $[^{14}\text{C}]\text{cis-DCE}$ to $[^{14}\text{C}]\text{NSR} + ^{14}\text{CO}_2$. Batchelor and Lee (7) examined the ability of pure magnetite to abiotically transform cis-DCE and observed only minor amounts of transformation to acetylene. However, they did not test for possible soluble products.
Ferrey et al. (2) observed transformation of cis-DCE and 1,1-DCE with sediment containing magnetite in live and autoclaved microcosms; VC was the only daughter product they measured. No non-volatile products or CO₂ were measured.

For this study, the presence of goethite in the autoclaved sandstone suggests that it too played a role in abiotic transformation of cis-DCE. When Costanza et al. (3) amended soil samples with 1% goethite, abiotic transformation of TCE to glycolate, formate, CO and CO₂ increased by one order of magnitude. Goethite also played a role in the abiotic reductive dechlorination of carbon tetrachloride in the presence of Fe(II) (24). This indicates that soils rich in iron in general, and goethite in particular, have an ability to abiotically transform chlorinated compounds. XPS analysis indicated the absence of goethite and magnetite in sandstone from the live treatment. However, this does not necessarily mean that these minerals are absent in the live sandstone, since XPS analysis is unable to detect ppm levels of minerals on the surface of the sandstone. Since autoclaving increased the surface area and pore diameter and volume of the sandstone, it was easier to detect the presence of minerals in rock from this treatment. The increased availability of minerals in the autoclaved sandstone correlates with the higher initial rate of transformation of [¹⁴C]cis-DCE to ¹⁴CO₂ + [¹⁴C]NSR that occurred with this treatment.

Several previous studies reported significant increases in extractable Mn in soils after autoclaving (10-12), although no such increase occurred with the sandstone used in this study. In soils with high Fe content, other studies observed no increase in Mn levels with autoclaving (25,26). In soils with a pH greater than 5.2, Mn is complexed to Fe-
oxides and, therefore, is not affected by autoclaving, which is consistent with the lack of an increase in Mn seen in the treated soils in this study, since the sandstone is rich in Fe.

VC, ethene, ethane and acetylene were identified as volatile products from cis-DCE transformation during this study. This is in contrast to the previous microcosm study using the same types of sandstone (1). Two differences in experimental approach likely contributed. First, the initial concentration of cis-DCE used in this study was considerably higher, making it easier to detect low levels of volatile products. Second, a higher ratio of sandstone to groundwater was used, thereby increasing the concentration of the surfaces involved in abiotic transformation, as well as the microbes involved in biotic activity. Among the treatments tested, all of the volatile products were detected in the live and propylene oxide bottles, while only acetylene was detected in the autoclaved sandstone. Furthermore, accumulation of acetylene was lower in the autoclaved bottles, indicating that the autoclaving process disrupted the mechanism of abiotic transformation of cis-DCE to acetylene. Accumulation of VC, ethene, ethane and acetylene in the treatment sterilized with propylene oxide suggest these transformations were abiotic. Transformation of cis-DCE to low concentrations of these volatile products is consistent with data from field measurements (15). This study underestimated the amount of volatile products that were formed, due to losses that occurred during removal of aqueous samples for analysis of $[^{14}\text{C}]\text{NSR} + ^{14}\text{CO}_2$.

In summary, this study demonstrated that typical sandstone from the site in southern California is capable of catalyzing abiotic transformation of cis-DCE to NSR + CO$_2$, and glycolate, formate, and acetate are components of the NSR. Autoclaving the
sandstone increased the rate of transformation but did not increase the extent of transformation. This sandstone has a finite capacity for transformation. Autoclaving increases the surface area, pore volume and pore diameter of the sandstone, increasing the number of active sites available for transformation, which may explain the higher initial rate of abiotic transformation. Autoclaving increased the availability of magnetite and goethite, which have been linked to abiotic transformation of chlorinated ethenes in previous studies. Sandstone treated with propylene oxide had a lower surface area, no major change in the volume of pores, and an increase in the diameter of pores. Nevertheless, these changes did not enhance abiotic transformation of cis-DCE. The lack of cis-DCE transformation seen in the propylene oxide treated sandstone in comparison to the live treatment may be due to residual carbon from the propylene oxide, preventing the surface catalyzed transformation of cis-DCE to NSR + CO₂.

3.5 References


(15) Pierce, A. A.; Isotopic and Hydrogeochemical Investigation of Major Ion Origin and Trichloroethene Degradation in Fractured Sandstone. MS Thesis, Earth Sciences, University of Waterloo, **2005**.


PCR-DGGE ANALYSIS of a TCE to cis-DCE DECHLORINATING ENRICHMENT CULTURE

4.0 Abstract

A fractured sandstone aquifer at an industrial site in southern California is contaminated with trichloroethene (TCE) to depths in excess of 244 m. Field monitoring data suggest that TCE is undergoing reduction to cis-DCE and that additional attenuation is occurring. Live microcosms prepared anaerobically with groundwater and sediment from the site and spiked with TCE exhibited transformation of TCE to cis-DCE within 30 days of incubation. The objective of this study was to identify the microbe responsible for TCE reduction to cis-DCE at the industrial site in southern California and compare this microbe to others that are known to mediate this reaction via chlororespiration. The TCE to cis-DCE dechlorinating microbe was enriched in the microcosms by repeatedly supplying TCE whenever it was reduced to cis-DCE. Samples from the microcosms were then transferred to a defined anaerobic mineral medium. Serial dilutions ranging from $10^{-2}$ to $10^{-9}$ were prepared, with activity observed though the $10^{-7}$ dilutions. These were used to inoculate a second set of serial dilutions, ranging from $10^{-2}$ to $10^{-4}$, all of which exhibited reductive dechlorination activity. Hydrogen and acetate were added as the electron donor and carbon source, respectively. DNA extracted from the microcosms and serial dilutions was amplified with specific primers for several microbes that are known to chlororespire TCE to cis-DCE (Dehalobacter, Desulfuromonas, Sulfurospirillum, Desulfitobacterium and Geobacter). These results indicated that the microorganism responsible for TCE to cis-DCE dechlorination was not one of the previously known
genera of TCE to cis-DCE dechlorinators. However, PCR with universal bacterial primers, followed by DGGE and sequencing of individual bands revealed *Pseudomonas stutzeri* as the microorganism in the community most likely responsible for the reduction of TCE to cis-DCE. PCR-DGGE analysis of DNA extracted from the serial dilutions yielded a sequence having a 100% match to *P. stutzeri*. In control cultures provided with lactate and acetate but not TCE, *P. stutzeri* was no longer present. In addition to the *P. stutzeri* identified in this study, only one other facultative anaerobe (strain MS-1) is known to dechlorinate TCE to cis-DCE. Confirmation of the presence of a *Pseudomonas* species in enrichments and dilutions provided with TCE was obtained with PCR using *Pseudomonas stutzeri* specific primers. *Desulfovibrio putealis*, a sulfate reducer, was also found to be present in the community, during growth in the presence and absence of TCE. Overall, the results indicate that reductive dechlorination of TCE to cis-DCE at the southern California industrial site is mediated by a facultative anaerobic microbe that has not previously been associated with this type of transformation activity.
4.1 Introduction

At an industrial site in southern California described previously by Darlington et al. \(1\) and Pierce (2), groundwater in fractured sandstone is contaminated with trichloroethene (TCE) from past discharges at the surface. The TCE has migrated as far as 244 m below the surface. Field data indicate that significant amounts of TCE have undergone reduction to \textit{cis}-dichloroethene (DCE). The strictly biotic nature of this activity has been confirmed in a laboratory microcosm study. The frequency of reductive dechlorination activity increased with samples taken from greater depth. Only minor amounts of vinyl chloride (VC) have been detected at the site and in the microcosm study, suggesting that \textit{cis}-DCE is the terminal product from biologically mediated reductive dechlorination.

Numerous microorganisms have been identified that are capable of dechlorinating TCE sequentially to \textit{cis}-DCE, VC, and ethene. Some microorganisms are capable of chlororespiring TCE to one or all of its daughter products, whereas others can only use TCE cometabolically \(3,4\). In order for TCE to be dechlorinated via chlororespiration, low redox conditions (likely below -100 mV) are required \(5\).

\textit{Dehalococcoides ethenogenes} strain 195 \(6\) and \textit{Dehalococcoides} sp. strain FL2 \(7\) are microorganisms that reduce TCE to VC metabolically, then cometabolically convert VC to ethene. The enrichment culture from which \textit{D. ethenogenes} strain 195 was isolated was first inoculated with sludge from a municipal anaerobic digester \(8\). Strain 195 uses hydrogen as its electron donor. Hydrogen is widely considered the universal electron donor for reductive dechlorination of chlorinated ethenes. \textit{D.}
*ethenogenes* strain FL2 originated in sediment from the Red Cedar River, for which there was no prior evidence of TCE contamination (7). *Dehalococcoides* strain GT (9) and strain VS (10) are capable of chlororespiring TCE completely to ethene, a harmless end product. Both strain VS and GT were obtained from sites contaminated with chlorinated solvents (9,11).

Other microorganisms have been isolated or enriched that chlororespire TCE to *cis*-DCE but are incapable of further dechlorination (Table 4.1). *Dehalospirillum* (now referred to as *Sulfurospirillum* multivorans) was isolated from activated sludge in the presence of pyruvate and tetrachloroethene (PCE). It is capable of dechlorinating PCE to *cis*-DCE, but no further (12-15). *Dehalobacter restrictus* chlororespires TCE using only hydrogen as an electron donor (16-18). This organism was enriched from a packed bed column containing Rhine River sediments and ground anaerobic granular sludge (18). Several strains of *Desulfitobacterium* (19-21) are capable of dechlorinating TCE to *cis*-DCE in the presence of pyruvate. Strain PCE1 was initially enriched from a soil sample that originated from a PCE-contaminated site (21). *Desulfuromonas* strain TT4B, another microbe that chlororespires TCE to *cis*-DCE, utilizes acetate as its electron donor rather than hydrogen (22). Known originally for its ability to reduce hexavalent chromium, *Geobacter lovleyi* strain SZ is the only *Geobacter* that can dechlorinate TCE, but only as far as *cis*-DCE. *Geobacter lovleyi* was isolated from a location without prior contamination and uses acetate as its electron donor. Strain MS-1 is a facultative aerobe that chlororespires TCE to *cis*-DCE (26) when grown under anaerobic conditions with several electron donors, including acetate, lactate and pyruvate (26).
Table 4.1. Summary of microbes known to chlororespire TCE to cis-DCE.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Strain</th>
<th>Source</th>
<th>e⁻ donors</th>
<th>Carbon sources</th>
<th>pH range</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sulfurospirillum multivorans</em></td>
<td>NA</td>
<td>sewage sludge</td>
<td>lactate, pyruvate, H₂, ethanol, formate glycerol</td>
<td>acetate</td>
<td>7.0-7.5</td>
<td>(12-14,23) 4)</td>
</tr>
<tr>
<td><em>Dehalobacter restrictus</em></td>
<td>PER-K23</td>
<td>anaerobic river sediment</td>
<td>H₂</td>
<td>acetate, CO₂</td>
<td>6.8-7.6</td>
<td>(18)</td>
</tr>
<tr>
<td><em>Dehalobacter restrictus</em></td>
<td>TEA</td>
<td>contaminated aquifer</td>
<td>H₂</td>
<td>acetate, CO₂</td>
<td>NR</td>
<td>(16)</td>
</tr>
<tr>
<td><em>Desulfitobacterium sp.</em></td>
<td>PCE-1</td>
<td>PCE contaminated soil</td>
<td>lactate, pyruvate, formate, ethanol</td>
<td>pyruvate lactate</td>
<td>NR</td>
<td>(21)</td>
</tr>
<tr>
<td><em>Desulfitobacterium sp.</em></td>
<td>PCE-S</td>
<td>PCE contaminated soil</td>
<td>formate, pyruvate</td>
<td>acetate</td>
<td>NR</td>
<td>(15)</td>
</tr>
<tr>
<td><em>Desulfuromonas chloroethenica</em></td>
<td>TT4B</td>
<td>contaminated anaerobic sediment</td>
<td>acetate, pyruvate</td>
<td>acetate</td>
<td>6.5-7.4</td>
<td>(24)</td>
</tr>
<tr>
<td><em>Desulfuromonas michiganensis</em></td>
<td>NA</td>
<td>pristine river sediment</td>
<td>acetate, lactate</td>
<td>Acetate</td>
<td>7.0-7.5</td>
<td>(25)</td>
</tr>
<tr>
<td>NA</td>
<td>MS-1</td>
<td>PCE contaminated site</td>
<td>acetate, lactate, ethanol</td>
<td>acetate</td>
<td>NR</td>
<td>(26)</td>
</tr>
<tr>
<td><em>Geobacter lovleyi</em></td>
<td>SZ</td>
<td>creek sediment</td>
<td>H₂</td>
<td>acetate</td>
<td>6.5-7.2</td>
<td>(9)</td>
</tr>
</tbody>
</table>

N/A = not available
NR = not reported
The objective of this study was to identify the microbe(s) responsible for TCE reduction to cis-DCE at the industrial site in southern California. Conditions at this site are distinct from the ones described above that have yielded microbes capable of chlororespiring TCE, i.e., it consists of fractured sandstone at considerable depths below the surface. It was, therefore, of interest to determine if the microbe responsible belongs to one of the known genera of TCE dechlorinating microbes, or if it belongs to a genus not previously associated with reductive dechlorination of TCE.

The approach taken in this study was to use samples from microcosms that exhibited reductive dechlorination of TCE as a source of inoculum to develop a TCE-dechlorinating enrichment culture, followed by sediment-free dilutions in a defined anaerobic medium. Using denaturing gradient gel electrophoresis (DGGE), the microbes in the enrichment were identified and compared to a companion enrichment culture that was grown in the absence of TCE. *Pseudomonas stutzeri* emerged as the microbe present in the enrichment that is most likely responsible for reductive dechlorination of TCE. This was unexpected, since pseudomonad species have not previously been associated with reductive dechlorination of TCE.

4.2 Materials and Methods

4.2.1 Chemicals

TCE (99%) was obtained from Fischer Scientific. Hydrogen gas (99%) was purchased from National Welders (Charlotte, NC). Sodium acetate anhydrous ACS reagent grade (99%) was purchased from VWR. All other chemicals were reagent grade or equivalent.
4.2.2 Analytical Methods

The amount of TCE and cis-DCE present in serum bottles was monitored by gas chromatographic (GC) analysis of headspace samples (0.5 mL), as previously described (1). A Hewlett Packard 5890 Series II GC was used, equipped with an FID and a 2.44-m by 3.175-mm column packed with 1% SP-1000 on 60/80 Carbopak B (Supelco). Hydrogen was monitored on a Hewlett Packard 5890 Series II GC, equipped with a thermal conductivity detector, Carbosieve SII column and N\textsubscript{2} as the carrier gas. A 150°C isothermal method was used, with a reference gas flow rate of approximately 50 mL/min. Acetate was measured on a Waters high performance liquid chromatograph (HPLC) equipped with an Aminex HPX-87H column (300 mm x 7.6 mm), H\textsubscript{2}SO\textsubscript{4} eluant (0.1 N, 0.6 mL/min), and a UV/Vis detector (model 490E) at 210 nm; 20 µL samples were injected onto the column.

4.2.3 Source of Inoculum

The enrichment process was started with inoculum from a microcosm prepared with sandstone and groundwater that reductively dechlorinated TCE to cis-DCE (1). The microcosm was first spiked with [\textsuperscript{14}C]TCE to assist in the identification of biotic and abiotic transformation products. Upon confirmation of the appearance of [\textsuperscript{14}C]cis-DCE, subsequent additions of TCE (using TCE-saturated water) were not \textsuperscript{14}C-labeled. Each new addition of TCE was increased by 50% over the previous addition; the highest amount of TCE added was 21 µmol per bottle, which is equivalent to 55 mg/L, assuming equilibrium between the headspace and aqueous phase within the serum bottles (160 mL). When transformation of TCE to cis-DCE ceased, hydrogen (20 µmol/bottle) was
added to serve as an electron donor. Whenever the hydrogen was consumed, the same amount was added again.

4.2.4 Initial Enrichment of TCE to cis-DCE Dechlorinators

An enrichment culture was developed using inoculum from a TCE dechlorinating microcosm and adding it to a reduced anaerobic medium, modified from Edwards and Grbic-Galic (27). The medium consisted of 10 mL of a phosphate buffer solution (52.5 g/L of K$_2$HPO$_4$); 10 mL of concentrated HCl; 2 mL of a magnesium sulfate solution (62.5 g/L MgSO$_4$$

7\text{H}_2\text{O}$); 10 mL of a salt solution containing (in grams per liter) NH$_4$Cl (53.5); CaCl$_2$$\cdot$$2\text{H}_2\text{O}$ (4.69) and FeCl$_2$$\cdot$$\text{H}_2\text{O}$ (1.78); 2 mL of a trace metal solution containing (in grams per liter) H$_3$BO$_3$ (0.30), ZnSO$_4$$\cdot$$7\text{H}_2\text{O}$ (0.21), NiCl$_2$$\cdot$$6\text{H}_2\text{O}$ (0.75), MnCl$_2$$\cdot$$4\text{H}_2\text{O}$ (1.0), CuCl$_2$$\cdot$$2\text{H}_2\text{O}$ (0.1), CoCl$_2$$\cdot$$6\text{H}_2\text{O}$ (1.5), Na$_2$SeO$_3$ (0.02) and Al$_2$(SO$_4$)$_3$$\cdot$$16\text{H}_2\text{O}$ (0.1); 1 mL of a 1 g/L resazurin solution; and 900 mL of distilled-deionized water, sterilized by autoclaving. The sterile medium was amended with 50 mL of a filter sterilized bicarbonate solution (8 g/L NaHCO$_3$) and 10 mL of a filter sterilized vitamin solution (28) containing the following (in per liter): biotin (0.05), folic acid (0.02), pyridoxine (0.1), riboflavin (0.05), thiamine (0.1), nicotinamide (0.55), cyanocobalamin (0.1), p-aminobenzoic acid (0.25), lipoic acid (0.05), and panthotenic acid (0.05). After mixing the components, the medium was placed in an anaerobic chamber and 0.14 g of FeCl$_2$$\cdot$$\text{H}_2\text{O}$ and 0.24 g of Na$_2$S$\cdot$$9\text{H}_2\text{O}$ were added. The medium was then stirred until it was reduced, as indicated by a change in the color of resazurin from blue to clear.

The enrichments consisted of 160 mL serum bottles with 50 mL of liquid present. The headspace was purged with N$_2$/CO$_2$ (70%:30% v/v), yielding a final pH of 7.4, and
they were sealed with Teflon-faced red rubber septa. The bottles were then amended with 5 mM acetate, 3 mL of inoculum, approximately 0.5 µmol of TCE (0.2 mg/L) and 20 µmol of hydrogen. They were incubated in an anaerobic chamber in the dark. Whenever TCE was consumed, the amount of the next addition was increased by 50%. pH was monitored using pH strips (6.0 to 7.7; JT Baker) and was adjusted (using 10 M NaOH) whenever the pH decreased below 6.8.

4.2.5 Further Enrichment

After the enrichment culture consumed approximately 20 mg/L of TCE, two sequences of dilutions were prepared. The first set (referred to as Phase I) consisted of the following serial dilutions, using the enrichment culture as the inoculum: $10^{-2}$, $10^{-4}$, $10^{-6}$, $10^{-7}$, $10^{-8}$ and $10^{-9}$. All of these were prepared in serum bottles with 50 mL of media, with five replicates per dilution. TCE, acetate and hydrogen were added at the same concentrations as described above for the enrichment culture. Along with the dilution bottles, a set of triplicate controls were prepared. These were initially fed with hydrogen but no acetate or TCE. When the rate of hydrogen consumption slowed, acetate was added.

Using one of the $10^{-7}$ Phase I dilutions that exhibited reductive dechlorination of TCE to cis-DCE as the inoculum, a second set of serial dilutions was prepared (referred to as Phase 2), at the following concentrations: $10^{-2}$, $10^{-3}$ and $10^{-4}$ (Appendix C, Figure C.1). These dilutions were monitored and maintained in the same manner as the Phase 1 bottles.
4.2.6 DNA Extraction and PCR

Genomic DNA was extracted from enrichments and dilutions that exhibited transformation of TCE to cis-DCE, as well as from the control that was prepared and fed only hydrogen and acetate. DNA was extracted using MoBio’s UltraClean Microbial DNA Isolation Kit. The DNA was then amplified in preparation for DGGE. The PCR reaction mixture consisted of 20 µL of an Eppendorf master mix (MgCl₂, 3.75 mM; Taq polymerase, 3.125U; and dNTPs, 500 µM), 1 µL each of a 10 µM solution of both the forward and reverse primers (in order to have 100 to 200 nM of primer in the reaction mix) and 5 µg of template DNA. The primers used were 1055f universal bacterial primer (5’-ATG GCT GTC GTC AGC T-3’) and 1406r universal bacterial primer (5’- CGC CCG CCG CCC GGC CCG CCG CCG CCC CAC GGG C GG TGT GTA C-3’) with a GC clamp added. The reverse primer was equipped with a GC clamp to allow the amplified DNA to be used for DGGE. The following touchdown thermocycling program was used: 5 min at 94°C, nine amplification cycles of denaturation (1 min at 94°C), annealing (1 min at 65°C, decreasing 1°C each cycle until touch down at 54°C), and elongation (3 min at 72°C), 24 additional amplification cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C) and elongation (3 min at 72°C) and a final elongation step of 72°C for 10 min. The amplified DNA was then used for DGGE. Direct PCR with Pseudomonas stutzeri specific primers fps 158 and rps 743 (29) was performed using the program described above. PCR products were confirmed on a 2% agarose gel and run alongside a 100 bp ladder to determine the size of the PCR products obtained.
4.2.7 Denaturing Gradient Gel Electrophoresis (DGGE)

The PCR products were purified using QIAquick PCR purification kit. PCR products were then separated using a CBS Scientific Company DGGE-2001 system. A polyacrylamide gel (6% acrylamide; 37.4:1% acrylamide:bis-arylamide; 2 % SB buffer) was prepared with a 0 to 100% and 0 to 60% linear gradient of denaturant. DGGE gels were run for 2-3 h at 40 mV and 60°C. The gels were stained with ethidium bromide and images were taken with a Biorad Molecular Imager Gel Doc XR system.

4.2.8 Nested PCR

DNA from enrichments and dilutions that exhibited the most transformation of TCE to cis-DCE was amplified for nested PCR. Initial amplification was done with a pair of universal bacterial primers 8f (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 1541r (5’- AAG GAG GTG ATC CAG CCG CA-3’) using the same conditions as described above. Specific primers for Dehalobacter, Desulfuromonas, Sulfurospirillum, Desulfitobacterium and Geobacter were then used in the second PCR (Table 4.2) using the amplified products as template (0.1 µL, 1 µL, 3 µL, 5µL). The same thermocycle program as described above was used. When necessary the annealing temperature was adjusted to the optimum (Table 4.2) for the specific primers. Positive controls for Dehalobacter, Desulfuromonas, Sulfurospirillum, Desulfitobacterium and Geobacter were obtained from Frank Loeffler at the Georgia Institute of Technology.
Table 4.2. Primers used for PCR and Nested PCR

<table>
<thead>
<tr>
<th>Primers Used</th>
<th>Primer Sequence</th>
<th>Length of fragment (bp)</th>
<th>Annealing Temperature (°C)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Universal Bacterial Primers 1</strong></td>
<td>F: 5'-ATG GCT GTC GTC AGC T-3'</td>
<td>352</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 5'- CGC CCG CCG CCG CCC GCG</td>
<td></td>
<td></td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td>CCC GGC CCG CCG CCC CCG CCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAC GGG CCG TGT GTA C-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Universal Bacterial Primers 2</strong></td>
<td>F (5'-AGA GTT TGA TCC TGG CTC AG-3')</td>
<td>1533</td>
<td>58</td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td>R (5'- AAG GAG GTG ATC CAG CCG CA-3')</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dehalobacter restrictus</strong></td>
<td>F (5'-CCT CTC CTG TCC TCA AGC CAT A-3')</td>
<td>562</td>
<td>53</td>
<td>(31)</td>
</tr>
<tr>
<td></td>
<td>R (5'-GTT AGG GAA GAA CGG CAT CTG T-3')</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Desulfuromonas michiganensis strain BB1</strong></td>
<td>F (5'-AAC CTT CGG GTC CTA CTG TC-3')</td>
<td>810</td>
<td>58</td>
<td>(32)</td>
</tr>
<tr>
<td></td>
<td>R (5'-GCC GAA CTG ACC CCT ATG TT-3')</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sulfurospirillum multivorans</strong></td>
<td>F (5'-GCT CTC GAA ACT GGT TAC CTA-3')</td>
<td>634</td>
<td>55</td>
<td>(33)</td>
</tr>
<tr>
<td></td>
<td>R (5'-GTA TCG CGT CTC TTT GTC CTA -3')</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Desulfitobacterium sp. Viet 1</strong></td>
<td>F (5'-AAT ACC GNA TAA GCT TAT CCC -3')</td>
<td>1199</td>
<td>55</td>
<td>(19)</td>
</tr>
<tr>
<td></td>
<td>R (5'-TAG CGA TTC CGA CTT CAT GTT C -3')</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aF – Forward primer  bR - Reverse Primer
4.2.9 Sequencing

Several bands obtained from the DGGE analysis were excised and amplified using the same universal bacterial primers that were initially used to amplify the DNA extract. The amplified DNA was confirmed on an agarose gel. The amplified DNA was cleaned using the Qiagen Qiaquick PCR Purification kit before sequencing. Sequencing was then performed by MWG Biotech Inc. (High Point, NC). The primer used was the 1055 forward universal primer (5'-ATG GCT GTC AGC T-3') without the GC clamp. Sequences were then identified using the NCBI BLAST database.

4.3 Results

4.3.1 Enrichments and Identification

Microcosms that transformed the initial addition of TCE were able to dechlorinate 78 µmol per bottle TCE to 68 µmol per bottle of cis-DCE over 365 days before transformation slowed and hydrogen had to be added as an electron donor to revive and maintain dechlorination. Small amounts of 1,1-DCE (0.232 mol/bottle) and VC (0.053 µmol per bottle) also accumulated. 1,1-DCE appeared after approximately 0.209 µmol of TCE had been consumed and VC appeared later, after 1.862 µmol of TCE had been consumed. The VC did not undergo further reduction.

The initial enrichments prepared with inoculum from one of the microcosms were able to dechlorinate repeated additions of TCE. The five enrichment bottles prepared dechlorinated 49±4 µmol per bottle of TCE over 350 days (Figure 4.1), producing a nearly stoichiometric amount of cis-DCE. Minor amounts of VC and 1,1-DCE formed in the enrichments (a maximum of 0.14 and 0.058 µmol per bottle, respectively). 1,1-DCE
Figure 4.1. Reductive dechlorination of TCE in a representative enrichment culture and serial dilution; a) TCE, cis-DCE and H$_2$ in an enrichment culture; b) 1,1-DCE and VC accumulation in an enrichment culture; c) a 10$^{-4}$ dilution of the enrichment culture fed TCE, H$_2$ and acetate; and d) a control dilution fed hydrogen and acetate (acetate data not shown).
appeared with the first appearance of cis-DCE, while VC appeared after 21 µmol per bottle of TCE was transformed to cis-DCE. The five enrichment bottles used 338±43 µmol per bottle of hydrogen in 313 days, more than ten times the amount required for stoichiometric reduction of the TCE consumed. After consuming approximately three additions of TCE, dechlorination activity decreased even when sufficient hydrogen was available. Acetate (5 mM) was then added to the enrichments on day 123 and reductive dechlorination resumed. Although the concentration of acetate in the enrichments was not monitored regularly, one measurement on day 256 showed that acetate had decreased to 0.1 mM; the formate concentration at that point was 0.18 mM.

The first addition of TCE to the Phase 1 dilutions was transformed to cis-DCE within 30 to 60 days. Subsequent additions (increased by 50%) were dechlorinated in a similar time frame. All five of the replicate Phase 1 dilutions at 10⁻² dechlorinated TCE (1.73 µmol/bottle) and consumed H₂ (178 µmol/bottle) during 212 days of incubation. Only one of the 10⁻⁴ dilutions dechlorinated repeated additions of TCE (0.64 µmol/bottle) and consumed hydrogen (125 µmol/bottle) during 205 days of incubation. Three of the 10⁻⁷ dilutions dechlorinated TCE to cis-DCE over a 212 day period (an average of 1.06 µmol/bottle) and consumed hydrogen (226 µmol/bottle). Dechlorination of TCE to cis-DCE was not accompanied by even minor amounts of 1,1-DCE or VC in the Phase 1 or Phase 2 dilutions, as occurred in the microcosm and initial enrichments. In replicates of the dilutions that did not dechlorinate TCE (i.e., all of the 10⁻⁶, 10⁻⁸ and 10⁻⁹ dilutions), hydrogen was consumed. Since TCE dechlorination activity occurred in two of the 10⁻⁷ dilutions, it is unclear why no activity occurred in any of the 10⁻⁶ series.
Phase 2 dilutions were prepared after Phase 1 dilutions had been incubated for 89 days. Four of the replicates at the $10^{-2}$ dilution, three at the $10^{-3}$ dilution, and one at the $10^{-4}$ dilution dechlorinated repeated additions of TCE (Appendix C, Figure C.1). Similar to the Phase 1 dilutions, reduction of TCE to cis-DCE was nearly stoichiometric and occurred without any accumulation of 1,1-DCE or VC. The amount of hydrogen consumed was well in excess of the amount needed for TCE reduction to cis-DCE, as described above. One of the triplicate controls that was fed H$_2$ used as much as 550 µmol per bottle in 80 days.

4.3.2 Nested PCR, PCR-DGGE and Sequencing

PCR and nested PCR were performed on DNA extracted from the initial enrichments and Phase 1 and 2 dilutions that transformed repeated additions of TCE to cis-DCE. The universal bacterial primers produced a fragment 1533 base pairs in length, which was used as the template for nested PCR. Both nested and direct PCR, using DNA that was extracted and amplified from the microcosms and dilutions with specific primers, did not yield any bands for five of the genera known to chlororespire TCE to cis-DCE. The positive control templates, however, did yield bands of the correct sizes corresponding to the specific primers used.

The universal bacterial primer pair with the GC clamp attached to the reverse primer yielded products 350 bp in size. DGGE analysis of the PCR products showed the presence of three to five species of bacteria in the community. Figure 4.2 shows a DGGE gel for the initial enrichments and Phase 1 dilutions. Lanes 4 and 5 represent duplicate enrichments; lanes 2 and 3 are amplified DNA from the $10^{-2}$ and $10^{-4}$ Phase 1 dilutions,
Figure 4.2. PCR-DGGE analysis of the 16S rDNA gene fragments of the TCE to cis-DCE enrichment dechlorinating culture. DGGE gel gradient 0 to 60%. Lane 1, enrichment control fed H₂ and acetate; Lane 2, Phase 1 dilution (10⁻⁴); Lane 3, Phase 1 dilution (10⁻²); Lanes 4 and 5, Initial enrichment culture used to prepare Phase 1 dilutions. Letters represent sequenced bands. Unlabeled bands were not sequenced.
respectively. The DNA in lane 1 was from a control that was fed H₂ and acetate. From the DGGE gel, two organisms appear to be dominant in this community. The bands labeled B, C, D and E represent one species that is present in each sample. The second dominant band is G, H, I, J and K (Figure 4.2), which is present in all the lanes and is the only band in the control fed hydrogen and acetate but no TCE. The organism represented by this band may be responsible for consumption of hydrogen and acetate that was consumed in excess of what was needed for reductive dechlorination of TCE. The other dominant band (labeled B, C, D, E and present in all of the lanes) was the most intense in lane 2, which is the most dilute series from Phase 2, and was either the most intense band or the second most intense band in the other lanes. This band is most likely the microbe responsible for reductive dechlorination of TCE to cis-DCE.

Sequences of bands B, C, D and E closely matched to *Pseudomonas stutzeri* (Table 4.3), which as indicated above, most likely represents the microbe responsible for reductive dechlorination of TCE. Sequences of bands G, H, I, J, and K matched 98% to the partial sequence of the 16S ribosomal RNA gene of *Desulfovibrio putealis* (Table 4.3). The sulfate concentration in the medium was 0.53 mM, most of which consisted of MgSO₄•7H₂O. Although this is a low concentration, it apparently provided a sufficient amount of sulfate to enrich for a sulfate reducing microbe. F most closely matched to an uncultured *Pseudomonas*. Although sequencing of band A was attempted several times, useable results were not obtained.

Confirmation of the presence of *Pseudomonas stutzeri* was obtained by direct PCR with specific *Pseudomonas stutzeri* primers (Figure 4.3). The expected length of the amplified
Table 4.3. DGGE bands sequencing results.

<table>
<thead>
<tr>
<th>Band</th>
<th>Best matches</th>
<th>Accession numbers</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>B, C, D, E</td>
<td><em>Pseudomonas stutzeri</em></td>
<td>EU275359, EU187488</td>
<td>100%</td>
</tr>
<tr>
<td>F</td>
<td>Uncultured <em>Pseudomonas</em></td>
<td>EU305596.1</td>
<td>99%</td>
</tr>
<tr>
<td>G, H, I, J, K</td>
<td><em>Desulfovibrio putealis</em></td>
<td>AY574979.1 AM056026.1</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td>Uncultured delta proteobacterium</td>
<td>DQ205193.1</td>
<td>96%</td>
</tr>
</tbody>
</table>

* Figure 4.2
**Figure 4.3.** Direct PCR with Universal Bacterial primers and specific *Pseudomonas stutzeri*
fragment was 585 bp, based on the specific forward and reverse primers used. The 100 bp DNA ladder, run concurrently with the amplified DNA, confirms the size of the amplified product obtained with the specific primers and template DNA from the enrichments and dilutions.

4.4 Discussion

A highly enriched culture was developed using inoculum from a positive microcosm that retained the ability to reductively dechlorinate TCE to cis-DCE, through several transfers. By comparing this community to one that was not provided with TCE, the microbe most likely associated with reductive dechlorination of TCE to cis-DCE is *Pseudomonas stutzeri*. Analysis of DNA extracts by direct and nested PCR using primers for five of the genera known to chlororespire TCE to cis-DCE confirmed that the microbe responsible for dechlorination in this study is not one of these. The presence of *P. stutzeri* was confirmed using specific primers. A microbe that was common to dilutions with and without TCE added was identified as a *Desulfovibrio* sp.

This appears to be the first report of a *Pseudomonas* sp. with the capability to reductively dechlorinate TCE to cis-DCE. Most of the known chlororespiring microbes are obligate anaerobes. However, at least one is a facultative aerobe, similar to *Pseudomonas*. Strain MS-1, isolated by Sharma and McCarty (26), is a facultative aerobe that chlororespires PCE to cis-DCE and is most closely related to *Enterobacter*. Although anaerobic growth of *Pseudomonas* is most commonly associated with nitrate-reducing conditions, some are known to also grow under fermentative conditions with
pyruvate as a substrate (34). In this study, it seems likely that acetate was used as the electron donor and carbon source by the enrichment culture to support growth of the *Pseudomonas* sp., since pseudomonades are not likely to use hydrogen as their sole electron donor. Some of the hydrogen consumption by the enrichments and dilutions may have yielded acetate via acetogenesis. Further work is needed to isolate the dechlorinator in order to firmly establish its identity and physiology.

*Pseudomonas stutzeri* is no stranger to anaerobic transformation of chlorinated aliphatic hydrocarbons. *P. stutzeri* is best known in this regard for its ability to transform carbon tetrachloride under denitrifying conditions (35). However, the transformation products are not those of reductive dechlorination (i.e., chloroform, dichloromethane, etc.) but rather CO₂ and a soluble, non-volatile product. In a landmark pilot-scale field study, Dybas et al. (36) were the first to demonstrate the potential for in situ bioaugmentation of chlorinated solvents by addition of *Pseudomonas stutzeri* strain KC to an aquifer in Michigan that was contaminated with carbon tetrachloride.

Schreiber et al. (34) identified a pyruvate fermentation pathway in *Pseudomonas aeruginosa*. This *Pseudomonas* was able to survive under anaerobic conditions without using an alternative anaerobic respiratory pathway. Although survival and not growth of the *Pseudomonas* occurred under these strict anaerobic conditions, this is a significant finding considering that previously *Pseudomonas* were known to only survive under aerobic and nitrate reducing conditions (37).

From the results of this study it is not yet clear if the microbe responsible for TCE reduction to *cis*-DCE does so via chlororespiration or cometabolism. The fact that
dechlorination activity was sustained across several transfers at very high dilutions favors
the likelihood of chlororespiration. In addition, the onset of activity tended to follow a
lag and increase in rate over time, rather than start quickly and then taper off, as is
common with cometabolism. Once an isolate is obtained it will be straightforward to
conclusively establish if the transformation is metabolic or cometabolic.

Transformation of TCE to cis-DCE was accompanied by minor amounts of 1,1-
DCE and VC. Kastner (38) provided evidence for the transformation of PCE and TCE to
cis-DCE with traces of 1,1-DCE from an aerobic culture that was transferred to an
anaerobic medium at -150 mV.

Since the enrichments utilized more than ten times the hydrogen necessary for
reductive dechlorination of TCE to cis-DCE and the southern California site is known to
be high in sulfate (1), it is not surprising that a sulfate reducer was present and became
dominant in this community. The species in this community whose sequence matched
Desulfovibrio putealis was also the only species present in the control that was fed
hydrogen. A Desulfovibrio putealis that closely aligns with the microbe in this study was
isolated from a deep subsurface aquifer and was able to grow on H2 only when acetate
was present (39). It is unclear if there is any type of syntrophic interaction between the
Pseudomonas sp. and the sulfate reducer, although this appears unlikely.

The results of this study suggest that more types of microbes are present in the
environment that are capable of using chlorinated ethenes as terminal electron acceptors
than what are currently known. Since the geological conditions at the southern California
site from which samples were obtained are quite different from other locations that have
yielded chlororespirators, it is not too surprising that a new type of TCE dechlorinator was identified. While the results thus far are intriguing, further work is needed to isolate the microbe responsible for TCE reduction and more conclusively establish its identity and physiology.

4.5 References


CONCLUSIONS

The results of this dissertation add insight into the natural attenuation of chlorinated ethenes. It has been shown that significant amounts of soluble products and CO$_2$ may be formed via abiotic and biotic transformations of trichloroethene (TCE) and cis-dichloroethene (DCE) in fractured sandstone. The results provide additional evidence that a facultative aerobe is capable of dechlorinating TCE to cis-DCE under low redox conditions. Furthermore, this research has shown that autoclaving, the most common method of sterilization used for abiotic experiments, alters the reactivity of sandstone. These results were obtained by conducting microcosm studies utilizing crushed sandstone and groundwater from a site contaminated with TCE and cis-DCE.

The specific conclusions from this study are:

1) TCE and cis-DCE underwent biotic and abiotic transformations in sandstone and groundwater samples from the southern California industrial site where TCE was released to the subsurface several decades ago. TCE reduction to DCE in the microcosms was a biotic process, based on the predominance of cis- over trans-DCE as the dechlorination proceeded, the lack of products from reductive dechlorination in autoclaved treatments, and the lag period that preceded a rapid onset of reduction in several of the microcosms. Sandstone from the site is also capable of abiotic transformation of TCE, cis-DCE and VC to soluble products (referred to as non-strippable residue, or NSR) and CO$_2$. Evidence for this abiotic transformation is based on statistically significant accumulation of $[^{14}\text{C}]$NSR and $^{14}$CO$_2$ in the autoclaved microcosms at all depths.
2) Pyrite is not the predominant catalyst for the abiotic transformation of *cis*-DCE to NSR + CO₂. The extent of *cis*-DCE transformation was four times higher in the treatment with typical sandstone in comparison to the treatment with pyrite-rich sandstone, and both were higher than the treatment with pure pyrite.

3) Autoclaving sandstone from this site altered the rate of abiotic transformation but not the extent. The fastest rate of transformation occurred in the treatment with autoclaved sandstone. Autoclaving increased the surface area, pore volume and pore diameter of the sandstone, increasing the number of active sites available for transformation, which may explain the higher initial rate of abiotic transformation. Autoclaving increased the availability of magnetite and goethite, which have been linked to abiotic transformation of chlorinated ethenes in previous studies.

4) The surface area normalized first order rate of transformation for *cis*-DCE was highest for the autoclaved sandstone (1.65E-05 L/m²d), followed by the live treatment (8.08E-06 L/m²d) and the propylene oxide treated sandstone. The rates obtained are comparable to those previously reported for abiotic transformation of chlorinated ethenes in soil. The rates reported here are an order of magnitude lower than those for pure minerals like pyrite and magnetite because normalizing the rate to surface area assumes that the reaction is taking place over the entire surface of the rock.

5) The microcosm responsible for the transformation of TCE to *cis*-DCE is not one of the previously known genera of TCE to *cis*-DCE dechlorinators. Analysis of DNA extracts by direct and nested PCR using primers for five of the genera known to
chlororespire TCE to cis-DCE confirmed that the microbe responsible for dechlorination in this study is not one of these.

6) The microorganism most likely responsible for the reductive dechlorination of TCE to cis-DCE was identified as *Pseudomonas stutzeri*. This appears to be the first report of a *Pseudomonas* that mediates this process.

Overall, this study has contributed to a deeper understanding of both the biotic and abiotic processes of transformation of chlorinated ethenes. It has provided an additional pathway by which chlorinated ethenes can be transformed abiotically. This is the first time that chlorinated ethenes are reported to be transformed by an anaerobic abiotic process to formate, glycolate, acetate and CO$_2$. This study also provided additional evidence that a facultative aerobe can dechlorinate TCE to cis-DCE. This study, therefore, supports the documentation of natural attenuation of chlorinated ethenes, in particular at the southern California site that was the focus of this research, and in fracture sandstone in general.
APPENDICES
Appendix A

A.1 Procedures for determining the distribution of $^{14}$C in microcosms

$^{14}$C-labeled volatile compounds were analyzed with a GC/combustion technique. Headspace samples were injected onto the 1% SP-1000 on 60/80 Carbopak B column and the well-separated compounds were routed to a catalytic combustion tube, where they were oxidized at 800°C to CO$_2$. As fractions eluted from the combustion tube, they were trapped in NaOH (0.5 M) and added to liquid scintillation cocktail. The $^{14}$C activity in a fraction was converted to $^{14}$C-activity per bottle based on the volumes of the sample, headspace, and liquid, as well as Henry’s law constant for each compound.

In some bottles a substantial amount of $^{14}$C in the headspace sample eluted off the Carbopack column in the first 2.5 min, an interval that was too narrow to obtain efficient resolution of the $^{14}$C. Possible compounds within this interval included methane, ethane, ethene, acetylene, and CO$_2$. To separate these, a second headspace sample was injected onto a Carbosieve SII column (2.44-m by 3.175-mm, operated with a temperature program of 150°C for 5 min, ramped to 200°C at 10°C/min, hold for 15 min), which separated these compounds efficiently.

$^{14}$CO$_2$ and $^{14}$C-labeled nonvolatile compounds were measured after analysis of the $^{14}$C-labeled volatile compounds was completed ($I$). Samples of the liquid phase (10 mL) were transferred to a test tube that was connected to a second test tube containing NaOH (0.5 M). Nitrogen gas was sparged into the first tube and passed into the second. The pH in the first tube was then lowered with HCl, to allow for stripping of CO$_2$, which was trapped in the second tube. The term given to the liquid remaining in the first tube
after acidic sparging is nonstrippable residue (NSR). The percentage of NSR associated
with particulates was determined by counting the level of $^{14}$C activity remaining after
centrifugation (10,000g for 10 min; Sorvall Evolution RC Centrifuge) and comparing this
to the total $^{14}$C-NSR activity.

The following test was conducted to evaluate the presumption that the absorption
chamber contained $^{14}$CO$_2$ and not other compounds that are soluble at high pH. Barium
hydroxide (1.54 g) was added to samples from the adsorption chamber (8 mL), shaken
vigorously. The amount of Ba(OH)$_2$ added was in considerable excess of the amount
needed to precipitate all of the carbonates present as highly insoluble BaCO$_3$. The
contents were then centrifuged and 2 mL of centrate was counted in liquid scintillation
cocktail. Overall, the average amount of $^{14}$C present in the centrate was 1.97% of the
total $^{14}$C in the absorption chamber. This indicated that the vast majority of the $^{14}$C
activity in the absorption chamber was $^{14}$CO$_2$.

Once it was determined that acetate, glycolate and formate constituted a
significant percentage of the NSR, another test was performed to specifically determine
the amount of acetate, glycolate and formate that might be transferred from the stripping
chamber to the absorption chamber. A standard containing 2 mM each of formate,
glycolate and acetate was placed in the stripping chamber and then subjected to the same
stripping conditions as the microcosm samples. From duplicate tests, the average percent
recovery of acetate, glycolate and formate remaining in the NSR were 99.3%, 100%, and
98.1%, respectively. This conclusively demonstrated that little or none of these three
organic acids are transferred to the absorption chamber during the stripping process.
A.2 Formate dehydrogenase method

An enzymatic method was used to confirm the presence of formate in one of the HPLC fractions obtained from the NSR concentrate. The following were combined: 160 µL of sample (adjusted to pH 7), 0.05 M NAD⁺ (20 µL; reduced form; Sigma-Aldrich), 0.5 M KH₂PO₄ buffer (800 µL) and 40 units per mL formate dehydrogenase (20 µL, 8.8 units/mg protein; Sigma Aldrich). In the presence of NAD⁺ and formate dehydrogenase, formate was converted to CO₂ + NADH; NADH was detected by UV at 340 nm. The absorbance was monitored for 60 min. Formate standards were tested along with the NSR fraction. The formation of ^14CO₂ during the assay was determined by inserting into the assay vial a small centrifuge tube containing 100 µL of 8 M NaOH and a 25-mm filter disc to wick up the NaOH. After the reaction was complete, the contents of the centrifuge tube were transferred to liquid scintillation cocktail and counted.
A.3 Mineral characteristics of the fractured sandstone used in this study

Mineral and organic matter analysis of the crushed rock from the four different depths was conducted. Analyses were performed by the Agricultural Services Laboratory at Clemson University.

Table A.1. Characteristics of the Rock Cores Used in the Microcosms.

<table>
<thead>
<tr>
<th>Component (units)</th>
<th>depth (m)</th>
<th>161</th>
<th>173</th>
<th>222</th>
<th>265</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (%)</td>
<td></td>
<td>0.009</td>
<td>0.020</td>
<td>0.005</td>
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</tr>
<tr>
<td>P (%)</td>
<td></td>
<td>0.021</td>
<td>0.034</td>
<td>0.020</td>
<td>0.019</td>
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<tr>
<td>K (%)</td>
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<td>0.170</td>
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<tr>
<td>Ca (%)</td>
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<td>Mg (%)</td>
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<td>0.660</td>
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<td>S (%)</td>
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<td>Fe (%)</td>
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<td>Organic matter (%)</td>
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<td>Zn (ppm)</td>
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<td>General characteristics</td>
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<td>biotite rich</td>
<td>typical sandstone</td>
<td>typical sandstone</td>
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</tr>
</tbody>
</table>
A.4 GC monitoring data used to determine TCE and cis-DCE transformation rates in Table A.1

This section of the Appendix presents the headspace monitoring data that were used to determine the zero-order rates of TCE and cis-DCE loss reported in Table A.1. The total amount of volatile organic compounds in all of the microcosms was evaluated by analysis of headspace samples on the GC. GC measurements were made much more frequently than the four time steps when the \(^{14}\)C distribution was determined and the decrease in TCE revealed by the GC data encompass all transformation processes, not just transformation to \(^{14}\)CO\(_2\) + \([^{14}\text{C}]\text{NSR}\).

Figure A-1 presents the headspace monitoring results for triplicate live, AC and WC bottles that received \([^{14}\text{C}]\text{TCE}\) and were incubated for the longest period of time. GC data for the microcosms that were sacrificed for \(^{14}\)C analysis at earlier time periods (i.e., at time steps 1, 2 and 3) were not used to determine rates of TCE loss, although they followed similar trends. Figure A-1a shows the WC data; Figures A-1b through e show results for the live bottles (red triangles, regression lines and text) and AC bottles (blue squares, regression lines and text correspond to the AC bottles), for each depth.

When a regression line is not shown (i.e., Figure A.1a; A.1e for the AC bottles), it means that the slope was not statistically significant (p>0.05) and therefore the rate of loss was zero. When the slope of the regression line was statistically significant (p<0.05), it was used to calculate the rate of loss. The TCE present is reported in terms of μmol per bottle, so the slope is μmol per bottle per day. These values were converted to μM/yr (as
reported in Table 1) by calculating the aqueous phase concentration based on the total mass per bottle and Henry’s law constant:

\[
C_l = \frac{M}{V_t + Hc V_g}
\]

where \(M\) = total amount of TCE per bottle (\(\mu\)mol); \(C_l\) = concentration in the aqueous phase (\(\mu\)M); \(V_t\) = volume of groundwater in the bottle (50 mL); \(V_g\) = volume of headspace in the bottle (99 mL); and \(Hc\) = Henry’s constant for TCE (dimensionless) at 23°C (calculated from (2)). The same equation was used for the cis-DCE results (with the appropriate \(Hc\)), presented below.

In the live bottles selected for measurement of TCE rates (3 bottles for each depth), reductive dechlorination was not a significant fate process. Decreases in TCE were presumptively due to other processes, including transformation to CO₂, NSR and possibly other compounds, as well as adsorption to the rock and diffusive losses.

Figure A.2 presents the headspace monitoring results for triplicate live, AC and WC bottles that received \([^{14}\text{C}]\text{cis-DCE}\) and were incubated for the longest period of time. GC data for the microcosms that were sacrificed for \(^{14}\text{C}\) analysis at earlier time periods (i.e., at time steps 1, 2 and 3) were not used to determine rates of cis-DCE loss, although they followed similar trends. As stated above for TCE, GC measurements were made much more frequently than the four time steps when the \(^{14}\text{C}\) distribution was determined and the decrease in cis-DCE revealed by the GC data encompass all transformation processes, not just transformation to \(^{14}\text{CO}_2 + [^{14}\text{C}]\text{NSR}\). Figure A.2a shows the WC data; Figures A-2b through e show results for the live bottles (red triangles, regression lines
and text) and AC bottles (blue squares, regression lines and text correspond to the AC bottles), for each depth. As indicated above for Figure S-1, when a regression line is not shown (i.e., Figure A.2c for the live bottles; A.2d for the AC bottles), it means that the slope was not statistically significant (p>0.05) and therefore the rate of loss was zero. When the slope of the regression line was statistically significant (p<0.05), it was used to calculate the rate of loss, using the same procedure described above.
Figure A.1. GC monitoring results for microcosms that received $^{14}$C\text{TCE} and were incubated the longest period of time; triplicate WC bottles (a); live and AC bottles prepared with rock from depths of 161 m (b); 173 m (c); 222 m (d, on the next page); and 265 m (e, on the next page).
Figure A.1, continued from the previous page.
Figure A.2. GC monitoring results for microcosms that received $[^{14}\text{C}]\text{cis-DCE}$ and were incubated the longest period of time; triplicate WC bottles (a); live and AC bottles prepared with rock from depths of 161 m (b); 173 m (c); 222 m (d, on the next page); and 265 m (e, on the next page)
Figure A.2, continued from the previous page.
A.5 Distribution of $^{14}$C in microcosms that received $[^{14}$C]VC

The distribution of $^{14}$C in microcosms that received $[^{14}$C]VC is shown in Figure A-3. VC represented the largest category of $^{14}$C remaining in all of the treatments at the time of analysis. An average of 0.9% of the total $[^{14}$C]VC added consisted of UAF volatiles. $^{14}$C losses averaged 12.1±5.3% in the live bottles, 14.3±5.5% in the ACs, and 17.3±4.5% in the WCs, with no apparent trend over time. Consistent with the headspace monitoring results, no ethene, ethane or methane was detected in the live treatments (Figure A-1a, A-1b).

$^{14}$C and NSR, the nonvolatile transformation products from $[^{14}$C]VC, were consistently higher than in the WCs, with CO$_2$ predominating in most of the live bottles. The exception was in microcosms for the 568 ft depth at time step 3 (Figure A-1c), in which $[^{14}$C]NSR dominated. In the ACs, $[^{14}$C]NSR (up to 11%) was the major fraction of nonvolatile transformation products (Figure A-1d). The sum of $^{14}$CO$_2$ + $[^{14}$C]NSR was generally higher in the ACs than in the live bottles. All of the AC treatments accumulated significantly more $^{14}$CO$_2$ + $[^{14}$C]NSR than the WCs (Student’s t-test, $\alpha = 0.05$). None of the treatments exhibited a statistically significant increase in $^{14}$CO$_2$ + $[^{14}$C]NSR over time (based on the slope of the trend line, $\alpha = 0.05$).
Figure A.3. Distribution of $^{14}$C compounds in microcosms with $[^{14}$C$]VC$ added; (a) volatiles in live treatments, (b) volatiles in ACs, (c) $CO_2$ + NSR in live treatments, and (d) $CO_2$ + NSR in ACs; WC results are shown in each panel for comparison. The numbers below the bars indicate the four time steps when microcosms were analyzed for $^{14}$C distribution (7-8, 11-12, 15-16, and 21-22 months). Error bars represent the standard deviation for the sum based on triplicate serum bottles. In (c) and (d), asterisk(s) above a bar indicate that the sum of $^{14}$CO$_2$ + $[^{14}$C$]$NSR is statistically greater than in the WCs at the 10% (*) or 5% (**) confidence level.
A.6 Matching of HPLC retention times to fractions containing $[^{14}C]$NSR

After concentrating several NSR samples from AC microcosms by lyophilization, the concentrate was run through the Biorad HPX-87H organic acids column and UV/Vis detection (210 nm) on the HPLC. Figure A-4a shows a chromatogram for the NSR before lyophilizing. The injection peak is visible, but no other peaks were detected. During the run, the eluant was collected in discrete intervals and the $^{14}$C activity present was measured. The arrows on Figure A-4a indicate the four fractions that contained the highest levels of $^{14}$C activity. Figure A-4b shows a chromatogram for the NSR after lyophilizing. In addition to the injection peak, three well-defined peaks were detected, at retention times of 12.819 min, 14.090 min, and 15.324 min. These peaks corresponded to three of the eluant fractions with the highest levels of $^{14}$C activity. Figure A-4c shows a chromatogram for a standard containing 5 mM of glycolate, formate, and acetate, with retention times of 12.932 min, 14.203 min, and 15.432 min. The close match in retention times between the peaks in the standard mixture, the peaks in the lyophilized NSR, and the corresponding eluant fractions containing $^{14}$C, provided one of the lines of evidence that three of the main components of NSR from AC microcosms are glycolate, formate, and acetate.
Figure A.4. Representative HPLC chromatograms for (a) NSR from AC samples before lyophilizing and (b) NSR from AC samples after lyophilizing and (c) a standard containing 5 mM of acetate, glycolate, and formate. Arrows in panel a) and b) indicate the eluant fractions that contained the highest amounts of $^{14}$C activity.
A.7 Ion chromatography results for lyophilized \[^{14}C\]NSR

The HPLC results described above and in the manuscript indicate that most of the \(^{14}C\) activity in the NSR is associated with glycolate, formate and acetate. Further evidence for the identity of these three compounds was sought by separating NSR from the AC treatments on an ion chromatograph, using a column and eluant with considerably different properties from the HPLC system. The difference in properties is reflected in a difference in the elution order of the compounds. On the HPLC, glycolate emerges first, followed by formate, and acetate. On the ion chromatograph (IC), the elution order for formate and acetate are reverse, i.e., acetate elutes before formate. As with the HPLC system, when eluant emerged from the IC, discrete fractions were collected and added to liquid scintillation cocktail for measurement of \(^{14}C\) activity. The times selected for collecting fractions was based on elution times for standards prepared with glycolate, formate and acetate. Fractions were also collected before and after these compounds. Glycolate and acetate overlapped slightly, so it is possible that a “tail” on glycolate may have been apportioned to acetate, while a leading part of the acetate peak may have been apportioned to glycolate.

Results are summarized in Table A-2, in the same manner as the HPLC results shown in Table 2 of the manuscript. The presence of glycolate, formate and acetate in the lyophilized NSR was confirmed based on separation on the IC system. The highest percentages recovered (i.e., \(^{14}C\) in a fraction eluting off the IC divided by the total \(^{14}C\) injected onto the IC system) were
40% for glycolate, 7.9% for formate, and 10% for acetate. These values are within the ranges observed from the HPLC analysis for glycolate (1-46%), formate (0-6.8%), and acetate (1-41%). Although there are some differences between the HPLC and IC results in terms of the percentages of each compound present in the AC NSR, the IC results do confirm the presence of glycolate, formate and acetate.

One notable difference between the HPLC and IC results for analysis of NSR was the lower percent recovery of $^{14}\text{C}$ in the fractions collected off the IC. With the HPLC system, the average percent recovery of $^{14}\text{C}$ (i.e., $^{14}\text{C}$ in a fraction eluting off the HPLC divided by the total $^{14}\text{C}$ injected onto the HPLC system) was 97% for NSR from microcosms that received [$^{14}\text{C}$]cis-DCE and 90% from microcosms that received [$^{14}\text{C}$]TCE (Table A-2). With the IC system, the average percent recovery of $^{14}\text{C}$ was 63% for NSR from microcosms that received [$^{14}\text{C}$]cis-DCE and 64% from microcosms that received [$^{14}\text{C}$]TCE. The difference in recovery appears to be related to the behavior on the $^{14}\text{C}$ fraction from the HPLC that was unretained (i.e., eluted at the same time as the injection peak). With the IC, there was no $^{14}\text{C}$ associated with the unretained fraction. Apparently this material did not elute from the IC system within the time frame of the analysis (30 min). It should also be noted that glycolate and acetate eluted very close together from the IC system, and this overlap may have resulted in some mis-assignment of $^{14}\text{C}$ activity to one compound or the other.

Figure A-5 shows an IC chromatogram for lyophilized NSR from AC microcosms that received [$^{14}\text{C}$]cis-DCE and another for a standard containing 5 mM glycolate, acetate and formate. Retention times for the standard were 5.283 min for glycolate, 5.683 min...
for acetate, and 6.417 min for formate. These matched well with the retention times for the three main peaks in the NSR: 5.247, 5.637, and 6.430 min, respectively. The arrows on Figure A-5b indicate the three fractions that contained the highest levels of $^{14}$C activity, corresponding to detection of glycolate, acetate and formate.

Figure A.5. Representative IC chromatograms for (a) NSR from AC samples after lyophilizing and (b) a standard containing 5 mM of acetate, glycolate, and formate. Arrows in panel a indicate the eluant fractions that contained the highest amounts of $^{14}$C activity.
Table A.2. Efficiency of NSR Lyophilization and Distribution of $^{14}$C in Lyophilized Samples from Autoclaved Control and Water Control Microcosms with $[^{14}$C]$\textit{cis}$-DCE and $[^{14}$C]$\textit{t}$-TCE Added as Determined by Ion Chromatography Separation

<table>
<thead>
<tr>
<th>Sample</th>
<th>NSR as a percent of total $^{14}$C added, before lyophilizing</th>
<th>% of $^{14}$C-NSR remaining after lyophilizing</th>
<th>% recovery of $^{14}$C-NSR in IC fractions</th>
<th>IC fractions ( % of lyophilized NSR)$^e$</th>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>unretained</td>
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<tr>
<td>AC microcosms with $[^{14}$C]$\textit{cis}$-DCE added</td>
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<td></td>
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</tr>
<tr>
<td>i</td>
<td>16</td>
<td>55</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>ii</td>
<td>8.7</td>
<td>67</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
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<tr>
<td>iv</td>
<td>16</td>
<td>151</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>v</td>
<td>13</td>
<td>62</td>
<td>66</td>
<td>0</td>
</tr>
<tr>
<td>average</td>
<td>13</td>
<td>68</td>
<td>63</td>
<td>0</td>
</tr>
</tbody>
</table>

| AC microcosms with $[^{14}$C]$\textit{t}$-TCE added |
| i       | 12                                                             | 80                                            | 59                                        | 0          | 5         | 1.7     | 1.7     | 51      |             |
| ii      | 9.9                                                            | 81                                            | 111                                       | 0          | 16        | 7       | 13      | 75      |             |
| iii     | 14                                                             | 43                                            | 69                                        | 0          | 6.3       | 7.9     | 5.9     | 49      |             |
| iv      | 14                                                             | 44                                            | 17                                        | 0          | 1.8       | 1.8     | 1.5     | 12      |             |
| average | 12                                                             | 62                                            | 64                                        | 0          | 7         | 4.6     | 5.5     | 47      |             |

$^a$ Same as Table 1: each sample was a composite of 25 mL of liquid from four autoclaved control or water control microcosms.

$^b$ Same as Table 1: amount of $[^{14}$C]NSR as a percentage of the total $[^{14}$C]TCE or $[^{14}$C]$\textit{cis}$-DCE added.

$^c$ Same as Table 1: (total $^{14}$C remaining after lyophilizing)/(total $^{14}$C present prior to lyophilization).

$^d$ $\Sigma$($^{14}$C in all IC fractions)/(total $^{14}$C of the lyophilized NSR injected onto the IC).

$^e$ ($^{14}$C in the fraction indicated)/(total $^{14}$C of the lyophilized NSR injected onto the IC).

$^f$ Same as Table 1: $^{14}$C activity not measured prior to lyophilizing.
A.8 Formate dehydrogenase results

Further confirmation for the identity of formate as a component of NSR was obtained using a formate dehydrogenase assay. The procedure is described in an earlier section of Supporting Information. A representative result is shown in Figure A-6. The positive control consisted of a 0.2 mM solution of formate. As expected, the absorbance of the solution increased over time, reflecting the accumulation of NADH. NADH formed as formate was oxidized to CO₂ and the resulting electrons reduced NAD⁺ to NADH. The negative control consisted of a reagent blank, i.e., all reagents were present except formate; there was no significant change in absorbance during the assay period. Two samples of lyophilized NSR were evaluated, from AC microcosms that received [¹⁴C]cis-DCE (indicated by footnote “f” in Table 2 of the manuscript). They showed a very similar increase in absorbance to the positive control. This provided definitive evidence for the presence of formate in the NSR, since the formate dehydrogenase assay is highly specific to reaction with formate. The results were further confirmed by measurement of ¹⁴CO₂ in assay vials containing NSR.
Figure A.6  Results for the formate dehydrogenase assay to confirm the presence of formate in NSR from the AC microcosms.
A.9 Gibbs free energy calculations for transformations of TCE and cis-DCE to glycolate, formate, and acetate

Gibbs free energy values ($\Delta G^\circ$) for transformation of TCE and cis-DCE to acetate, formate and glycolate were calculated at standard conditions (i.e., concentrations of reactants and products at 1 M or 1 atm, and temperature of 25°C) adjusted to pH 7. If the transformation involves a reduction or oxidation, H$_2$ was used as a reactant or product, respectively. As shown in Table A-3, all of the reactions are thermodynamically favorable. The most favorable reactions for TCE and cis-DCE were transformation to acetate. For the purposes of comparison, the Gibbs free energy for reduction of TCE to cis-DCE is also shown. Transformations of TCE to acetate, formate and glycolate are considerably more thermodynamically favorable than reductive dechlorination.

Since H$_2$ was assumed to serve as the electron carrier in several of the reactions, Gibbs free energies were also calculated using a lower concentration ($10^{-4}$ atm) than standard conditions, to better reflect the likely in situ H$_2$ concentration. Reactions in which H$_2$ appears as a reactant (e.g., reduction of TCE to acetate) become less favorable while reactions with H$_2$ as a product (e.g., oxidation of cis-DCE to glycolate) become more favorable. Regardless, changing the H$_2$ concentration does not significantly alter the free energy values.

In summary, abiotic transformation of TCE and cis-DCE to non-volatile products such as acetate, formate and glycolate is a thermodynamically favorable process.
Table A.3. Changes in Gibbs Free Energy for Transformation of TCE and cis-DCE.

<table>
<thead>
<tr>
<th>Reaction Description</th>
<th>Reaction</th>
<th>$\Delta G^{\circ,a}$</th>
<th>$\Delta G^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCE $\rightarrow$ acetate</td>
<td>$\text{C}_2\text{HCl}_3 + \text{H}_2 + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + 4\text{H}^+ + 3\text{Cl}^-$</td>
<td>-469.9</td>
<td>-447.1</td>
</tr>
<tr>
<td>TCE $\rightarrow$ formate</td>
<td>$\text{C}_2\text{HCl}_3 + 4\text{H}_2\text{O} \rightarrow 2\text{HCOO}^- + 5\text{H}^+ + 3\text{Cl}^- + \text{H}_2$</td>
<td>-368.1</td>
<td>-390.9</td>
</tr>
<tr>
<td>TCE $\rightarrow$ glycolate</td>
<td>$\text{C}_2\text{HCl}_3 + 3\text{H}_2\text{O} \rightarrow \text{C}_2\text{H}_3\text{O}_3^- + 4\text{H}^+ + 3\text{Cl}^-$</td>
<td>-394.3</td>
<td>-394.3</td>
</tr>
<tr>
<td>TCE $\rightarrow$ cis-DCE</td>
<td>$\text{C}_2\text{HCl}_3 + \text{H}_2 \rightarrow \text{C}_2\text{H}_2\text{Cl}_2 + \text{H}^+ + \text{Cl}^-$</td>
<td>-169.8</td>
<td>-147.0</td>
</tr>
<tr>
<td>cis-DCE $\rightarrow$ acetate</td>
<td>$\text{C}_2\text{H}_2\text{Cl}_2 + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + 3\text{H}^+ + 2\text{Cl}^-$</td>
<td>-300.1</td>
<td>-300.1</td>
</tr>
<tr>
<td>cis-DCE $\rightarrow$ formate</td>
<td>$\text{C}_2\text{H}_2\text{Cl}_2 + 4\text{H}_2\text{O} \rightarrow 2\text{HCOO}^- + 4\text{H}^+ + 2\text{Cl}^- + 2\text{H}_2$</td>
<td>-198.3</td>
<td>-244.0</td>
</tr>
<tr>
<td>cis-DCE $\rightarrow$ glycolate</td>
<td>$\text{C}_2\text{H}_2\text{Cl}_2 + 3\text{H}_2\text{O} \rightarrow \text{C}_2\text{H}_3\text{O}_3^- + 3\text{H}^+ + 2\text{Cl}^- + \text{H}_2$</td>
<td>-224.5</td>
<td>-247.3</td>
</tr>
</tbody>
</table>

$a$ Calculated using the aqueous Gibbs free energies of formation in Table A-4. All reactants and products at 1 M or 1 atm except $\text{H}^+$, $\text{pH} = 7.0$, $T = 25^\circ\text{C}$.  

$b$ $\Delta G$ calculated using the Nernst equation from $\Delta G^{\circ}$ and $[\text{H}_2] = 10^{-4}$ atm.
Table A.4. Data used for Gibbs Free Energy Calculations Presented in Table A-3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \Delta G^o_f )</th>
<th>Source</th>
<th>Henry’s law constant (H)</th>
<th>Source</th>
<th>( \Delta G^o_{f(aq)} ) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kJ/mol</td>
<td></td>
<td>atm-m$^3$/mol</td>
<td></td>
<td></td>
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<td>TCE</td>
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<td>0.009446</td>
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<td>(3)</td>
<td>0.003769</td>
<td>(2)</td>
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<td>Acetate</td>
<td>-369.41</td>
<td>(4)</td>
<td>-</td>
<td>-</td>
<td>-369.41</td>
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<tr>
<td>Formate</td>
<td>-351.04</td>
<td>(4)</td>
<td>-</td>
<td>-</td>
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<td>Glycolate</td>
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<td>(4)</td>
<td>-</td>
<td>-</td>
<td>-237.17</td>
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<tr>
<td>Chloride</td>
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<td>(5)</td>
<td>-</td>
<td>-</td>
<td>-131.30</td>
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<tr>
<td>H$^+$ (pH = 7)</td>
<td>-39.83</td>
<td>(4)</td>
<td>-</td>
<td>-</td>
<td>-39.83</td>
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<tr>
<td>H$_2$</td>
<td>0.00</td>
<td>(4)</td>
<td>-</td>
<td>-</td>
<td>0.00</td>
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</tbody>
</table>

$^a \Delta G^o_{f(aq)} = \Delta G^o_f + RT(\ln H)$
A.10 References


(3) Thermodynamics Research Center *TRC Thermodynamic Tables, Non-Hydrocarbons*; The Texas A&M University System: College Station, TX, 1986; Vol. VII.


Appendix B

B.1 Design of bottle used in experiment

In the microcosm study that preceded this study (1) losses of cis-DCE from the water controls were significant. In order to reduce losses a bottle was designed that ensures that during the incubation period the contents of the bottle are exposed to unpunctured septa, therefore, preventing cis-DCE from being lost from the bottles through a hole in the septa. The bottle was prepared by Glass Warehouse. See Figure B.1 for further details on the bottle design.

![Diagram of bottle design](image)

**Figure B.1. Design of bottle for experiments I and II.**
B.2 Small scale experiments

To obtain adequate time steps to determine a rate of transformation of \textit{cis}-DCE the typical sandstone treatment of Experiment 1 was scaled down by a factor 7.67 (due to the greater availability of 300 mL bottles), and the appearance of transformation products monitored initially every two days (Appendix B, Figure B.2a). There was no trend in the formation of products, so even shorter time frames were used in the next experiment (0.5, 1, 2, 4, 6, 12 and so on) (Appendix B, Figure B.2b). These time steps were sufficient to observe a trend in the formation of products and were used in Experiment II.

The size fraction of typical sandstone used was then varied and the transformation to NSR and CO$_2$ monitored. Sizes < 0.075 mm, > 0.25 mm and < 0.85 mm was tested (Appendix B Figure B.2c, d, e). For experiment II, the size < 0.85 mm was used because the size represented the entire mass of rock crushed and, therefore, incorporates all phases of the rock into the experiment.
Figure B.2. CO$_2$ and NSR formed from *cis*-DCE in the smaller scale preliminary tests; a) 0.075 to 0.25 mm sandstone, longer product analysis time intervals, b) 0.075 to 0.25 mm sandstone shorter product analysis time intervals, c) < 0.075 mm, d) > 0.25 mm, and e) <0.850 mm. No error bars are present since the tests were done with single bottles.
B.3 Response Factor Determination

In order to monitor the formation of soluble products formed from the transformation of cis-DCE, liquid had to be removed from the bottles at each time step. Since cis-DCE partitions between the headspace and the liquid, changes in the headspace-to-liquid ratio necessitate a change in the response factor used to quantify the concentration of cis-DCE measured by the GC-FID headspace analysis. In order to correct for this, a series of standards were prepared at three to five different headspace to liquid ratios and the response factor or slope of the line for each liquid volume was determined. A linear relationship between the different response factors vs. the different liquid volumes was found and the equation of that line was used to determine the response factor of the instrument to different liquid to volume ratios and therefore distribution of cis-DCE in the bottles. A conversion factor was then determined by plotting the response factors versus the different volumes of liquid in the standards. A linear relationship between response factor and volume in liquid in each standard was obtained for the DCE standards but a linear relationship was not obtained for the gas standards because the response factors for each volume was very similar. Figure B.3 shows the linear relationship between response factor and volume of liquid for standards corresponding to the a) water controls and standards corresponding to the b) samples with sediment. Standards prepared to obtain response factors for sediment controls were prepared with glass beads and water to simulate a sediment control.
Figure B.3. Graphs used to determine GC response factors for cis-DCE at different volumes in a) water controls and b) samples.
B.4 Rate of loss of cis-DCE from the treatments in Experiment II

Figure B.4. Plots of ln C vs. time for Experiment 2 to determine first order rates of reaction in a) Live sandstone, b) Autoclaved sandstone, c) Propylene oxide treated sandstone.
### Table B.1. Decreases in volatile compounds during removal of liquid samples.

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<th></th>
<th>cumulative % loss after sampling event #</th>
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<th>3</th>
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<td></td>
<td></td>
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*N/A Measurement not taken*
B.4 Reference

Appendix C

Figure C.1 Genealogy of enrichments and dilutions