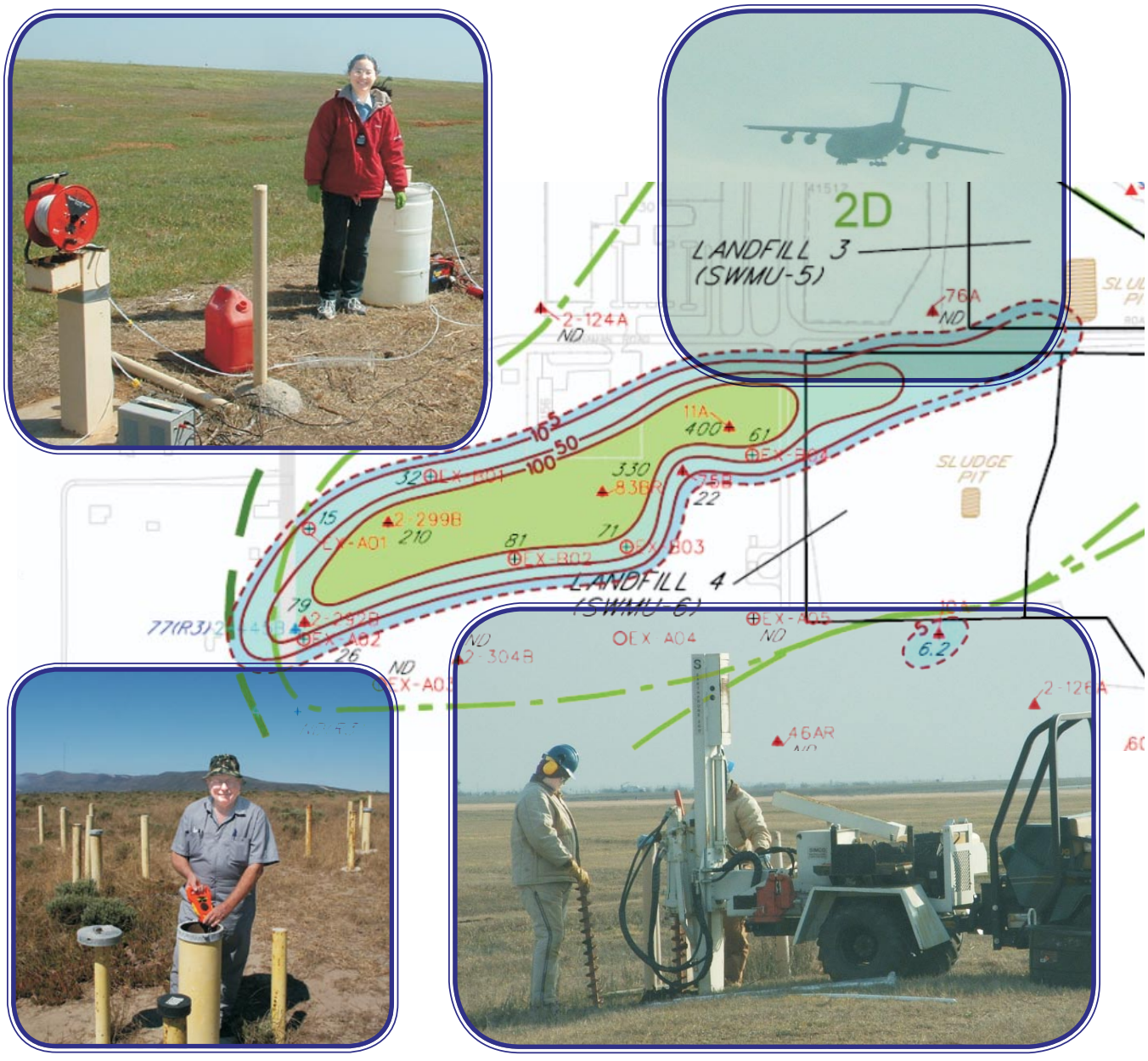
United States Environmental Protection Agency

Evaluation of the Role of *Dehalococcoides* Organisms in the Natural Attenuation of Chlorinated Ethylenes in Ground Water



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Xiaoxia Lu
National Research Council Post Doctoral Associate
tenable at the
U.S. Environmental Protection Agency
Office of Research and Development
National Risk Management Laboratory
Ada, Oklahoma 74820

Donald H. Kampbell, and John T. Wilson
U.S. Environmental Protection Agency
Office of Research and Development
National Risk Management Laboratory
Ada, Oklahoma 74820

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Project Officer
John T. Wilson
Ground Water and Ecosystems Restoration Division
National Risk Management Research Laboratory
Ada, Oklahoma 74820

National Risk Management Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

Notice

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Foreword

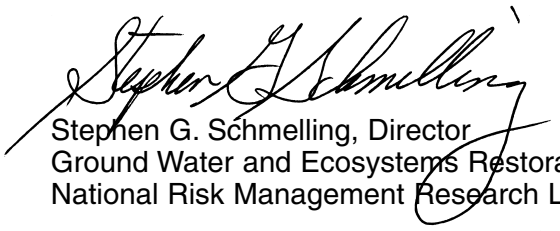
The U.S. Environmental Protection Agency is charged by Congress with protecting the Nation's land, air, and water resources. Under a mandate of national environmental laws, the Agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, EPA's research program is providing data and technical support for solving environmental problems today and building a science knowledge base necessary to manage our ecological resources wisely, understand how pollutants affect our health, and prevent or reduce environmental risks in the future.

The National Risk Management Research Laboratory is the Agency's center for investigation of technological and management approaches for preventing and reducing risks from pollution that threatens human health and the environment. The focus of the Laboratory's research program is on methods and their cost-effectiveness for prevention and control of pollution to air, land, water, and subsurface resources; protection of water quality in public water systems; remediation of contaminated sites, sediments and ground water; prevention and control of indoor air pollution; and restoration of ecosystems. NRMRL collaborates with both public and private sector partners to foster technologies that reduce the cost of compliance and to anticipate emerging problems. NRMRL's research provides solutions to environmental problems by: developing and promoting technologies that protect and improve the environment; advancing scientific and engineering information to support regulatory and policy decisions; and providing the technical support and information transfer to ensure implementation of environmental regulations and strategies at the national, state, and community levels.

This publication has been produced as part of the Laboratory's strategic long-term research plan. It is published and made available by EPA's Office of Research and Development to assist the user community and to link researchers with their clients.

Chlorinated solvents such as tetrachloroethylene and trichloroethylene are an important category of contaminants in ground water at hazardous waste sites. Frequently, these compounds are subject to natural anaerobic biodegradation in ground water. During anaerobic biodegradation they undergo a sequential biological reductive dechlorination to produce *cis*-dichloroethylene, then vinyl chloride, and finally ethylene or ethane. Although *cis*-dichloroethylene is less hazardous than trichloroethylene or tetrachloroethylene, vinyl chloride is more hazardous. In contrast, ethylene or ethane is not hazardous to humans. If the biological reductive dechlorination is complete, with ethylene or ethane as the final product, then monitored natural attenuation can be used a remedy for the ground water contamination.

In recent years, bacteria that can dechlorinate dichloroethylene to ethylene or ethane have been isolated and characterized. All the strains that can dechlorinate vinyl chloride to ethylene or ethane belong to the genus *Dehalococcoides*. A biochemical assay for DNA specific to the genus *Dehalococcoides* is commercially available. This report provides technical recommendations on the interpretation of the biochemical assay and on the contribution of bacteria in the *Dehalococcoides* group to monitored natural attenuation of chlorinated solvents in ground water.



Stephen G. Schmelling, Director
Ground Water and Ecosystems Restoration Division
National Risk Management Research Laboratory

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Abstract

At most hazardous waste sites where monitored natural attenuation (MNA) of chlorinated solvents in ground water is successful as a remedy, the chlorinated solvents are biologically degraded to harmless end products such as ethylene or ethane. Many organisms can degrade chlorinated solvents such as tetrachloroethylene or trichloroethylene, to dichloroethylene and vinyl chloride. This contributes little to risk reduction because vinyl chloride is more toxic and more carcinogenic than tetrachloroethylene or trichloroethylene. The only organisms known to degrade dichloroethylenes and vinyl chloride to ethylene or ethane are members of the *Dehalococcoides* group. As a result, these organisms have a critical role in the evaluation of MNA at chlorinated solvent sites. In recent years, biochemical assays for the presence of DNA from the organisms have become commercially available. These assays are based on the polymerase chain reaction (PCR) for the amplification of DNA extracted from ground water. They are very sensitive and can be very specific.

This report is designed for technical staff in the EPA Regions and in state agencies that require information on the contribution of *Dehalococcoides* bacteria to MNA of chlorinated solvents, and information on the proper application and interpretation of the assays in an evaluation of MNA. This report includes sections on the role of biotransformation in evaluation of MNA of chlorinated solvents, the ecology of microorganisms that transform chlorinated solvents, tools to assay microorganisms that transform chlorinated solvents, the relationship between *Dehalococcoides* DNA in ground water and rates of natural attenuation at field scale, the relationship between geochemical parameters and the occurrence of *Dehalococcoides* DNA in ground water, and the relationship *Dehalococcoides* DNA in ground water and behavior of chlorinated solvents in laboratory treatability studies or microcosm studies done with water from the plume.

Section 1.
Role of Biotransformation in Evaluation of MNA of Chlorinated Solvents

Perchloroethylene (PCE) and trichloroethylene (TCE) are among the most pervasive chlorinated solvents in ground water at hazardous waste sites. PCE and TCE are subject to biological and chemical processes that may further transform them to *cis*-dichloroethylene (*cis*-DCE), *trans*-dichloroethylene (*trans*-DCE), 1,1-dichloroethylene (1,1-DCE), vinyl chloride, ethylene, and ethane. Vinyl chloride is the most hazardous of the transformation products. Ethylene and ethane are essentially harmless. A wide variety of microorganisms can transform PCE or TCE to produce the dichloroethylenes. To date, only microorganisms from a specific and relatively uncommon group (the *Dehalococcoides* group) have been shown to have the capability to transform *cis*-DCE to vinyl chloride, and then transform vinyl chloride to ethylene. Complete transformation of PCE or TCE to ethylene can be an important process contributing to the monitored natural attenuation of PCE and TCE at hazardous waste sites.

In the past ten years, very sensitive and specific biochemical tools have been developed that can recognize DNA from *Dehalococcoides* organisms. To carry out an assay for *Dehalococcoides* organisms, DNA is isolated from ground water or sediment. Then short pieces of DNA that are complementary to gene sequences that are unique to *Dehalococcoides* organisms are added to the extract. These short pieces bind to the *Dehalococcoides* DNA, and allow the DNA to be copied by an enzyme called DNA polymerase. The short pieces that bind to the *Dehalococcoides* DNA are often referred to as primers for the DNA polymerase reaction. The process is repeated for a number of cycles. Each time the DNA that was synthesized in the previous reaction becomes the template for the subsequent reaction. In each cycle of the chain reaction, the concentration of *Dehalococcoides* DNA is doubled. Finally, the concentration of *Dehalococcoides* DNA is high enough that it can be identified and analyzed by other molecular biology procedures. The entire process is referred to as the polymerase chain reaction (PCR).

A recurrent feature in the journal, *Remediation,* is the Monitored Natural Attenuation Forum: A Panel Discussion. In a recent issue (Borden et al., 2003), a panel of three experts responded to the question:

> Recently, there has been a lot of discussion about the need for *Dehalococcoides ethenogenes* to completely break down chlorinated solvents such as TCE in groundwater. Should natural attenuation studies automatically include a test for the microbe? Does the presence of *cis*-DCE and vinyl chloride preclude the need for microbe testing? Is suitable groundwater geochemistry or the detection of this bacterium in groundwater more valuable in natural attenuation studies?

Their responses are a good summary of the state of knowledge with respect to the use of PCR tools to evaluate the contribution of *Dehalococcoides* bacteria to natural attenuation of chlorinated ethylenes in ground water (Borden et al., 2003).

One panel member noted that due to the complexity of most field sites, we rely on multiple lines of evidence to evaluate the behavior of plumes. The panel member observed that:

> "While geochemical data are usually the best indicators of the potential for complete dechlorination in an aquifer, definitive information on the presence or absence of *Dehalococcoides* can provide very useful information in determining whether MNA is an appropriate approach for a specific site. At present, we do not understand enough about the distribution and activity of the currently available assays to use these results as a primary indicator for the presence/absence of complete reductive dechlorination at a site."

A second panel member noted the assay as currently practiced has a high rate of false positives and

false negatives. Based on the cost of the PCR assay compared to the cost for conventional geochemical parameters, the panel member concluded,

> "But clearly the cost benefit analysis of DSA [*Dehalococcoides* specific PCR assays] makes it comparatively less attractive, and due to limitation in sensitivity and interpretation, it should not be a required analysis at this time."

The third panel member noted that the significance of PRC data depended on the specific procedure that was used. She also noted that:

> "Another reason that limits the usefulness of microbial characterization at field sites has to do with the difficulty associated with using these data for assessing the efficacy of natural attenuation. Understanding the microbial population, its activity, and its diversity provides insight into the biodegradation processes and pathways but does not yield data that can be used to estimate concentration declines, generation of by-products, of clean-up times."

Intended Use of the Report

This report is intended to facilitate the use of DNA analysis to document the role of *Dehalococcoides* organisms in the natural attenuation of PCE, TCE, and their transformation products in ground water. The report contains seven sections. This section describes several innovative genetic tools for evaluating microbial communities that degrade chlorinated hydrocarbons. It also describes the relationship between the new genetic tools and previous technical recommendations published by EPA/ORD in the *Technical Protocol for Evaluating Natural Attenuation of Chlorinated Solvents in Ground Water* (Wiedemeier et al., 1998).

A short review of the nutritional ecology and physiological diversity of organisms that can degrade chlorinated solvents in ground water is provided in *Section 2. Ecology of Microorganisms that Transform Chlorinated Solvents*. The new techniques in genetic analysis that are available to identify and enumerate specific microorganisms in ground water or aquifer sediment are described in *Section 3. Tools to Assay Microorganisms that Transform Chlorinated Solvents*. These sections provide background and context for the detailed discussion of *Dehalococcoides* organisms in the remainder of the report. A reader who is familiar with these topics can skip Sections 2 and 3.

An evaluation of Monitored Natural Attenuation (MNA) as defined by U.S. EPA in the OSWER Directive (U.S. EPA, 1999) requires a quantitative understanding of the behavior of the plume of contamination over time and space. The critical parameter is the rate of attenuation of concentration of the contaminant over time and with distance away from the source. To contribute to an evaluation of MNA, an assay for the presence or activity of microorganisms must be associated with the field scale behavior of the plume containing the microorganisms. *Section 4. Dehalococcoides DNA and Rates of Natural Attenuation at Field Scale* compares the achieved rates of natural attenuation of PCE, TCE, *cis*-DCE, and vinyl chloride at several chlorinated solvent plumes to estimates of the concentration of *Dehalococcoides* DNA in ground water provided by the polymerase chain reaction (PCR) assay. In general, concentrations of *Dehalococcoides* DNA that were high enough to be detected by a commercially available assay were associated with rates of attenuation that are useful for MNA.

At most field scale plumes where MNA has been proposed as a remedy for chlorinated solvents in ground water, biotransformation of the solvents to harmless end products is an important part of the remedy. The expected contributions of other processes (such as sorption or dilution and dispersion) are usually not adequate to be protective of human health and the environment. As a consequence, the *Technical Protocol for Evaluating Natural Attenuation of Chlorinated Solvents in Ground Water* (Wiedemeier et al., 1998) put a heavy emphasis on biotransformation as a process to achieve natural attenuation.

The *Technical Protocol* (1998) used a number of geochemical parameters in a scoring system to predict whether ground water contained microorganisms that could biologically transform chlorinated solvents. The scoring system was criticized by the Committee on Intrinsic Remediation of the National Research Council (NRC, 2000). The Committee recommended that the scoring system should not be used to evaluate prospects for MNA.

In Table 2.3 of the *Technical Protocol* (Wiedemeier, 1998), scores are assigned based in part on analyses for oxygen, nitrate, iron II, sulfate, methane, molecular hydrogen, oxidation reduction potential (ORP), pH, total organic carbon, chloride, BTEX compounds, temperature, and alkalinity. An assay for the presence of *Dehalococcoides* DNA provides direct evidence of an organism that can completely transform chlorinated ethylenes. The presence or absence of *Dehalococcoides* DNA can be used to evaluate the information provided by the geochemical parameters.

In *Section 5. Geochemical Parameters and Occurrence of Dehalococcoides DNA,* statistics are used to identify the conventional geochemical parameters that are associated with the presence of *Dehalococcoides* DNA in ground water. The distribution of the values for ORP, and the concentrations of nitrate and methane were significantly different between samples of ground water where *Dehalococcoides* DNA was detected and samples where *Dehalococcoides* DNA was not detected. As for the other parameters, there were no significant differences. In Section 5, a statistical technique is used to derive a formula for the probability that *Dehalococcoides* is present in ground water knowing the concentrations of nitrate and methane, and the oxidation/reduction potential. The formula is offered as a replacement for the scoring system in the Technical Protocol (Wiedemeier et al., 1998).

The process of evaluating MNA as an option at chlorinated solvent sites often involves a laboratory study to determine whether the contaminated aquifer harbors microorganisms that can entirely transform the contaminants. These studies are often referred to as engineering treatability studies or microcosm studies. Laboratory enrichment studies were conducted with ground water from plumes where the long-term monitoring data clearly indicated that biotransformation processes were responsible for the observed natural attenuation of the plume. In *Section 6. Dehalococcoides DNA and Laboratory Studies of Biodegradation*, the results of the laboratory studies were compared to the distribution of *Dehalococcoides* DNA in the contaminated aquifer.

If the presence of *Dehalococcoides* DNA indicates the presence of organisms that will dechlorinate contaminants to ethylene, then all the cultures that were established with ground water containing amplifiable *Dehalococcoides* DNA would be expected to dechlorinate PCE or TCE to ethylene. If false positives for the assay are defined as water samples where *Dehalococcoides* DNA was detected, but ethylene was not detected in the enrichment culture, then the proportion of false positive predictions as evaluated against the results of all the enrichment cultures that were constructed was 55%.

An absence of *Dehalococcoides* DNA would suggest that these organisms were absent from the ground water. Because the only organisms known to dechlorinate *cis*-DCE and vinyl chloride belong to the *Dehalococcoides* group, the absence of *Dehalococcoides* DNA would indicate that dechlorination would not proceed to vinyl chloride or ethylene. If false negatives for the assay are defined as water samples where *Dehalococcoides* DNA was not detected, but vinyl chloride or ethylene was detected in the enrichment culture, then the proportion of false negative predictions as evaluated against the results of all the enrichment cultures that were constructed was 15%. The proportion of false negative predictions as evaluated against the results of only those enrichment cultures that showed dechlorinating activity was 43%.

Although the number of false determinations was high, the assay can be useful, particularly when the overall evaluation of natural attenuation is based on a variety of tests and conditions. Not all strains of *Dehalococcoides* can dechlorinate *cis*-DCE to vinyl chloride or ethylene (Duhamel et al., 2004). However, the unequivocal presence of *Dehalococcoides* DNA in a ground water sample strongly suggests, although it does not prove, that chlorinated ethylenes are being dechlorinated to ethylene in the aquifer. The determination is much stronger if it is supported by other information that would be consistent with dechlorination to ethylene. A failure to detect *Dehalococcoides* DNA in a sample of ground water should not be taken to mean that dechlorination in the aquifer will stop at the level of dichloroethylene, and that *cis*-DCE and vinyl chloride will not be degraded.

Section 7. Recommendations to Evaluate Biotransformation of Chlorinated Solvents provides recommendations on the interpretation of data on the concentration of DNA in ground water samples, and on the sampling protocol.

State of Practice and Emerging State of the Science

Laboratory research and collection of field data to prepare this report on the *Evaluation of the Role of Dehalococcoides Organisms in the Natural Attenuation of Chlorinated Ethylenes in Ground Water* began in December 2002. At that time, the literature suggested that organisms in the *Dehalococcoides* group were primarily responsible for complete dechlorination of chlorinated solvents to ethylene, both in the field and in laboratory cultures. This report is intended to facilitate the use of DNA analysis to document the role of *Dehalococcoides* organisms in the natural attenuation of PCE, TCE, and their transformation products in ground water at hazardous waste sites.

Future evaluations at U.S. EPA enforcement actions will be carried out by contractors and consultants to the responsible parties using commercially available services for the assay for *Dehalococcoides* DNA. To make our research findings consistent with results that would be obtained at other field sites, we obtained the

assay for *Dehalococcoides* DNA from a commercial vendor, instead of doing the assay in-house with EPA research staff. To generate data that met our data quality objectives for comparability, all the assays for *Dehalococcoides* DNA reported in this study were conducted by the same commercial vendor using the same protocol. In December 2002, we were aware of only one commercial laboratory in the world that could assay ground water for the presence of *Dehalococcoides*. Since that time, a number of laboratories have entered the commercial market.

The assay provided by our vendor was based on the polymerase chain reaction, and the assay used primers for the 16S-rRNA gene. This gene codes for a structural component of the ribosome. As a consequence the nucleotide sequences in the gene tend to be conserved as the organisms evolve over time. This makes it possible to recognize organisms that are currently widely distributed, but which had a common ancestor. However, the 16S-rRNA gene is not directly related to metabolism of chlorinated ethylenes, and it is possible that the assay detected organisms that belonged to the *Dehalococcoides* group, but were not able to metabolize *cis*-DCE and vinyl chloride. Genes have been identified in the *Dehalococcoides* group for enzymes that dechlorinate *cis*-DCE and vinyl chloride (Krajmalnik-Brown et al., 2004; Magnuson et al., 1998; Müller et al., 2004), and PCR assays for genes encoding for a Vinyl Chloride Reductase enzyme are now commercially available. There may be many Vinyl Chloride Reductase genes, and an assay for only one of the Vinyl Chloride Reductase genes might fail to identify the capacity to reduce vinyl chloride to ethylene in the mixed microbial community in a contaminated plume. However, if a PCR assay detects DNA for a Vinyl Chloride Reductase, it is very likely that the microbial community has the capacity to degrade vinyl chloride.

The U.S. Federal Government has provided substantial funding for research to development new tools for genetic analysis of organisms that are capable of degrading chlorinated organic contaminants in ground water. Many talented people work in this field, their research is bearing fruit, and new applications are coming into the market place. This report is based on the state of commercial practice in 2004. It is already out of date as far as new applications or potential applications of genetic analysis. However, use of the polymerase chain reaction to assay for the 16S-rRNA gene of *Dehalococcoides* is still widespread, and there is need of a report that documents the performance of the assay to evaluate natural attenuation of chlorinated solvents in ground water.

Section 2.
Ecology of Microorganisms that Transform Chlorinated Solvents

The ecology of organisms that biologically transform chlorinated ethylenes has recently been reviewed (Bradley, 2003). This Section is a short summary of the available literature. It is intended to provide the necessary background for subsequent sections of this report. Bradley (2003) is recommended to any reader who is interested in more information on the ecology of organisms that degrade chlorinated ethylenes.

Chlorinated solvents such as PCE and TCE are xenobiotic compounds. They have low molecular weight and are lipophilic, volatile, and nonflammable. This makes them effective solvents for oil and grease. The commercial use and production of PCE and TCE began in the 1920s and peaked in the 1970s. Use declined thereafter because they were suspected to be carcinogens.

The evolutionary origin of microbes capable of transforming chlorinated solvents is uncertain. Many chlorinated hydrocarbons occur naturally in the environment, including TCE and PCE (Gribble, 1994) and vinyl chloride (Keppler et al., 2002). As a result, microbial enzymes may have evolved that are specialized for degrading organochlorine compounds (Lee et al., 1998). However, the concentrations of chlorinated ethylenes in plumes of contaminated ground water are much higher than their natural concentrations. Chlorinated solvents such as PCE and TCE have been in the environment at high concentrations for only a short time. The dechlorinating bacteria may have evolved to degrade other substrates and by chance have the capability to degrade chlorinated solvents. Alternatively, in only a few decades, enzymes that were originally evolved to degrade other substrates may have adapted to degrade chlorinated solvents.

The mechanisms involved in microbial metabolism of chlorinated solvents can be broadly classified into two categories: oxidation reactions and reductive dechlorination. Oxidation is a process where the chlorinated solvents are oxidized to carbon dioxide or other benign compounds. Reductive dechlorination is a process where a chlorine atom is removed and replaced with a hydrogen atom. Typically, reductive dechlorination occurs under anaerobic conditions. An electron donor is required to carry out reductive dechlorination.

PCE and TCE are highly oxidized compounds and therefore are most susceptible to reductive dechlorination. In general, reductive dechlorination of PCE or TCE occurs by sequential dechlorination from PCE to TCE to DCE isomers to VC to ethylene. Depending upon environmental conditions, this sequence may be interrupted and not go all the way to ethylene (Wiedemeier et al., 1998). Reductive dechlorination may be performed by bacteria that couple their growth with the dechlorination of the chloroethylene or by bacteria that do not benefit from the dechlorination. In the former case, the process is known as halorespiration or dehalorespiration, and the bacteria are referred to as halorespiring bacteria. In the latter case, the process is a co-metabolic reaction where the growth of the bacteria is supported by metabolism of other compounds.

During reductive dechlorination, all three isomers of DCE can theoretically be produced. However, Bouwer (1994) reported that under the influence of biotransformation, *cis*-DCE is a more common intermediate than *trans*-DCE and 1,1-DCE. Compared with PCE and TCE, the dichloroethylene isomers and vinyl chloride are not as highly oxidized. Conventional wisdom holds that they are not as readily reduced in chemical reactions. As a consequence, the biological reductive dechlorination of dichloroethylene and vinyl chloride should be slower and less extensive than is the case for PCE and TCE. This perspective was based on a comparison of the Gibbs free energy for complete dechlorination of each chlorinated ethylene; however, the chlorinated ethylenes are dechlorinated in a step-wise fashion. A better way to evaluate the energy yield is to compare the yield of each separate dechlorination reaction. The Gibbs free energy for reductive dechlorination of PCE to TCE, of TCE to DCE, of DCE to vinyl chloride, and vinyl chloride to ethylene is -171.8, -166.1, -144.8, and -154.5 kilo Joules per mole of chlorinated ethylene, respectively (Dolfing, 2000). The energy yields for each successive dechlorination are essential equivalent. The rates

of reductive dechlorination of PCE, TCE, *cis*-DCE and vinyl chloride should be equivalent. Cupples et al. (2004b) compared dechlorination of *cis*-DCE and vinyl chloride by *Dehalococcoides* strain VS. The maximum growth rates were equivalent, the maximum rate of utilization of vinyl chloride was within 75% of the maximum rate of utilization of *cis*-DCE, and half saturation constants were equivalent.

The energy yield for reductive dechlorination is high relative to other anaerobic processes. The Gibbs free energy for nitrate reduction, sulfate reduction and methanogenesis is -112, -38, and -33 kilo Joules per mole of substrate consumed (Dolfing, 2000).

The DCE isomers and vinyl chloride also have the potential to undergo oxidation to carbon dioxide or acetate (Bradley and Chapelle, 1998). Aerobic growth on PCE and TCE as an electron donor has never been reported. However, Ryoo et al. (2000) reported that PCE can be degraded co-metabolically under aerobic conditions by oxygenase enzymes, and the co-metabolic oxidation of TCE has been well demonstrated (Wilson and Wilson, 1985; Nelson et al., 1988; Ensley, 1991; Chang and Alvarez-Cohen, 1994). In ground water, different organisms with different metabolic pathways may share the responsibility for natural attenuation of chlorinated solvents. The following sections describe the microorganisms that may be involved in biotransformation of chlorinated ethylenes.

Bacteria that Gain Energy from Reductive Dechlorination (Halorespiring Bacteria)

The halorespiring bacteria couple reductive dechlorination to growth. They were first described by Holliger and his co-workers (1993) who obtained a highly purified enrichment culture that was able to grow by the reduction of PCE to *cis*-DCE using hydrogen as the electron donor. The active organism was named *Dehalobacter restrictus*. Soon after, many different genera of bacteria capable of respiring chlorinated ethylenes were isolated. Table 2.1 lists many of the organisms that have been reported in the literature.

Some of the halorespiring organisms can only grow on a very limited range of substrates. They use only hydrogen as the electron donor and couple growth only to the reduction of chlorinated compounds. Examples are *Dehalobacter restrictus*, Strain TEA, *Dehalococcoides ethenogenes* strain 195, and *Dehalococcoides* sp. strain BAV1. Other halorespiring organisms are less restricted. They are able to use a number of different electron donors and acceptors for growth. Examples are *Dehalospirillum multivorans* (Sulfospirillum) and various *Desulfitobacterium* strains including *Desulfitobacterium* strain PCE1, *Desulfitobacterium* strain PCE-S, and *Desulfitobacterium frappieri* TCE1.

The Place of Dehalorespiring Bacteria in the Diversity of Life

To understand the diversity of microbial communities and the evolutionary relationships between various kinds of microbes, microbiologists have compared the DNA sequences of genes that encode for enzymes that carry out the metabolic processes of interest and the DNA sequences of genes that encode for important structural components of the microbial cell. The gene sequence that encodes for 16S rRNA is commonly used to compare evolutionary relationships between bacteria. All living cells on earth contain an organelle called a ribosome that assembles proteins from amino acids. Because 16S rRNA is an important component of ribosomes, portions of the 16S rRNA sequence are highly conserved due to the pivotal role of protein synthesis in cell metabolism. Based on the similarity of 16S rRNA sequences, a phylogenetic tree of microbial groups has been constructed (Figure 2.1). All life on earth is divided into three great domains; the organisms with a cell nucleus (eukarya), the bacteria, and the archaea. The archaea are single-celled and of similar size as the bacteria but are more closely related to the eukarya in their genetics and biochemistry. The archaea include the methanogens and other members that live in extreme environments. Most common soil and ground water bacteria that degrade organic contaminants are bacteria and fall into the division Proteobacteria, the Bacteroides/Cytophaga group, and the Gram positive bacteria. The cell wall of the Gram positive bacteria has a characteristic structure that can be recognized using a specific staining technique, the Gram stain. The Gram positive bacteria are divided into a high C+G group and a low C+G group based on the relative proportion of the bases guanine and cytosine in their DNA.

Many halorespirers (*Dehalobacter*, *Desulfitobacterium*) fall within the group of low C+G Gram positives; however, some halorespirers are found within very distantly related phylogenetic groups. This distribution indicates that the ability to dechlorinate is not restricted to one phylogenetic cluster and may have evolved separately. On the other hand, no halorespiring organisms have yet been isolated that belong to the archaeal domain (Middeldorp et al., 1999).

Of the organisms described to date, only *Dehalococcoides* species, which are phylogenetically located within the green non-sulfur bacteria, are capable of

Table 2.1. Diversity of Bacteria that can Reductively Dechlorinate Ethylenes*

Isolate or Culture	Closest Phylogenetic Affiliation	Dechlorination Steps Performed	Reference
Dehalobacter restrictus	Low G+C Gram positive bacteria	PCE to *cis*-DCE	Holliger et al., 1993
Dehalospirillum multivorans, renamed *Sulfospirillum*	Proteobacteria, ε subdivision	PCE to *cis*-DCE	Scholz-Muramatsu et al., 1995
Desulfitobacterium strain PCE1	*Desulfitobacterium* a Gram positive bacterium	PCE to TCE	Gerritse et al., 1996
Desulfuromonas chloroethenica	*Geobacter*	PCE to *cis*-DCE	Krumholz et al., 1996
strain MS-1	*Enterobacteriaceae*	PCE to *cis*-DCE	Sharma and McCarty 1996
strain TEA	Gram positive bacteria Low G+C	PCE to *cis*-DCE	Wild et al., 1996
Desulfitobacterium sp. strain PCE-S	*Desulfitobacterium* a Gram positive bacterium	PCE to *cis*-DCE	Miller et al., 1997
Dehalococcoides ethenogenes strain 195	Green, nonsulfur bacteria	PCE to ethylene	Maymó-Gatell et al., 1997
Desulfitobacterium frappieri TCE1	*Desulfitobacterium* a Gram positive bacterium	PCE to *cis*-DCE	Gerritse et al., 1999
Clostridium bifermentans strain DPH-1	*Clostridium*	PCE to *cis*-DCE	Chang et al., 2000
Dehalococcoides sp. strain CBDB1	*Dehalococcoides ethenogenes*	PCE to *trans*-DCE	Adrian et al., 2000
Desulfitobacterium sp. strain Y51	*Desulfitobacterium* a Gram positive bacterium	PCE to *cis*-DCE	Suyama et al., 2002
Desulfitobacterium metallireducens	*Desulfitobacterium* a Gram positive bacterium	PCE to *cis*-DCE	Finneran et al., 2003
Desulfuromonas michiganenis	*Geobacter*	PCE to *cis*-DCE	Sung et al., 2003
Dehalococcoides sp. strain BAV1	*Dehalococcoides etheneogenes*	*cis*-DCE to ethylene	He et al., 2003a,b

* *Extracted from Major et al. (2003).*

dechlorinating lower chlorinated ethylenes (i.e., dichloroethylene and vinyl chloride) and coupling growth with the dechlorination. For instance, *Dehalococcoide ethenogenes* strain 195 obtains energy from all dechlorination steps except the final step from vinyl chloride to ethylene (Maymó-Gatell et al., 1999, 2001). *Dehalococcoides* sp. strain BAV1 grows on vinyl chloride and all the dichloroethylene isomers (He et al., 2003a, 2003b). All laboratory mixed cultures that dechlorinate PCE or TCE beyond *cis*-DCE have been found to contain organisms in the *Dehalococcoides* phylogenetic group (Adamson and Parkin, 2000; Ellis et al., 2000; Fennell et al., 2001; Duhamel et al., 2002; Richardson et al., 2002; Cupples et al., 2003; Dennis et al., 2003). Certain stains of *Dehalococcoides* can also partially degrade polychlorobiphenyls (PCBs), chlorobenzenes and dioxins (Fennell et al., 2004) and dichloroethane and dibromoethane (Thomson and Vidumsky, 2003). There is a group of Chloroflexi bacteria that is closely related to *Dehalococcoides* (but is not the same genus) that also dechlorinates PCBs, chlorobenzenes, and PCE (Miller et al., 2005; Watts et al., 2005; Wu et al., 2002a , 2002b).

If natural attenuation is to be a remedy for ground water contamination with chlorinated ethylenes, the chlo-

rinated ethylenes must be completely dechlorinated to harmless products. Because the *Dehalococcoides* group is the only known group of organisms that can grow by carrying out the reductive dechlorination of dichloroethylene or vinyl chloride, it has a critical role in any evaluation of monitored natural attenuation in anaerobic ground water.

Section 3 of this report discusses the use of techniques for genetic analysis to recognize members of the *Dehalococcoides* group in ground water and aquifer sediment. The *Dehalococcoides* group is unusual in that its sequences of ribosomal RNA group with sequences shared by the green non-sulfur bacteria (i.e., the *Chloroflexi*). The green non-sulfur bacteria are green because they contain a form of chlorophyll. They are phototrophic, meaning that they gain energy from light and grow in the presence of light, but they do not produce oxygen during photosynthesis.

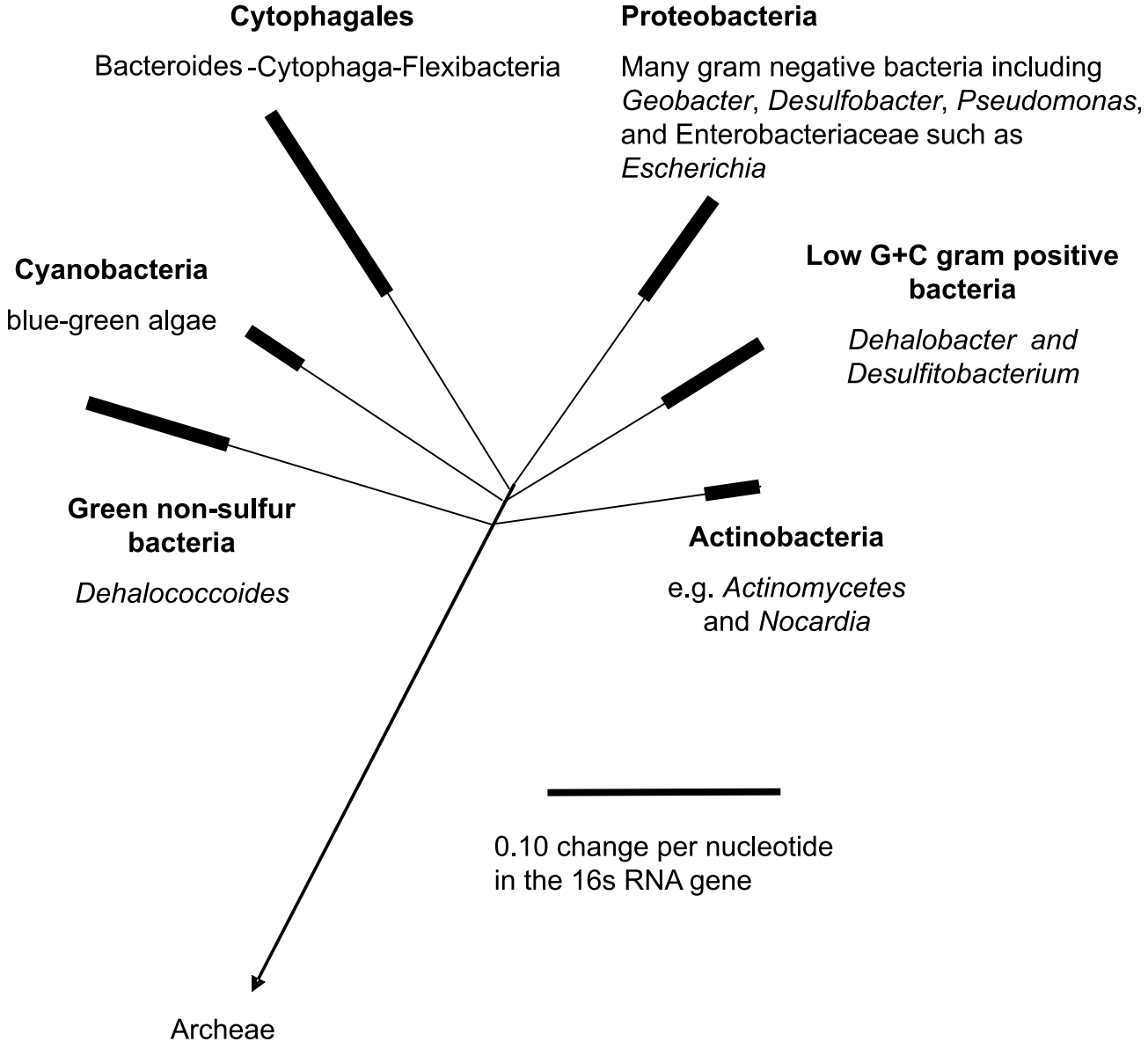

Figure 2.1. *A phylogenetic tree based on comparisons of sequences in the 16s Ribosomal RNA. The length of the thin lines is roughly proportional to the evolutionary distance between groups of organisms. The thick lines represent the range within a particular group. Redrawn and simplified from Figure 1 in Hugenholtz et al., 1998.*

Organisms that Oxidize Chlorinated Ethylenes under Anaerobic Conditions

Oxidation usually occurs in the presence of molecular oxygen; however, oxidation of *cis*-DCE and vinyl chloride may also occur under some anaerobic conditions (Bradley and Chapelle, 1998, 2000a, 2000b; reviewed in Bradley, 2003). Bradley and Chapelle (2000b) showed that vinyl chloride can be oxidized to acetate by an interesting class of anaerobic bacteria called acetogens. They oxidize the organic compound solely as part of their energy metabolism. They do not use the chlorinated ethylene as a substrate to build microbial cell constituents. Although their role in natural attenuation of chlorinated ethylenes in anaerobic aquifers may be as significant as that of the *Dehalococcoides* group, very little is known about them, and there are no techniques that are currently commercially available to estimate their contribution.

Organisms that Co-Metabolize Chlorinated Ethylenes

Under some circumstances, dechlorination of chlorinated compounds is not coupled to growth. The reaction is catalyzed by an enzyme or cofactor that is fortuitously produced by the microbes for other purposes. When this occurs, the process is called co-metabolism. Chlorinated ethylenes can be co-metabolized under aerobic conditions and under anaerobic conditions.

PCE and TCE can be co-metabolically dechlorinated by many types of anaerobic organisms, including certain species of methanogens such as *Methanosarcina mazei* (Fathepure and Boyd, 1988) and certain acetogens such as *Acetobacterium woodii* (Egli et al., 1988) and *Sporomusa ovata* (Terzenbach and Blaut, 1994); and sulfate-reducing bacteria (Bagley and Gossett, 1989). Only a small fraction of the total reducing equivalents derived from the oxidation of electron donors is used to reduce the chlorinated compounds.

Some bacteria contain certain enzymes that can fortuitously catalyze the oxidation of partially chlorinated compounds. These monoxygenase or dioxygenase enzymes require molecular oxygen as a substrate. Oxygen and a primary substrate are required for growth of the organisms. When these bacteria grow on primary substrates like methane, propane, propene, methanol, toluene, or phenol, they can co-metabolically oxidize partially chlorinated ethylenes such as TCE, *cis*-DCE, or vinyl chloride (Wilson and Wilson 1985; McCarty and Semprini, 1994; Reij et al., 1995; Fitch et al., 1996). The only plausible co-substrates in a contaminated aquifer are methane or aromatic hydrocarbons such as toluene. If oxygen is available, bacteria can grow rapidly on the primary substrates, and exhaust supplies of either the primary substrate or oxygen. Ground water in a contaminated aquifer can be expected to have the primary substrate or oxygen, but not both. Aerobic co-oxidation might occur at the fringe of an anaerobic plume where contaminated ground water containing methane mixes with ground water containing oxygen. However, the process is unlikely to make a substantial contribution to natural attenuation of chlorinated ethylenes.

Aerobic Growth on Chlorinated Ethylenes

There is no report on microbial growth supported by the oxidation of PCE or TCE. However, DCE and vinyl chloride can be directly oxidized by some bacteria. For instance, aerobic bacteria such as *Actinomycetales* sp. (Phelps et al., 1991), *Mycobacterium* sp. (Hartmans and de Bont, 1992), *Rhodococcus* sp. (Malachowsky et al., 1994), *Pseudomonas* sp. (Verce et al., 2000), and *Nocardioides* sp. (Coleman et al.; 2002a, 2002b) can grow on vinyl chloride as the sole carbon source. Similarly, *cis*-DCE has been shown to be utilized as the sole carbon source by some microorganisms (Bradley and Chapelle, 2000a, 2000b; Coleman et al., 2002a, 2002b; Olaniran et al., 2004).

Coleman et al. (2002b) determined the distribution of aerobic bacteria that could metabolize vinyl chloride in ground water from monitoring wells at chlorinated solvent spill sites. They were able to isolate vinyl chloride oxidizing strains from 15 of 24 samples of ground water or aquifer sediment. Aerobic organisms that degrade vinyl chloride are widely distributed. Reducing conditions are required to produce vinyl chloride or *cis*-DCE from TCE or PCE in ground water, yet oxygen is required for the further aerobic metabolism of *cis*-DCE or vinyl chloride. Degradation of *cis*-DCE and vinyl chloride by aerobic microorganisms can only be important at the fringe of a plume where DCE and vinyl chloride, produced in the plume by anaerobic microbial processes, are mixed with uncontaminated ground water containing oxygen. Aerobic biodegradation is probably not important in ground water plumes, unless the plume is very long. However, conditions conducive to aerobic biodegradation occur in the bed sediments of streams and lakes where plumes of contaminated ground water discharge to aerobic surface water.

Section 3.
Tools to Assay Microorganisms that Completely Transform Chlorinated Solvents

The only organisms that are known to completely transform chlorinated ethylenes to harmless products are members of the *Dehalococcoides* group of bacteria. This section discusses an analytical procedure called the polymerase chain reaction (PCR) that is used to amplify DNA from the *Dehalococcoides* group of bacteria when they are present in samples of ground water or aquifer sediment, and procedures used to detect and measure the amplified DNA produced by PCR. This section discusses the application of PCR technology to evaluate the presence and distribution of *Dehalococcoides* bacteria in ground water or aquifer sediments, and discusses limitation of the PCR assays for detecting *Dehalococcoides* bacteria.

The identification and enumeration of the members of a microbial community have traditionally been achieved by cultivation techniques such as plate counting. However, it has been estimated that the portion of microbes obtained by traditional cultivation techniques amounts to only 0.1 to 1 % of the total diversity (Amann et al., 1995). As a result, non-plating techniques, including molecular genetic methods, have been developed that can detect and identify microbes in their natural environment based on variations in the sequences of base pairs in their DNA and RNA (e.g. Akkermans et al., 1995). Most of these techniques are based on amplification of DNA that encodes the gene for 16S rRNA, using the polymerase chain reaction (PCR). The amplified DNA is detected or characterized by a variety of techniques including denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993) and real time PCR (Lee et al., 1993; Livak et al., 1995).

Polymerase Chain Reaction (PCR) Assays for Genetic Analysis of the Microbial Communities

The development of PCR is a major step forward in the study of microorganisms in the environment (Erlich, 1989). To prepare for the assay, a target organism is identified or selected. It is necessary to know the DNA sequences from the target organism. Based on that knowledge, short sequences of DNA are synthesized ("primers") that are complementary to the gene sequences in the target organism. To carry out a PCR assay using the primers, DNA is isolated from ground water or sediment samples. Then, the primers are combined in a reaction mixture and bind to the target DNA, if it is present, and allow the DNA to be copied by an enzyme called DNA polymerase. The process is repeated for a number of cycles, usually 30 to 40. Each time the DNA that was synthesized in the previous reaction becomes the substrate for the subsequent reaction. In each cycle of the chain reaction, the concentration of target DNA is doubled. Finally, the concentration of target DNA (the amplicon) is high enough that it can be identified and analyzed by optical or chemical procedures. The entire process is referred to as a polymerase chain reaction (PCR).

Electrophoresis is a technique to separate large molecules such as DNA based in part on their ionic charge. If a direct current is imposed across a gel containing the large molecules in a solution, the molecules will migrate to one electrode or the other depending on their charge.

The migration of the large molecules through the electric field is controlled by the strength of the field, the ionic charge of the large molecules, and the size and shape of the molecules. Because DNA has a net negative charge, DNA will migrate through the gel toward the positive electrode.

In cells, DNA molecules occur as a duplex or double helix of two separate strands of DNA. The two strands of the double helix are held together by hydrogen bonds between the two strands. The DNA duplex or double helix is folded into a three-dimensional structure that is held together by hydrogen bonding and interactions between hydrophobic regions in the DNA molecule. The two strands of DNA in the double helix can be separated from each other by chemicals and heat.

DNA molecules are commonly separated by a variation of electrophoresis called denaturing gradient gel electrophoresis (DGGE). The gel contains a gradient of chemicals that cause the DNA double helix to unfold into single strands. As a DNA molecule moves through

the gradient in the gel, the two strands of DNA start to separate or unfold. The unfolded DNA assumes a different shape, which produces more drag and reduces the mobility of the DNA molecule through the gel. Eventually, the partially unfolded DNA molecule will become so entangled in the gel matrix that it will stop moving. Small differences in the genetic sequence have a strong effect on the position in the gradient where the DNA molecule unfolds. As a result, small differences can cause the DNA molecule to take up different positions along the gradient in the gel. The fragments of DNA separate from each other and take up unique positions along the electrical gradient in the gel. When the DNA in the electrophoresis gel is stained, it shows up as bands (See Figure 3.1).

To identify the organism that supplied the DNA that was extracted from an environmental sample or an enrichment culture, the position of the band of unknown DNA is compared to the positions of the bands of DNA from known organisms. Because the DNA from a particular organism has been amplified by PCR before analysis by DGGE, the combination of the two techniques can detect very sparse populations of individual organisms in mixed microbial communities. The DNA from bands can be excised, reamplified by PCR, and sequenced, and the source of the DNA can be identified by comparing the sequences of the DNA isolated from the environmental sample to the sequence in known organisms.

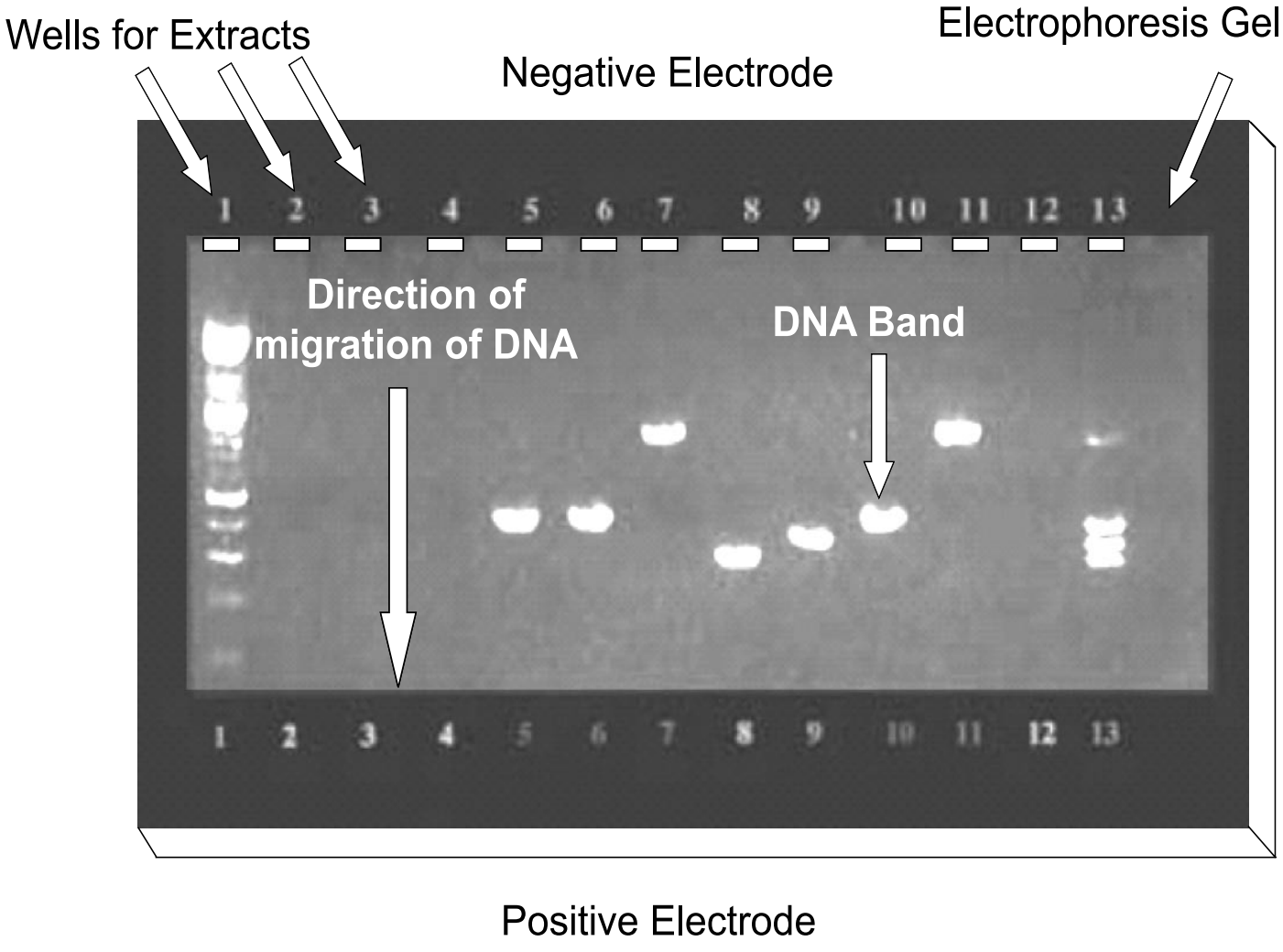

Figure 3.1. *Separation of DNA by Denaturing Gradient Gel Electrophoresis (DGGE). Wells are cut into a rectangular piece of gel. A different extract containing DNA is placed in each well. The DNA dissolves from the extract into the gel. An electrical current is imposed across the gel, causing the DNA molecules to migrate. Based on their ionic charge, size, and three-dimensional shapes, different pieces of DNA move different distances, causing the pieces of DNA to separate.*

Detection of *Dehalococcoides* Species by the PCR Assay

Although many microbial groups are involved in reductive dechlorination of chlorinated ethylenes, *Dehalococcoides* is the only group known to completely dechlorinate PCE/TCE to ethylene (Maymó-Gatell et al., 1997; Major et al., 2003). A recent paper reports that 17 putative reductive dehalogenases and five hydrogenase complexes are encoded in the genes of *Dehalococcoides ethenogenes*, indicating this organism is highly evolved to utilize halogenated organic compounds and hydrogen (Seshadri et al., 2005). Hence, detection of *Dehalococcoides* species may be a useful tool for assessing the efficiency of natural attenuation or engineered bioremediation at chlorinated solvent contaminated sites.

Currently (as of 2005), a polymerase chain reaction (PCR) assay based on primers for genes encoding for 16S-rRNA is the major tool for the detection of *Dehalococcoides* species (Löffler et al., 2000; Fennell et al., 2001; Hendrickson et al., 2002; He et al., 2003a, 2003b).

Löffler et al. (2000) were the first to publish an assay for the density of *Dehalococcoides* bacteria based on PCR. In their study, they used a two-step nested primer PCR approach using universal bacterial primers followed by a second PCR with the *Dehalococcoides*-targeted primers. The *Dehalococcoides*-targeted primers were designed based on the 16S rRNA sequence of *Dehalococcoides* sp. strain FL2, which was closely related to *Dehalococcoides ethenogenes* (96.9% sequence similarity). Since *Dehalococcoides* sp. strain FL2 was not available in pure culture, the sensitivity of the *Dehalococcoides*-targeted primers was evaluated using serially diluted plasmid DNA containing the 16S rRNA gene of strain FL2. One to 10 copies of the 16S rRNA gene of strain FL2 were found to be sufficient to yield the expected PCR product. This nested PCR approach was used to detect *Dehalococcoides* populations in river and aquifer sediments, and the results were confirmed by microcosm studies.

Fennell and her co-workers designed a *Dehalococcoides* primer set based on the 16S rRNA gene of *Dehalococcoides ethenogenes* strain 195 and used it for the detection of *Dehalococcoides* at a TCE-contaminated site (Fennell et al., 2001). In their study, separate PCR reactions were performed with universal primers and with *Dehalococcoides* primers. If no product was obtained directly with the specific primers, a nested approach was performed using the products from the universal PCR as the template for PCR with the specific primers. The detection limits were found to be approximately 10^3 cells per 0.5 g soil for the direct PCR and 5 to 10 cells per 0.5 g soil for the nested approach. The results of PCR analysis were supported by field data and microcosm studies.

Hendrickson and his co-workers developed a PCR assay to conduct an extensive survey for the presence of *Dehalococcoides* at multiple chloroethylene-contaminated sites (Hendrickson et al., 2002). In their study, seven *Dehalococcoides* primer sets were designed based on the 16S rRNA gene sequence of *Dehalococcoides ethenogenes* strain 195, which was originally isolated at Cornell University, and *Dehalococcoides* group sequences found in enrichment cultures originally isolated from sites at Victoria, Texas, and Pinellas, Florida. The sensitivities of the primer sets ranged from 10 to 1,000 copies of the gene per reaction mixture. The developed PCR assay was applied to ground water samples and soil samples collected from 24 sites. Positive results were obtained at 21 sites where full dechlorination of chloroethylenes to ethylene occurred. Phylogenetic analysis of the amplicons confirmed that *Dehalococcoides* sequences formed a unique 16S rRNA group, which could be divided into three subgroups (Pinellas, Victoria, and Cornell) based on specific base substitution patterns in variable regions 2 and 6 of the *Dehalococcoides* 16S rRNA gene sequence.

In general, the direct PCR technique using gel electrophoresis to detect the amplified gene product is a qualitative or semi-quantitative method. More recently, real time PCR (RT-PCR) has been developed to precisely quantify the density of the *Dehalococcoides* population (Lendvay et al., 2003; He et al., 2003a, 2003b). RT-PCR does not involve the use of gel electrophoresis to detect the amplified DNA, but it still makes use of the same principles of amplification as standard PCR. In RT-PCR amplification, a fluorescently labelled probe targeting 16S rRNA gene sequences of *Dehalococcoides* was included in the reaction. The fluorescence of the dye attached to the amplified DNA is measured during the “extension” phase of the polymerase chain reaction, allowing “real-time” monitoring of the accumulation of the replicated DNA by the instrument during each cycle of the polymerase chain reaction. The number of cycles that are required to accumulate a certain amount or so-called “threshold” concentration of copied DNA is used to quantitatively determine the starting concentration of DNA. This technique has been applied to measure the abundance of a *Dehalococcoides* population in a chloroethylene-contaminated aquifer undergoing active remediation (Lendvay et al., 2003).

Limitations of the PCR Assay for *Dehalococcoides* DNA

Although *Dehalococcoides* organisms are closely related phylogenetically, their capacity to transform chlorinated ethylenes can be quite different (Duhamel et al., 2004; He et al., 2003a, 2003b). *Dehalococcoides* strain CBDB1 can dechlorinate chlorobenzenes, but cannot grow on PCE or TCE (Adrian et al., 2000). *Dehalococcoides ethenogenes* strain CBDB1 and *Dehalococcoides* sp. strain 195 have 98% identity over 1,422 nucleotides of the 16S rRNA gene sequence. However, strain CBDB1 can only convert PCE to *trans*-DCE, while strain 195 converts PCE to vinyl chloride and ethylene (Fennell et al. 2004). *Dehalococcoides ethenogenes* 195 and *Dehalococcoides* strain FL2 both grow on TCE or dichloroethylenes, but cannot grow on vinyl chloride, while *Dehalococcoides* sp. strain BAV1 can grow on vinyl chloride (He et al., 2003b). Although these strains are closely related and are capable of dechlorinating some of the same substrates, they did not share the capacity to grow using vinyl chloride, which is critical to complete transformation of chlorinated ethylenes to harmless products.

The PCR primers used in commercially available assays are designed to detect as many D*ehalococcoides* strains as possible. They are not designed to distinguish between the different strains. It would be nearly impossible to design primers for 16S rRNA genes that could distinguish strains that dechlorinate dichloroethylenes and vinyl chloride from strains that do not. Even if the PCR products were cloned and sequenced from strains that did or did not dechlorinate dichloroethylenes and vinyl chloride, it is unlikely that the relatively short amplicons that are typically obtained would contain enough information to distinguish between strains (personal communication, Donna Fennell, Rutgers University).

One alternative to searching for a structural gene associated with the *Dehalococcoides* group is to create primers for the genes for the dehalogenase enzymes that actually carry out the transformation of TCE or vinyl chloride to ethylene. Some strains of D*ehalococcoides* are known to express an enzyme that can dechlorinate TCE to ethylene (Magnuson et al., 1998, Fennell et al., 2004) or an enzyme that can dechlorinate vinyl chloride to ethylene (Müller et al., 2004, Krajmalnik-Brown et al., 2004). Genes for these enzymes have been sequenced, and a quantitative PCR assay for these gene sequences is commercially available**.** This is an area of active research, and new dehalogenase primers are being developed. Until a comprehensive catalogue of primers for dehalogenase genes can be developed, there is a strong chance of a false negative in interpreting the absence of the dehalogenase gene. Although a particular gene may be absent, other dehalogenase genes that were not recognized by the primer may be expressed in the microbial population. However, the interpretation of the detection of PCR product from dehalogenase genes is straightforward. If the product is amplified by the primer, the gene is present in the population.

Current State of Practice of PCR Tools to Evaluate Biotransformation of Chlorinated Solvents

In 2005, the Strategic Environmental Research and Development Program (SERDP) and the Environmental Security Technology Certification Program (ESTCP) of the U.S. Department of Defense organized a workshop to evaluate the application of molecular biological tools to environmental remediation. The report of the workshop is a useful summary of the state of practice and needs for technology development (Alleman et al., 2005). Table 3.1 presents the consensus opinion of the experts that participated in the workshop concerning the advantages and disadvantages of PCR tools that might be used to evaluate biotransformation of chlorinated solvents in ground water, and Table 3.2 presents the consensus opinion on the applications of the PCR tools and the frequency at which they are used.

Table 3.1 Comparison of Advantages and Disadvantages of PCR Tools to Evaluate Biotransformation of Chlorinated Solvents in Ground Water. Summarized from Table 2 of Alleman et al. (2005)

Tool	Perceived Advantage	Perceived Disadvantage
Direct PCR	Easy to perform	False negatives
Nested PCR	Unsurpassed sensitivity	Requires two PCR steps
Quantitative PCR for 16s rRNA gene	Provides information on presence/ absence/ abundance of organisms of interest; nearly reaches the sensitivity of nested PCR; commercially available for few key organisms (e.g. *Dehalococcoides* spp.); estimates of total bacterial numbers are possible	Does not provide confirmation of activity; sampling, handling, and analysis are not standardized
Quantitative PCR for functional genes	Provides information on presence/ absence/ abundance of functional gene of interest; commercially available for few key genes (e.g. reductase dehalogenase genes)	For DNA, does not provide conformation of activity; sampling, handling, and analysis are not standardized
Quantitative PCR for messenger RNA	Provides information on gene expression (i.e. activity); quantitative approaches under development	Relative instability of RNA presents sampling and preservation challenges; not commercially available to a significant extent; sampling, handling, and analysis are not standardized

Table 3.2 Applications of PCR Tools to Evaluate Biotransformation of Chlorinated Solvents in Ground Water. Summarized from Table 2 of Alleman et al. (2005)

Tool	Current Applications	Current Relative Frequency of Use	Comments
Direct PCR	Screening tool for presence/absence [of DNA for putative active organisms]	Moderate	Replaced by quantitative PCR
Nested PCR	Screening tool for presence/absence [of DNA for putative active organisms]	Moderate	Replaced by quantitative PCR
Quantitative PCR for 16s rRNA gene	Screening tool for presence/absence of desired or indicator organisms; monitoring of growth and distribution of individual organisms	High	[Would benefit from] expansion to wider range of organisms; standardized procedures; availability of standards
Quantitative PCR for functional genes	Screening tool for presence/absence of target functional genes; monitoring of distribution and proliferation of individual genes	Low	Needs wider range of functional genes; extension to mRNA; standardized procedures; availability of standards
Quantitative PCR for messenger RNA	A few experimental applications for confirming expression of functional genes.	Low	Needs wider range of genes of interest; standardization of approach; clarification of how mRNA abundance relates to activity

Section 4.
Dehalococcoides DNA and Rate of Natural Attenuation

Many evaluations of chlorinated solvent contamination in ground water use mathematical models to project the future behavior of the plume. The models are very sensitive to the rate constants for degradation (Newell et al., 2002). Biological reductive dechlorination can be an important mechanism for the removal of chlorinated solvents from many anoxic aquifers; however, there appears to be a significant variation in the rates and extent of dechlorination from one plume to the next (Suarez and Rifai, 1999).

Hendrickson et al. (2002) reported that there was a strong association between the presence of *Dehalococcoides* DNA and complete dechlorination to ethylene. Lendvay et al. (2003) further demonstrated a quantitative relationship between *Dehalococcoides* DNA and ethylene production. To determine if there is a valid association between *Dehalococcoides* DNA in ground water and the observed rates of dechlorination at field scale, a survey was conducted at selected field sites. Rate constants for attenuation of chlorinated solvents at field scale were extracted from the monitoring data, and then the rate constants were compared to the presence or density of *Dehalococcoides* DNA in water from monitoring wells at the sites.

As is discussed under the subsection titled, **Rates of Natural Attenuation and Density of PCR Products from Dehalococcoides DNA**, the monitoring wells did not efficiently sample the *Dehalococcoides* organisms in the aquifer, and the number of *Dehalococcoides* cells recovered in a liter of well water was a small fraction of the number of cells that were exposed to a liter of ground water in the aquifer. Most of the *Dehalococcoides* cells were probably attached to sediment particles. As a result, there was not a quantitative relationship between the rates of natural attenuation at field scale and the density of *Dehalococcoides* cells in ground water from monitoring wells.

A Definition of "Generally Useful" Rates of Biological Reductive Dechlorination

There is no legally mandated time frame for monitored natural attenuation of contaminants in ground water (U.S. Environmental Protection Agency, 1999). For purposes of discussion, a time frame for remediation of 30 years will be assumed. The Maximum Contaminant Levels (MCLs) for PCE and TCE in drinking water are 5 µg/L. McNab et al. (2000) evaluated the distribution of contaminants in more than 200 chlorinated solvent plumes. Figure 4.1 shows the cumulative frequency distribution of the maximum concentrations of each separate chlorinated hydrocarbon in each of the plumes and the first order rates of degradation that are necessary to reduce the maximum concentrations to meet a MCL of 5 µg/L in 30 years.

Fifty percent of the plumes had maximum concentrations of chlorinated hydrocarbons greater than 8,000 µg/L. The rate of natural biodegradation necessary to reduce concentrations from 8,000 µg/L to 5 µg/L in 30 years would be 0.23 per year (calculated by a first-order kinetic model). Similarly, the rates of degradation necessary to reduce 10% and 90% of the plumes in the survey of McNab et al. (2000) from their maximum concentrations to their MCLs within 30 years would be 0.043 per year and 0.32 per year, respectively. For purposes of evaluating the data at our study sites, a rate of 0.3 per year can be considered a "generally useful" rate constant for monitored natural attenuation of chlorinated ethylenes in ground water. This "generally useful" rate should not be applied at other sites without due consideration of site specific conditions. If the initial concentrations of chlorinated ethylenes are low, or if the time allowed to reach MCLs is long, a slower rate might be acceptable at a particular site.

Site Selection

Eight sites at six locations were selected for the survey because they had good records of long-term monitoring. This data made it possible to extract rate constants for attenuation of the chlorinated solvents. These sites are the Western Processing Site at Kent, Washington; Landfill Number 3 (LF3) and Fire Training Area Number 2 (FTA2) at Tinker Air Force Base, Oklahoma; the North Beach Site at the U.S. Coast Guard Support Center in Elizabeth City, North Carolina; Spill Site Number 17 (SS-17) at Altus Air Force Base, Oklahoma; the Target Area 1 Site at Dover Air Force Base, Delaware; and Area 800 and Area 2500 at the former England Air Force Base, Louisiana. See Figure 4.2 for a map showing the locations of the sites within the United States.

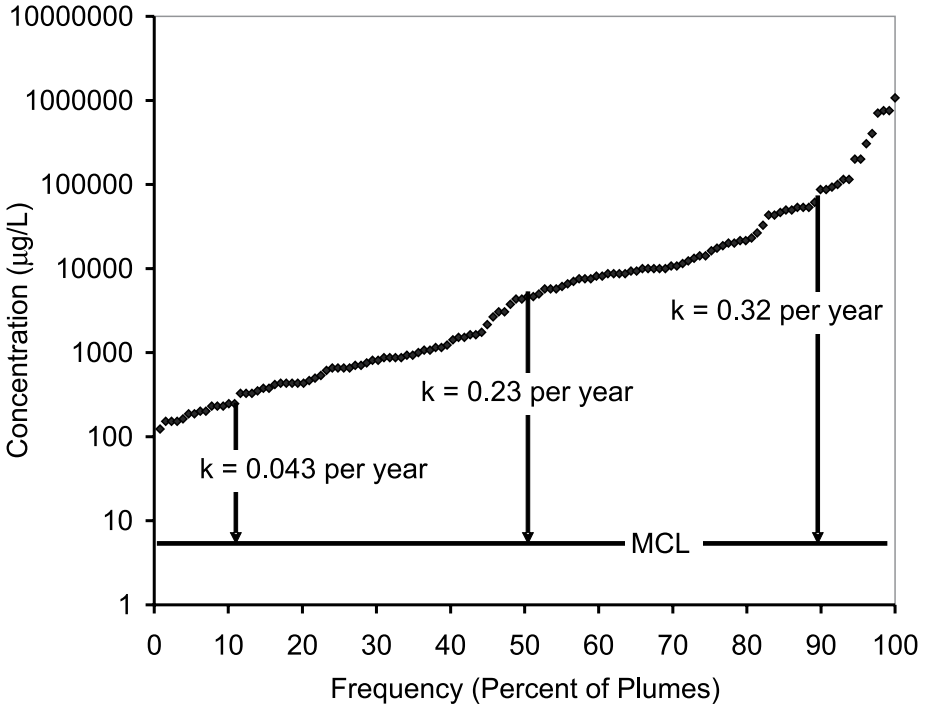

Figure 4.1. *The frequency distribution of the maximum concentration of chlorinated solvents and their transformation products at Department of Defense Sites in the United States (from McNab et al., 2000). The arrows identify the first order rates of biotransformation that are required to reach a Maximum Contaminant Level of 5 µg/L within thirty years at 10% of sites, at 50% of sites, and at 90% of sites.*

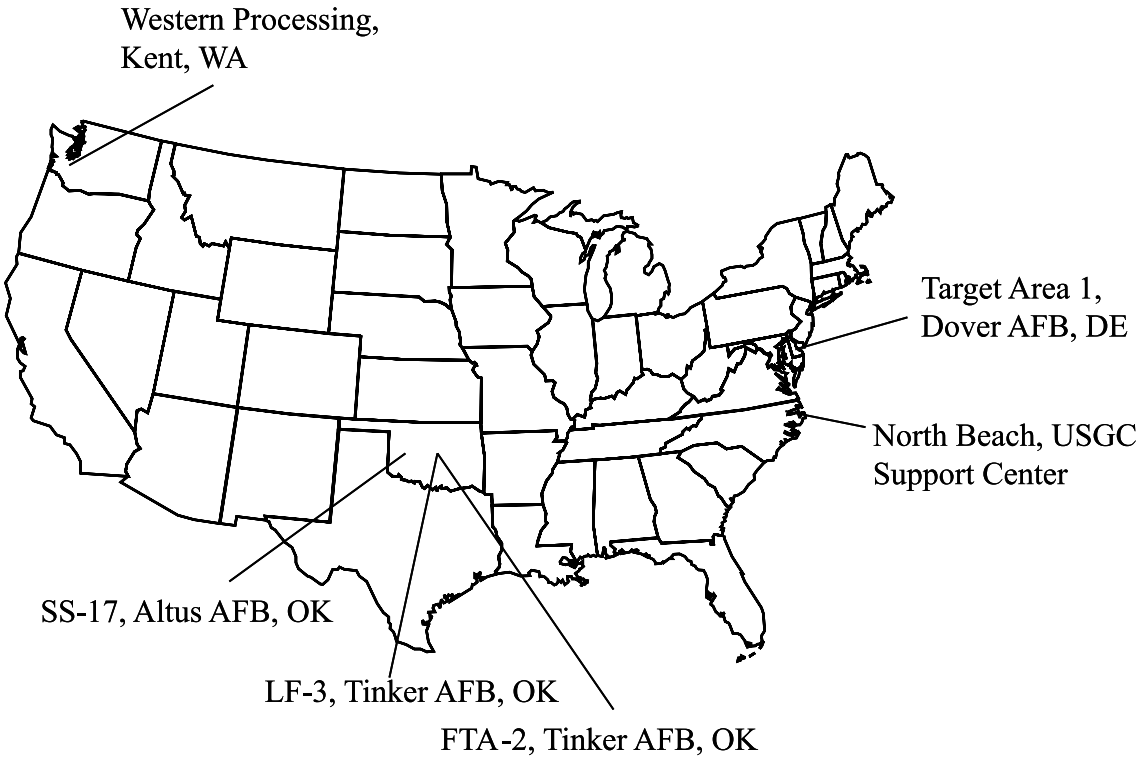

Figure 4.2. *Location of sites used to survey the relationship between Dehalococcoides DNA in ground water and the rate of natural attenuation of chlorinated ethylenes in field scale plumes.*

The plume at the Western Processing Site was extracted with a pump and treat system from September 1988 through April 2000. The plume at site LF3 has been extracted by a pump and treat system since early 1999. The plume at site SS-17 has been extracted with a two-phase vacuum extraction system since September 1996. At the North Beach Landfill Site, the source was removed in 1999. There are no engineered remediation actions at the other sites. To avoid errors in fitting rate constants for natural attenuation, the rate constants were fit to data that were collected prior to initiation of any engineered remedies.

Ground Water Sampling

At least two monitoring wells were sampled in the plume at each site. One well was located at the "hot spot" and one down gradient. See Table 4.1 for the number of wells sampled at each site. In most cases, the water samples were collected with a peristaltic pump at the well head using a polyethylene plastic tube inserted into the well. Occasionally, the water samples were collected using the dedicated submersible pump in the wells. Each well was purged for approximately one-half hour; at least two casing volumes were purged before samples were collected.

The dissolved hydrogen in ground water was sampled using a Microseeps Cell following the bubble stripping method (McInnes and Kampbell, 2000). After sampling for dissolved hydrogen, the effluent from the pump was directed to pass through an over-flow cell for measuring pH, temperature, conductivity, and oxidation reduction potential (ORP) against a Ag/AgCl reference electrode. Then, the effluent from the pump was collected to determine the concentrations of dissolved oxygen and ferrous iron using colorimetric field test kits. Finally, the effluent from the pump was directed to fully fill various sample containers for analysis of *Dehalococcoides* DNA, chlorinated solvents, dissolved gases (methane and ethylene), inorganic ions (nitrate plus nitrite, sulfate and chloride), and total organic carbon (TOC) in the laboratory.

The samples for chlorinated solvents and the samples for dissolved gases were preserved with 1% trisodium phosphate. The samples for nitrate plus nitrite were preserved with acid (five drops of 50% sulfuric acid to 50 ml of water sample). The samples for *Dehalococcoides* DNA were packed in ice and stored in coolers prior to shipment for analysis.

Chemical Analysis

Ground water samples for the analysis of chlorinated solvents were prepared in an automatic static headspace sampler (U.S. EPA, 1988). The samples were analyzed by gas chromatography with a mass spectrometer detector. The reporting limits were 1.0 µg/L for all the analytes. The concentrations of ethylene and methane in ground water samples were determined using a headspace equilibration technique (Kampbell and Vandegrift, 1998). The gaseous components in the headspace were separated by gas chromatography and then measured with a thermal conductivity detector. The reporting limits were 1 µg/L in the original aqueous phase for both gases. The concentration of dissolved H_2 was measured on a RGA3 Reduction Gas Analyzer equipped with a 60/80 molecular sieve 5A column and a reduction gas detector. The reporting limit was 1.0 nM in the water originally sampled. Nitrate plus nitrite were analyzed using Lachat Flow Injection Analyses. The reporting limit was 0.1 mg/L as nitrogen. Sulfate and chloride were analyzed using Waters Capillary Electrophoresis. The reporting limits were 0.5 mg/L for sulfate and 1.0 mg/L for chloride. Total Organic Carbon was determined by a Dohrman DC-80 Carbon Analyzer with a reporting

Table 4.1. Location of Specific Sites in the Survey and the Number of Wells Sampled at Each of the Sites

Location	Site	No. Wells Sampled
Altus Air Force Base, Oklahoma	Spill Site 17 (SS-17)	6
Dover Air Force Base, Delaware	Target Area 1	8
U.S. Coast Guard Support Center, Elizabeth City, North Carolina	The North Beach Landfill Site	6
England Air Force Base, Louisiana	Area 800	4
	Area 2500	6
Tinker Air Force Base, Oklahoma	Landfill Number 3 (LF-3)	5
	Fire Training Area Number 2 (FTA-2)	2
Kent, Washington	The Western Processing NPL Site	6

limit of 0.5 mg/L. The dissolved oxygen, temperature, pH, ORP, and conductivity were measured by corresponding electrodes and meters. The concentration of ferrous iron, hydrogen sulfide, and alkalinity were determined in the field using a Chemetrics Kit Model K-6010D for ferrous iron, a Hach Kit Model HS-C for sulfide, and a Hach Kit Model ALAPMG-L for alkalinity. The reporting limits for ferrous iron, sulfide, and alkalinity were 0.1, 0.5, and 20 mg/L, respectively.

Detection of Dehalococcoides by Polymerase Chain Reaction Analysis

Dehalococcoides DNA in ground water samples was analyzed by SiREM (Guelph, Ontario) using their Gene-Trac Test. The test employs the polymerase chain reaction using primer sets specific to DNA sequences in the 16S rRNA gene of the *Dehalococcoides* group (see Section 3 for a discussion). Each test used four primer sets, three of which were designed to target various sequences that were specific to the *Dehalococcoides* 16S rRNA gene. A fourth primer set amplified sequences that were shared by most members of the True Bacteria (see Figure 2.1) and was used as a control to confirm that DNA extracted from the sampled ground water could be amplified successfully by the polymerase chain reaction.

Bacteria were filtered from 1.0 liter of the ground water sample using a 0.45 µm sterile nylon filter, and DNA was extracted from the bacteria. The DNA was extracted and prepared in 55 µL of water that was free of DNAse and pyrogens. Then, 1 µL of the DNA extract was used in the PCR reaction mixture. The PCR product was separated by gel electrophoresis and then stained to visualize the PCR products produced from the amplification specific for *Dehalococcoides* 16S rRNA gene and from the amplification for the general (universal) bacterial 16S rRNA gene.

The presence of *Dehalococcoides* DNA in the samples was assessed as either "Detected" or "Not Detected" based on interpretation of an electronic image of the stained band of DNA in the electrophoresis gel (see Figure 3.1). Detects (gel bands) were quantified using densitometry software and assigned a "band intensity percentage" using the relative intensity of the strongest bands obtained to the intensity of the positive control containing 10^5 gene copies. Workers with experience with PCR are generally reluctant to make a quantitative association between the quantity of PCR product and the number of gene copies in the original sample. As a consequence, SiREM assigned a "test intensity score" as follows: if the value was 0% of positive control, a score of (-) was assigned; if the value was smaller than 3% of positive control, a score of (+/-) was assigned; if the value was in the range of 4% to 33% of positive control, a score of (+) was assigned; if the value was in the range of 34% to 66% of positive control, a score of (++) was assigned; if the value was in the range of 67% to 100% of positive control, a score of (+++) was assigned; and if the value was larger than 100% of positive control, a score of (++++) was assigned.

SiREM has compared the semi-quantitative "intensity score" with the results of quantitative PCR analyses done on the same samples. When the score was (+), the approximate range of gene copies was 10^3 to 10^5 per liter; when the score was (++), the approximate range of gene copies was 10^4 to 10^6 per liter; when the score was (+++), the approximate range of gene copies was 10^5 to 10^6 per liter; and when the score was (++++), the approximate range of gene copies was 10^6 to 10^8 per liter. The effective detection limit of the PCR reaction for *Dehalococcoides* DNA using gel electrophoresis was 500 to 5,000 gene copies of *Dehalococcoides* DNA per liter of ground water extracted.

The density of *Dehalococcoides* cells in the water samples was determined using Quantitative Real-time PCR of 16S rRNA genes. The assays were performed in the laboratory of Elizabeth Edwards at the University of Toronto, Ontario, Canada. The quantitation was performed using primers identical to those used in a commercial test (Quantitative Gene-Trac, SiREM, Guelph, Ontario). The primers are specific for variable regions of the *Dehalococcoides* 16S rRNA gene and produce an amplicon of 512 base pairs in length and are similar to those described by Hendrickson et al. (2002) and protected under US patent US6894156B2 (Hendrickson and Ebersole). Real-time quantitative PCR (q-PCR) reactions (50 µl) were performed in duplicate using 25 µl of 2X DyNAmo SYBR Green qPCR Master Mix (MJ Research Inc., MA), 1.0 µl of water containing 25 pmol of each primer and 19 µl of DNase and RNase-free water (Sigma) and 4 µl of template DNA which were gently mixed at room temperature and transferred into a 96 well plate (Opticon™ Systems) and sealed with 8-strip Ultraclear caps (MJ research Inc., Waltham, MA).

Real-time PCR was performed with a DNA Engine Opticon 2 System (MJ Research Inc., MA) with initial denaturation at 94 °C for 10 min; 45 cycles of 94 °C for 45 seconds, annealing at 60 °C for 45 seconds, and extension of 72 °C for 50 seconds. Standard curves of C_t versus $\log_{10}$ 16S rRNA gene copy number were produced using known quantities of cloned *Dehalococcoides* 16S rRNA genes. The standard curves were used to estimate the number of 16S rRNA gene copies in the ground water samples. Verification of the

specificity and identity of the PCR products was determined by melting curve analysis performed between 72 °C and 95 °C using the Opticon Monitor Software. The detection limit is near 2,000 gene copies per liter of ground water extracted.

As mentioned above, SiREM uses primers patented by DuPont. Hendrickson et al. (2002) sequenced amplicons of the primers to determine the specificity of the primers for *Dehalococcoides*. Occasionally SiREM will sequence the amplicons at the request of their clients. In every case, the amplicons have been highly similar (percent similarity >90%) to known *Dehalococcoides* organisms (personal communication, Philip Dennis, SiREM, Guelph, Ontario, Canada). Amplicons from samples provided in this study were not sequenced.

Purified sterile water was processed with every ten samples to serve as negative controls or "DNA blanks." If the controls produced a visible band, the test results were repeated or invalidated. In some cases where the bands from the controls were very weak, that fact was noted in the case narrative, and the test results were reported.

If no *Dehalococcoides* DNA was recovered, PCR was conducted with a universal primer to determine if amplifiable concentrations of bacterial DNA were present in the sample. Genomic DNA from *E. coli* was used as the positive control for the assays with the universal primer. The sensitivity of the assay with the universal primer has not been explicitly determined, but it should be the same or slightly lower than the assay with *Dehalococcoides* primers based on the fact that fewer cycles of PCR are performed and that the amplification products, for the most part, are longer (personal communication, Philip Dennis, SiREM, Guelph, Ontario, Canada). The amplification of the bacterial primer was scored as "detected," "trace," or "not detected."

Calculation of Dechlorination Rates from Monitoring Data

The BIOCHLOR decision support system was used to calculate the rates of reductive dechlorination of chlorinated ethylenes (available at http://www.epa.gov/ada/csmos.html) (Aziz et al., 2000). The BIOCHLOR software simulates remediation of dissolved solvents by natural attenuation at chlorinated solvent release sites. It is based on the Domenico analytical solutions to the solute transport equation and has the ability to simulate one-dimensional advection, three-dimensional dispersion, linear adsorption, and sequential biotransformation of chlorinated ethylenes by reductive dechlorination. It assumes biotransformation follows a pseudo-first order rate law. Parameters used to calibrate BIOCHLOR to the plumes are listed in Table 4.2. A detailed example of one of the calibrations is provided at the end of this section.

At six sites in five locations (see Table 4.3), the contamination in ground water formed a conventional plume. Each plume had a region of high contamination associated with the source and a region with lower concentrations extending away from the source in the direction of ground water flow. BIOCHLOR was calibrated to field data on contaminant concentrations from single sampling events. Site-specific information was collected from reports or papers on the study sites (Acree and Ross, 2003; Altus Air Force Base, 2002; Dover Air Force Base, 2003; Landau Associates, 1995, 2002; Tinker Air Force Base, 1999, 2002; Wilson et al., 1997). To avoid errors in fitting rate constants for natural attenuation, the rate constants were fit to data that were collected prior to initiation of any engineered remedies.

At two sites (Area 800 and Area 2500) at England AFB, Louisiana, there was no discernable overall direction of ground water flow in the plumes. The sites were overlaid by a bayou that communicated with the contaminated aquifer. Ground water in the aquifer flowed toward the bayou or away from the bayou depending on the seasons and recent precipitation events. As a result, concentration isopleths of TCE, *cis*-DCE, and vinyl chloride were arranged in concentric circles that were centered about the source area. Therefore, time series data on contaminant concentrations and dummy variables for hydrologic properties (seepage velocity 100 ft/yr, longitudinal dispersivity 10 ft and retardation factor 1) were inserted into BIOCHLOR to extract the rate constants of dechlorination over time in particular wells.

Calculation of Dechlorination Rates in Conventional Plumes

Table 4.3 shows the relationship between the concentrations of chlorinated ethylenes in the ground water plumes and their apparent rates of dechlorination along a flow path in the aquifer. A wide variety of concentrations were represented in the survey; however a relatively narrow range of rate constants was deduced. There did not appear to be any consistent relationship between the concentration of the chlorinated ethylenes and their first order rate of biotransformation.

At the Western Processing Site, field data before implementation of a pump and treat system were used for calibrating BIOCHLOR. The rate constants compared very favorably with the rate constants de-

Table 4.2. Calibration Parameters for BIOCHLOR

Parameter	Kent, WA	Tinker AFB, OK			Altus AFB, OK	USCG Support Center, Elizabeth City, NC		Dover AFB, DE
	Western Processing	Landfill 3		FTA2	SS17	North Beach		Target Area 1
	1988	Sept. 1997	Nov. 2002	Aug. 1997	March 2003	1997	Oct. 2002	July 1997
Seepage Velocity (m/yr)	20	29	29	29	8.3	8.5		37
Coefficient Dispersion (m)	2.4	7.6	7.6	45	45	0.6	0.8	7.6
Ratio Lateral to Longitudinal Dispersion	0.2	0.1	0.1	0.1	0.1	0.2	0.2	0.2
Ratio Vertical to Longitudinal Dispersion	0.2	none	none	none	none	0.2	0.2	0.2
Retardation Factor	1.1	3	3	3	1.3	1.1	1.1	1.0
Simulation Time (years)	30	60	60	30	30	60	60	60
Modeled Area Width (meters)	120	150	150	150	180	120	120	300
Modeled Area Length (meters)	240	760	760	460	460	60	76	760
Source Thickness (meters)	9	6	6	6	6	9	9	15
Source Width (meters)	60	76	76	76	60	60	60	150

rived from that site by other methods (Lehmicke et al., 2000, data cited in Table 4.3).

At the other sites, when possible, field data from independent sampling events were used to calibrate BIOCHLOR. This was done to evaluate the variability over time of the rate constants that were extracted from the field data. There was agreement in the rate constants extracted for the LF3 plume from data collected in 1997 before the operation of an extraction system and in 2002 after the operation of the extraction system. The rate constant we extracted from data on the FTA2 plume was faster than a rate constant extracted at the same site in 1999 by a contractor for the U.S. Air Force (Parsons, Inc., cited in Tinker Air Force Base, 1999). There was agreement in the rate constants extracted from the North Beach Site using data collected in 1997 before source removal efforts and data collected in 2002 after source removal.

The lower plume in the Target Area 1 is currently attenuating more rapidly near the source areas than in the far field. As a result, the current profile of concentrations with distance from the source is inverted. Therefore, we chose to calibrate BIOCHLOR to historical data collected in 1997. There was good

Table 4.3. Relationship between the Concentrations of Chlorinated Ethylenes in Ground Water and Their Apparent Rates of Dechlorination along an Inferred Flow Path in the Aquifer

Facility/ Location	Date	PCE	TCE	*cis*-DCE	VC	PCE	TCE	*cis*-DCE	VC
		Concentration near source (µg/L)				Pseudo-first order rate constant for dechlorination (per year)			
Western Processing Kent, WA	1988 [a]			10000	460			0.6	3
	1999			26	44			0.6 [b]	1 [b]
	4/2003			0.34	1.59				
LF3 (landfill) Tinker AFB, OK	9/1997 [c]	5.3	97	38000	23000			1	3
	11/2002	8	28	28400	20400			1	3
North Beach, USCG Support Center Elizabeth City, NC	1997 [d]	2000	105	74	30.8	0.1	1	0.3	1
	10/2002	561	52	25	<1	0.1	1	0.3	1
FTA2 (fire training) Tinker AFB, OK	8/1997	6.1	9440	1200	1.7		0.1 [e]		
	11/2002	7.5	9330	977	2.9		0.3		
SS17 Site Altus AFB, OK	3/2003	38.5	8160	264	4.28		0.01		
Target Area 1 (Lower Plume) Dover AFB, DE	7/1997	680	2400	560	<1	0.1	0.1	0.1	0.3
	1997 [f]	Transect TA to TB				0.26	0.23	0.31	0.3
		Transect TB to TC				0.18	0.07	0.27	
		Wells TA to TB				0.14	0.06	0.08	0.2
		Wells TB to TC				0.48	0.22	0.28	

[a] data collected before the operation of pump and treat system; [b] data calculated from the half-lives for cis-DCE (1.1 years) and VC (0.55 year) obtained by Lehmicke et al. (2000); [c] data collected before the operation of extraction system, except that data near source were from the earliest date available (August 2001); [d] data collected in May or December of 1997 before source removal; [e] rate estimate obtained by Parsons (Tinker Air Force Base, 1999); [f] data obtained by Ei et al. (2002).

agreement between the rate constants extracted using BIOCHLOR and rate constants previously extracted by Ei et al. (2002) using a transect approach or well-to-well comparisons (Table 4.3).

Relationship between *Dehalococcoides* DNA and Dechlorination Rates at Conventional Plumes

Dehalococcoides DNA was measured and determined to be present in 24 contaminated wells at six sites where the contamination in ground water formed conventional plumes. In nine wells at three of the sites, *Dehalococcoides* DNA was unequivocally detected. Table 4.4 compares the concentration of *Dehalococcoides* DNA as determined by the semi-quantitative PCR test using gel electrophoresis (Gene-Trac Test), and the concentration determined by quantitative real time PCR, to the concentrations of chlorinated ethylenes and ethylene in individual wells at the three sites.

Figure 4.3 compares the locations of the monitoring wells at the Western Processing Site in Kent, Washington, to the distribution of *cis*-DCE in the plume in 1994. At the Western Processing Site, reductive dechlorination was essentially complete when the wells were sampled for *Dehalococcoides* DNA in 2003 (Table 4.4). Wells at the original source area of the plume and a well down gradient in the plume had detectable concentrations of ethylene (Table 4.4). One of the source area wells and the down gradient well had detectable concentrations of vinyl chloride.

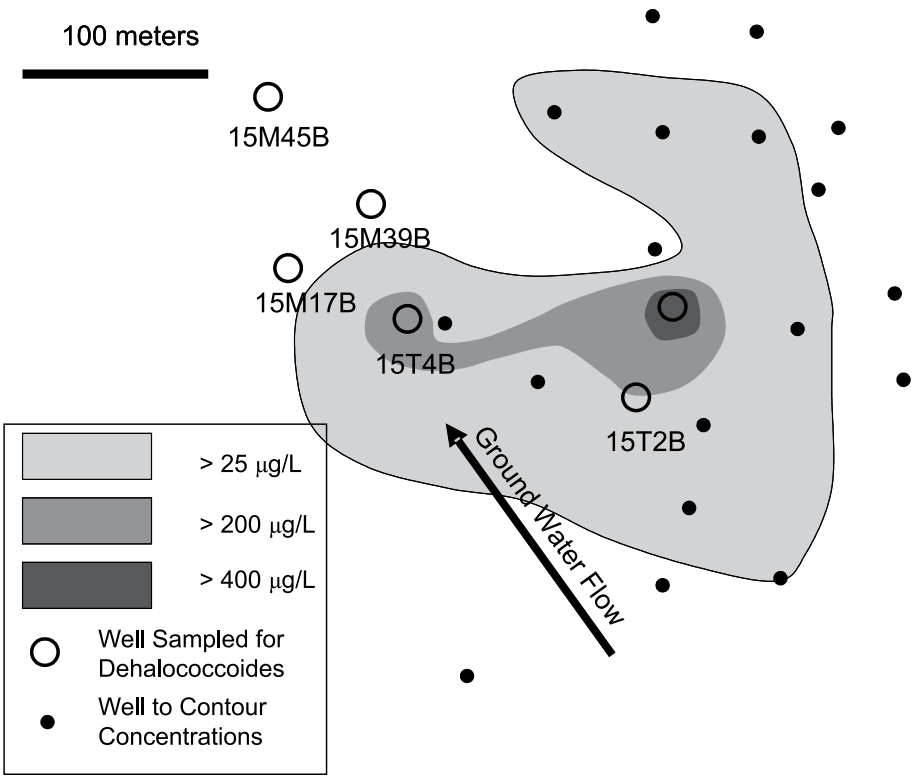

Figure 4.3. *Location of monitoring wells and distribution of cis-DCE at the Western Processing Site in Kent, Washington, fourth quarter 1994.*

The wells in the former source area and the down gradient well had intermediate concentrations of *Dehalococcoides* DNA based on the PCR assay with gel electrophoresis, and concentrations of *Dehalococcoides* cells ranging from 10^5 to 10^7 per liter based on quantitative real time PCR. Ethylene, vinyl chloride, and *Dehalococcoides* DNA were not detected in the three background wells that had never experienced the contaminants.

Figure 4.4 compares the location of the monitoring wells at the Landfill 3 Site at Tinker AFB, Oklahoma, to the distribution of *cis*-DCE in 2000. Four of the wells were originally considered to be in the plume (wells 2-259B, 83BR, 2-299B, and 2-292B), and well 2-304B was originally considered to be a background well. Dechlorination was less extensive than at the Western Processing Site, but the concentrations of chlorinated ethylenes were reduced to a major extent along the flow path (Table 4.4). When sampled as part of this survey, the background well 2-304B had low concentrations of *cis*-DCE and vinyl chloride. *Dehalococcoides* DNA was detected in all the wells, including the background well. The estimated cell density varied from 10^8 per liter in the most contaminated well at the source to 10^6 per liter in the down gradient wells (Table 4.4).

Figure 4.5 compares the distribution of wells at the North Beach Site at the U.S. Coast Guard Support Center at Elizabeth City, North Carolina, to the distribution of PCE in 1997, before excavation of a portion of the source. In 1996 and 1997, the concentrations of PCE in the most contaminated well (GW 3-30 in Figure 4.5) varied from 2,000 µg/liter to 2,900 µg/liter (data not shown). When the plume was sampled in 2002 for *Dehalococcoides* DNA, the concentration of PCE in well GW 3-30 was 561 µg/liter (Table 4.4).

Dechlorination at the North Beach Site was less extensive than at the Western Processing Site or the Landfill 3 Site (Table 4.4). Vinyl chloride was only detected in one well, and ethylene was not detected in any of the wells. The well where vinyl chloride was detected (MW1) was down gradient of the well in the source area. *Dehalococcoides* DNA was only detected in the down gradient well where vinyl chloride was detected.

Table 4.4. Distribution of Chlorinated Ethylenes and *Dehalococcoides* DNA at Sites that Form Conventional Plumes

Well	Position	PCE	TCE	*cis*-DCE	Vinyl Chloride	Ethylene	DNA Score*	Cell Density
		µg/L						cells/L
Western Processing Site 4/29/2003								
15T2B	Source	<1	<1	<1	<MDL	8	++ (63%)	2.3×10^6
6M6B	Source	<1	<1	<1	<1	3	++ (54%)	3.4×10^7
15T4B	Down Gradient	<1	<1	<1	1.6	6	+ (25%)	2.5×10^5
15M17B	Back-ground	<1	<1	<1	<1	<1	-	
15M39B	Back-ground	<1	<1	<1	<1	<1	-	
15M45B	Back-ground	<1	<1	<1	<1	<1	-	
Landfill 3 Site, Tinker AFB, Oklahoma 11/27/2002								
2-259B	Source	7.95	28.4	28,400	20,400	5.3	++ (46%)	2.0×10^8
83BR	Mid-point	0.52	267	13.1	4.0	<1	++++ (158%)	2.7×10^6
2-299B	Down Gradient	0.42	234	0.75	<1	<1	+ (24%)	4.8×10^6
2-292B	Down Gradient	0.43	224	0.97	0.68	<1	+ (21%)	1.3×10^6
2-304B	Back-ground	ND	0.48	0.47	<1	<1	+ (15%)	6.7×10^5
North Beach Site, USCG Support Center, Elizabeth City, North Carolina 10/22/2002								
GM 3-30	Source	561	52.1	25.3	<1	<1	-	
GP23D	Mid- Point	397	74.3	27.9	<1	<1	-	
MW6	Down Gradient	208	18	14.5	<1	<1	-	
MW1	Down Gradient	382	80.5	79.3	12	<1	++++ (166%)	1.8×10^5
MW5	Back-ground	<1	<1	<1	<1	<1	-	

** maximum score detected by any of the Dehalococcoides primers. 0% of positive control = (-), <3% = (+/-), 4% to 33% = (+), 34% to 66% = (++), 67% to 100% = (+++), >100% of positive control = (++++).*

Table 4.5 shows the relationship between the apparent rates of dechlorination along the flow path and the detection of bacterial DNA and *Dehalococcoides* DNA in water samples from a monitoring well in the plume. At sites where *Dehalococcoides* DNA was detected in at least one monitoring well (Western Processing, LF3, and North Beach), the dechlorination rates of *cis*-DCE and vinyl chloride were equal to 0.3 per year, or were greater than 0.3 per year.

"Generally useful" rates of dechlorination also occurred in a plume where *Dehalococcoides* DNA was not detected. In the lower plume at Target Area 1, the extracted rate of attenuation of vinyl chloride was 0.3 per year, but *Dehalococcoides* DNA was not detected (Table 4.5). Bacterial DNA was detected in water from this plume even though *Dehalcoccoides* DNA was not detected.

The SS-17 Site was oxic, and reductive dechlorination was not expected. This site was included in the survey as a control. The extracted rate constant was very low, 0.01 per year. As would be expected, *Dehalococcoides* DNA was not detected.

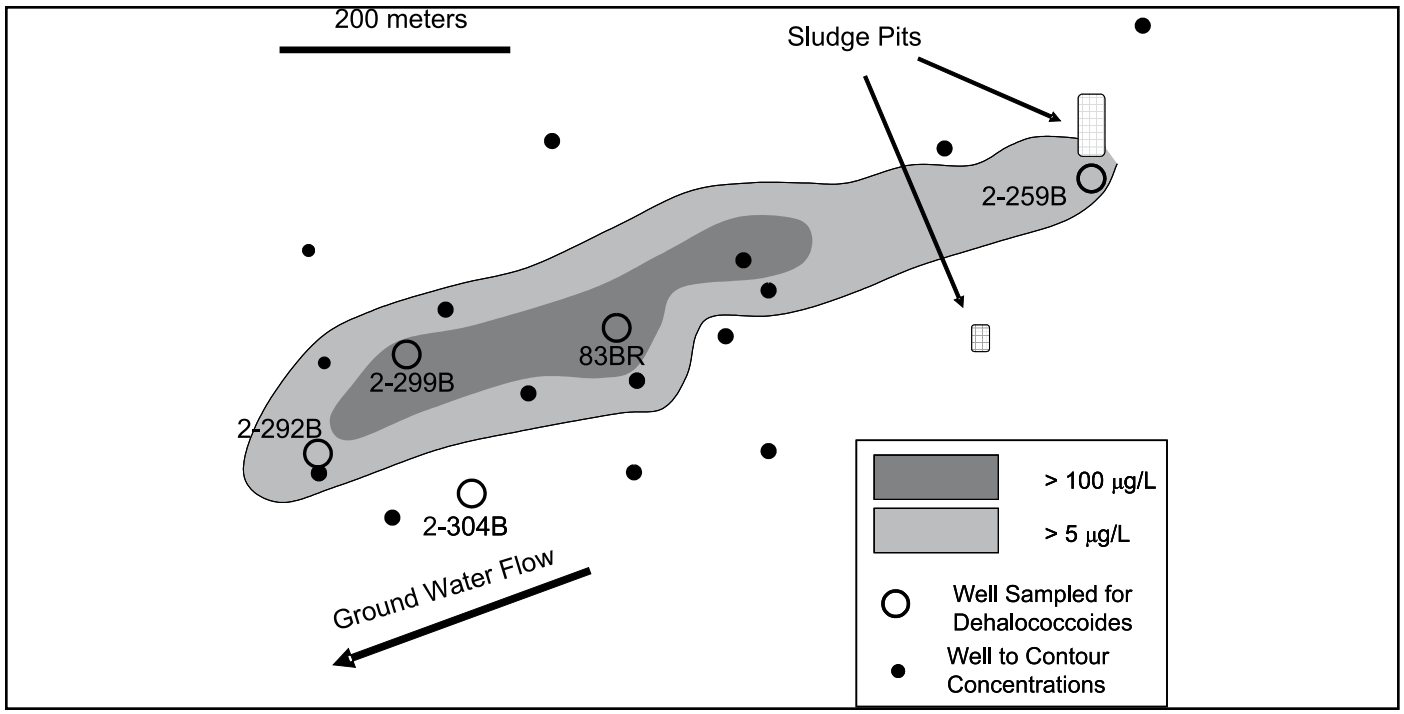

Figure 4.4. *Location of monitoring wells and distribution of cis-DCE in the Upper Saturated Zone Aquifer at the Landfill 3 Site, Tinker AFB, OK, in 2000.*

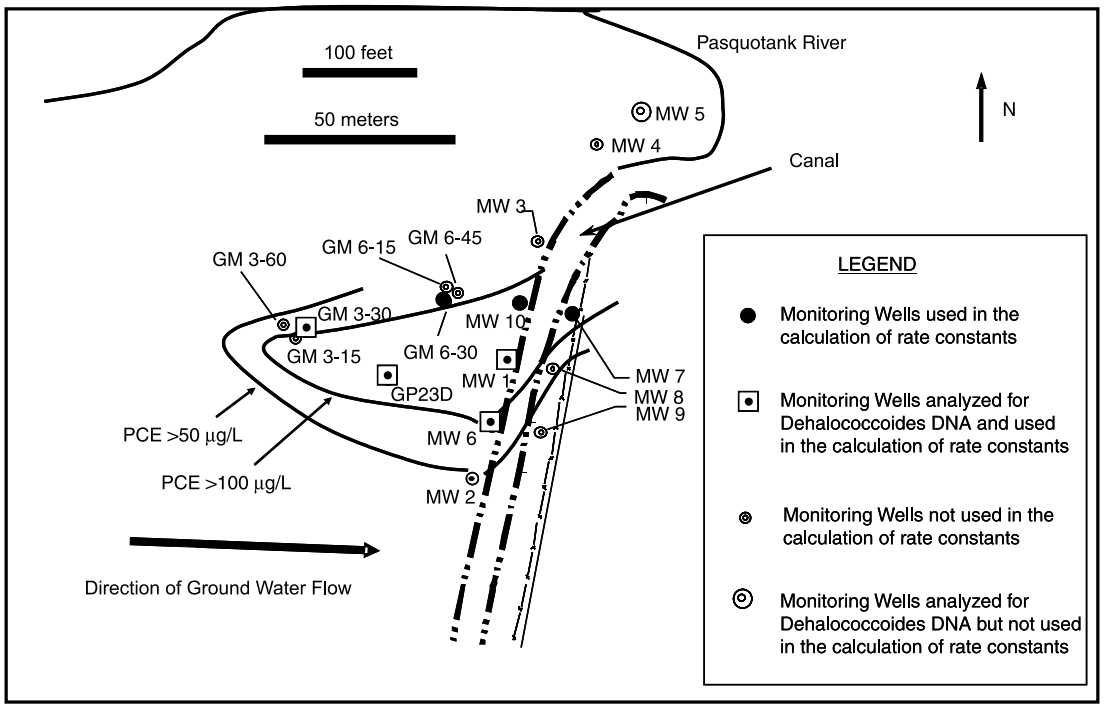

Figure 4.5. *Comparison of the locations of monitoring wells at the North Beach Site at the U.S. Coast Guard Support Center in Elizabeth City, North Carolina, to the distribution of PCE in ground water.*

Table 4.5. Relationship between the Apparent Rates of Dechlorination along a Flow Path in the Aquifer and the Detection of Bacterial DNA and *Dehalococcoides* DNA in Water Samples from a Monitoring Well in the Plume

Facility/ Location	Date	PCE	TCE	*cis*-DCE	VC	Bacterial DNA Primer	Dehalococcoides DNA	
		Rate of Dechlorination (per year)					Primers Detected	Maximum Score*
Western Processing Kent, WA	1988 [a]			0.6	3			
	4/2000					Not Used		+++ [b]
	4/2003					Not Used	3 of 3	++
LF3 (landfill) Tinker AFB, OK	9/1997 [c]			1	3			
	11/2002			1	3	Not Used	3 of 3	++++
North Beach, USCG Support Center Elizabeth City, NC	1997 [d]	0.3	1	1	3			
	10/2002	0.1	1	0.3	1	Not Used	2 of 3	++++
FTA2 (fire training) Tinker AFB, OK	8/1997		0.1 [e]					
	11/2002		0.3			Detected	0	
SS17 Site Altus AFB, OK	3/2003		0.01			Trace	0	
Target Area 1 (Lower Plume) Dover AFB, DE	7/1997	0.1	0.1	0.1	0.3	Detected	0	

** maximum score detected by any of the Dehalococcoides primers. 0% of positive control = (-), <3% = (+/-), 4% to 33% = (+), 34% to 66% = (++), 67% to 100% = (+++), >100% of positive control = (++++).*

[a] data collected before the operation of pump and treat system; [b] data obtained by Hendrickson et al. (2002, personal communication); [c] data collected before the operation of extraction system, except that data near source were from the earliest date available (August 2001); [d] data collected in May or December of 1997 before source removal; [e] rate estimate obtained by Parsons (Tinker Air Force Base, 1999); [f] data obtained by Ei et al. (2002).

Dehalococcoides DNA and Dechlorination Rates over Time in Particular Wells

At the two sites at England Air Force Base, there was no detectable net direction of ground water flow. The site is in the floodplain of the Red River, and the direction of flow varies widely with the seasons. The direction of flow also varies with the elevation of water in a body of surface water (Le Tig Bayou) that lies above the contaminated ground water. The distribution of contaminant concentrations forms a "bull's eye" pattern around the two sources of contamination. Figure 4.6 compares the location of water wells in two separate plumes to the distribution of *cis*-DCE in March, 2002. Wells at one site (Area 2500) showed extensive dechlorination, and wells at the other site (Area 800) showed very limited dechlorination (Table 4.6). However, *Dehalococcoides* DNA was detected in both plumes.

Table 4.7 compares the rates of attenuation over time in individual wells to the concentrations of chlorinated ethylenes. Two values are entered under "Date Collected" in Table 4.7. The first date is the earliest date in the data set used to extract rate constants. The second date is the last date used to extract rate constants and the date that the samples were collected to assay for *Dehalococcoides* DNA. In many wells, it was not possible to calibrate BIOCHLOR and extract rate constant because the chlorinated ethylene was absent, or because concentrations increased over time. When it was possible to extract rate constants, there was not a consistent relationship between the concentrations of chlorinated ethylenes in ground water and the rates of dechlorination over time that were extracted by calibrating BIOCHLOR to the monitoring data (Table 4.7).

Seven wells were sampled for analysis of *Dehalococcoides* DNA (Tables 4.7 and 4.8). There was also no consistent relationship between the presence of *Dehalococcoides* DNA and the trend in concentrations of chlorinated ethylenes over time (Table 4.8). Well A39L010PZ at Area 2500 behaved like the wells in the conventional plumes discussed above. *Dehalococcoides* DNA was present, and the dechlorination rates of *cis*-DCE and vinyl chloride were both 0.3 per year. In contrast, Well A39L011PZ at Area 2500 contained *Dehalococcoides* DNA, but the concentration of vinyl chloride increased over time rather than decreased. Well A39L009PZ at Area 800 contained *Dehalococcoides* DNA, but the rate of degradation of TCE and *cis*-DCE was very slow, and Well #23 at Area 2500 contained *Dehalococcoides* DNA, but the concentrations of both *cis*-DCE and vinyl chloride were increasing over time.

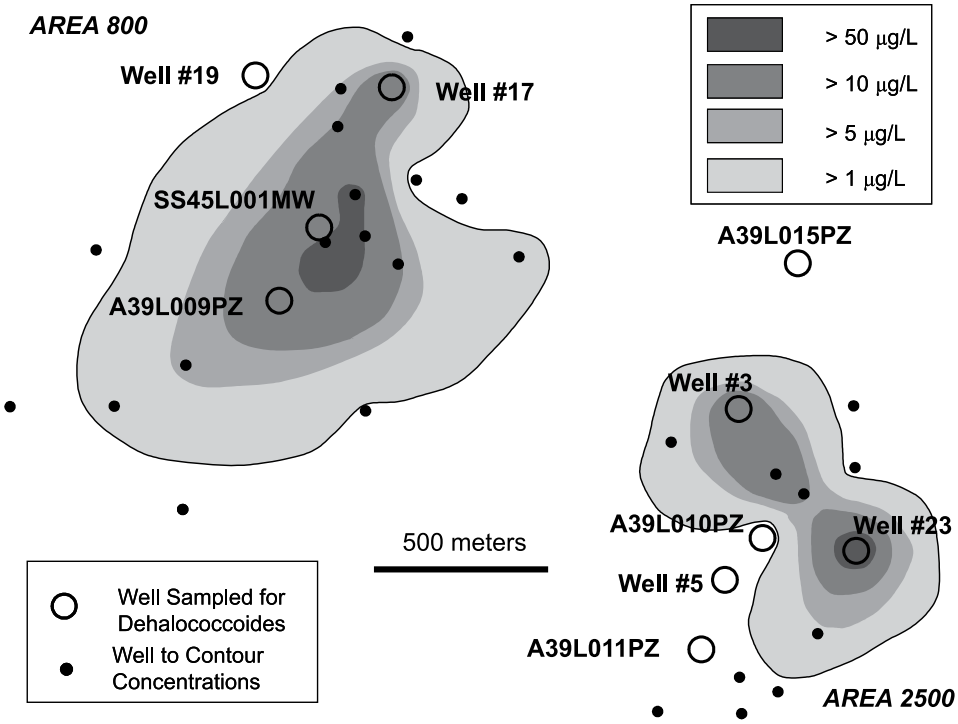

Figure 4.6. *Location of monitoring wells and distribution of cis-DCE in the Intermediate Ground –Water Zone at Spill Site-4, the former England AFB, Louisiana, March 2002.*

Table 4.6. Distribution of Chlorinated Ethylenes and *Dehalococcoides* DNA at Sites at England AFB, which do not Form Conventional Plumes

Well	PCE	TCE	*cis*-DCE	Vinyl Chloride	Ethylene	DNA Score*	Cell Density
	µg/L						cells/L
Area 800							
SS45L001MW	<1	249	126	4.46	0.02	-	
A39L009PZ	<1	20.8	52.6	5.21	<1	+ (10%)	6.7×10^5
WELL # 17	<1	20.5	30.1	3.88	<1	-	
WELL # 19	<1	<1	<1	3.85	<1	-	
Area 2500							
WELL # 005	<1	<1	0.58	166	0.014	-	
A39L011PZ	<1	<1	<1	102	0.02	+++ (88%)	4.0×10^6
A39L010PZ	<1	<1	<1	95.5	0.895	++ (37%)	1.2×10^6
WELL # 23	<1	<1	92	60.6	<1	+ (30%)	4.8×10^5
WELL #3	1.3	<1	25.6	39.1	0.007	-	
A39L015PZ	<1	<1	<1	0.395	<1	-	

** maximum score detected by any of the Dehalococcoides primers. 0% of positive control = (-), <3% = (+/-), 4% to 33% = (+), 34% to 66% = (++), 67% to 100% = (+++), >100% of positive control = (++++).*

Table 4.7. Relationship between the Concentrations of Chlorinated Ethylenes in Ground Water and Their Apparent Rates of Dechlorination over Time in Water Samples from Monitoring Wells at England AFB, Louisiana

Location	Well	Date Collected	TCE	*cis*-DCE	VC	TCE	*cis*-DCE	VC
			Concentration (µg/L)			Rate of Dechlorination (per year)		
Area 2500	A39L011PZ	9/1997 4/2003	<1 <1	189 <1	59.2 102	No TCE	1	VC ↑
Area 2500	A39L010PZ	6/1997 4/2003	<1 <1	9.5 <1	549 95.5		0.3	0.3
Area 2500	Well #23	5/2000 4/2003	<1 <1	<1 92	<1 60.6		*cis*-DCE ↑	VC↑
Area 800	A39L009PZ	6/1997 4/2003	35.8 20.8	80.3 52.6	<1 5.2	0.1	0.1	1
Area 800		6/1997 4/2003	451 249	4.3 126	0.5 4.5	0.1	1	3
Area 800	Well #17	3/1999 4/2003	9.8 20.5	<1 30.1	<1 3.9	TCE ↑	*cis*-DCE ↑	VC ↑
Area 800	Well #19	3/1999 4/2003	<1 <1	<1 <1	<1 3.8	No TCE	No *cis*-DCE	VC ↑

Table 4.8. Relationship between the Apparent Rates of Dechlorination over Time and the Detection of Bacterial DNA and *Dehalococcoides* DNA in Water Samples from Monitoring Wells at England AFB, Louisiana

Plume	Well	Date Collected	TCE	*cis*-DCE	VC	Bacterial DNA Primer	*Dehalococcoides* DNA	
			Rate of Dechlorination (per year)					Cell Density cells/L
Area 2500	A39L011PZ	9/1997 4/2003	No TCE	1	VC↑	Not Used	+++ 3 of 3	4.0×10^6
Area 2500	A39L010PZ	6/1997 4/2003		0.3	0.3	Not Used	++ 3 of 3	1.2×10^6
Area 2500	Well #23	5/2000 4/2003		*cis*-DCE ↑	VC↑	Not Used	+ 2 of 3	4.8×10^5
Area 800	A39L009PZ	6/1997 4/2003	0.1	0.1	1	Not Used	+ 1 of 3	6.7×10^5
Area 800		6/1997 4/2003	0.1	1	3	Detected	0	
Area 800	Well #17	3/1999 4/2003	TCE ↑	*cis*-DCE ↑	VC↑	Detected	0	
Area 800	Well #19	3/1999 4/2003	No TCE	No *cis*-DCE	VC↑	Detected	0	

** maximum score detected by any of the Dehalococcoides primers. 0% of positive control = (-), <3% = (+/-), 4% to 33% = (+), 34% to 66% = (++), 67% to 100% = (+++), >100% of positive control = (++++).*

Again, "generally useful" rates of dechlorination also occurred in some wells where *Dehalococcoides* DNA was not detected. In Well SS45L001MW at Area 800, the dechlorination rates of *cis*-DCE and vinyl chloride were rapid (1 and 3 per year, respectively), but no *Dehalococcoides* DNA was detected even though bacterial DNA was detected. This can be considered a false negative for the PCR assay. The assay may have failed to detect *Dehalococcoides* bacteria that were present in the aquifer, or the transformation was carried out by a strain of *Dehalococcoides* bacteria that was not recognized by the PCR primer, or the transformation may have been carried out by other bacteria all together.

As would be expected, *Dehalococcoides* DNA was absent in oxygenated ground water where chlorinated ethylene concentrations were for the most part below detection limits (Well #19 at area 800).

The rate of attenuation of concentrations of chlorinated ethylenes over time in monitoring wells is strongly influenced by the rate of physical and chemical weathering of the residual contamination in the source area of the plume (Newell et al., 2002). This residual can be TCE present as a non-aqueous phase liquid, or TCE that is sorbed to aquifer material. Apparently, the concentration of chlorinated ethylenes in the wells at England AFB was controlled by the rate of dissolution or desorption of the chlorinated ethylenes from the source material, and not by biotransformation of the chlorinated ethylenes dissolved in ground water. When the concentrations of chlorinated ethylenes are controlled by dissolution and sorption, a PCR assay for *Dehalococcoides* DNA should not be expected to predict the rate of attenuation in concentration over time. The PCR assay is not useful to predict the rate of natural attenuation in the source areas of plumes.

Rates of Natural Attenuation and Density of PCR Products from *Dehalococcoides* DNA

This section compares the rates of natural attenuation at field scale to the density of PCR products from *Dehalococcoides* DNA in monitoring wells. The comparison indicates that samples of ground water from wells do not adequately represent the density of *Dehalococcoides* bacteria in aquifers.

For a number of reasons, any association between *Dehalococcoides* DNA and rates of natural attenuation at field scale must be purely empirical. As discussed in the sections above, *Dehalococcoides* cells may be present in the aquifer, but not sampled in the ground water produced by a monitoring well. *Dehalococcoides* cells may be present in the aquifer, but they may be dead or inactive. *Dehalococcoides* DNA for the 16S rRNA gene may be extracted from stains of *Dehalococcoides* that are not capable of biological transformation of *cis*-DCE or vinyl chloride.

To evaluate the effects of any sampling bias caused by sampling monitoring wells as opposed to sampling the aquifer sediment, the density of *Dehalococcoides* DNA in monitoring wells and rates of attenuation at field scale were compared to rates in a field study of active anaerobic bioremediation (Lendvay et al., 2003) and the rates in a laboratory culture of *Dehalococcoides* (Cupples et al., 2004b). Lendvay et al. (2003) compared the density of *Dehalococcoides* DNA in sediment as measured by the quantitative real time polymerase chain reaction (q-PCR) to rates of removal of chlorinated ethylenes during in-situ bioremediation of a PCE plume. In a demonstration plot that was inoculated with a *Dehalococcoides*-containing culture, the initial concentration of *cis*-DCE was near 970 µg/L. This concentration is higher than the half saturation constant of 320 µg/L determined for the Victoria culture of *Dehalococcoides* (Cupples et al., 2004a). However, the removal of cis-DCE in the data presented by Lendvay is roughly first order on concentration. The plume of PCE was converted to a plume that was almost entirely *cis*-DCE in 8 days, and then 36% of the *cis*-DCE plume was converted to ethylene in an additional 12 days, 70% was converted to ethylene in 28 days, and 92% was converted to ethylene in 35 days. This corresponds to pseudo-first order rate constants for degradation of *cis*-DCE of 13.6, 15.7, and 26.3 per year, respectively. The average rate was near 19 per year.

Lendvay et al. (2003) acquired sediment samples from three depths in the center of demonstration plot (the injection zone) and from three depths near the extraction wells at the periphery of the demonstration plot. Averaged across the six samples, the aquifer sediment in the plot that was inoculated contained 8 x 10^5 *Dehalococcoides* cells per gram wet sediment. If the sediment contained 0.11 ml of pore water per gram wet weight (water filled porosity 25%), the density of *Dehalococcoides* DNA in the sediment corresponds to 7 x 10^9 cells in or exposed to a liter of pore water.

Substrate was supplied to a control plot, but the ground water was not inoculated with *Dehalococcoides* organisms. However, the aquifer contained native strains of *Dehalococcoides,* and *cis*-DCE was degraded to ethylene. The initial concentration of *cis*-DCE was near 1,500 µg/L. Transformation of cis-DCE began after a period of acclimation lasting 87 days. There was 27% conversion of *cis*-DCE to ethylene in the subsequent 27 days and 76% conversion in the subsequent 34 days, corresponding to first order rates of 8.2 and 15.3 per year. The average rate was near 12 per year. The aquifer sediment in the plot that was not inoculated contained on average 3.6 x 10^4 *Dehalococcoides* cells per gram which corresponds to 3.2 x 10^8 cells in or exposed to a liter of pore water.

The first order rates of attenuation of *cis*-DCE are compared to the density of *Dehalococcoides* cells in Figure 4.7. There was a little more than one-order of magnitude difference in the density of *Dehalococcoides* cells that achieved a rate of degradation near 20 per year in the in-situ bioremediation study of Lendvay et al. (2003). This is reasonably good agreement between two independent field-scale estimates of the effect of the density of *Dehalococcoides* cells on the rate of transformation of *cis*-DCE in the aquifer. This estimate will be compared to the rate of natural biotransformation at the natural attenuation sites. It is important to note that the estimate is for only two pilot-scale plots at one site. We could not find another study in the literature that compared the rates of biodegradation of *cis*-DCE to the density of *Dehalococcoides* cells in the sediment. Until other studies are reported, there is no benchmark to determine whether the rates achieved by Lendvay et al. (2003) are typical.

If the rate of biotransformation of *cis*-DCE by an individual cell of *Dehalococcoides* is pseudo-first order on concentration, then the overall rate of transformation should be proportional to the number of active cells. Two cells would degrade *cis*-DCE twice as fast as one cell, and so on. The grey shape in Figure 4.7 bounds the area where the rate of biotransformation is proportional to the cell density in the plot that was bioaugmented with an active culture of *Dehalococ-*

coides, and the plot that relied on the native strains of *Dehalococcoides*. The density of *Dehalococcoides* cells in two of the monitoring wells at the MNA sites fell within the grey shape, indicating that the performance of the native *Dehalococcoides* stains in the plumes undergoing natural attenuation was roughly equivalent to the performance of the *Dehalococcoides* stains at the active in-situ bioremediation plots. Based on the concentration of cells in the water from the monitoring wells, the performance in the other wells was substantially better than in the bioremediation demonstration plots. The rates were up to one order of magnitude faster than would be expected from the density of *Dehalococcoides* cells.

The solid square in Figure 4.7 is the first order rate of transformation of *cis*-DCE by a laboratory culture of *Dehalococcoides* strain VS (Cupples et al., 2004b). The culture was growing at 20° C with optimal concentrations of molecular hydrogen. The first order rate of *cis*-DCE transformation was calculated by dividing the maximum rate of transformation of *cis*-DCE by the half saturation constant for *cis*-DCE transformation. The dotted line in Figure 4.7 extrapolates the performance of the laboratory culture to cell densities that were determined by quantitative PCR in monitoring wells at the study sites. Based on the concentration of *Dehalococcoides* cells in ground water from monitoring wells, the *Dehalococcoides* organisms in the aquifer at the field sites performed as well or better than the culture growing under optimal conditions in the laboratory.

It is possible, but unlikely, that the native *Dehalococcoides* organisms at our survey sites were more efficient than the organisms reported by Lendvay et al. (2003) and Cupples et al. (2004b). It is possible, but unlikely, that other organisms were primarily responsible for natural attenuation of chlorinated ethylenes at our study sites. The most likely explanation is that the monitoring wells did not efficiently sample the *Dehalococcoides* organisms in the aquifer, and that the number of *Dehalococcoides* cells recovered in a liter of well water was a small fraction of the number of cells that were exposed to a liter of ground water in the aquifer. Most of the *Dehalococcoides* cells were probably attached to sediment particles.

If the relationship between the density of *Dehalococcoides* cells and the rates of *cis*-DCE degradation in

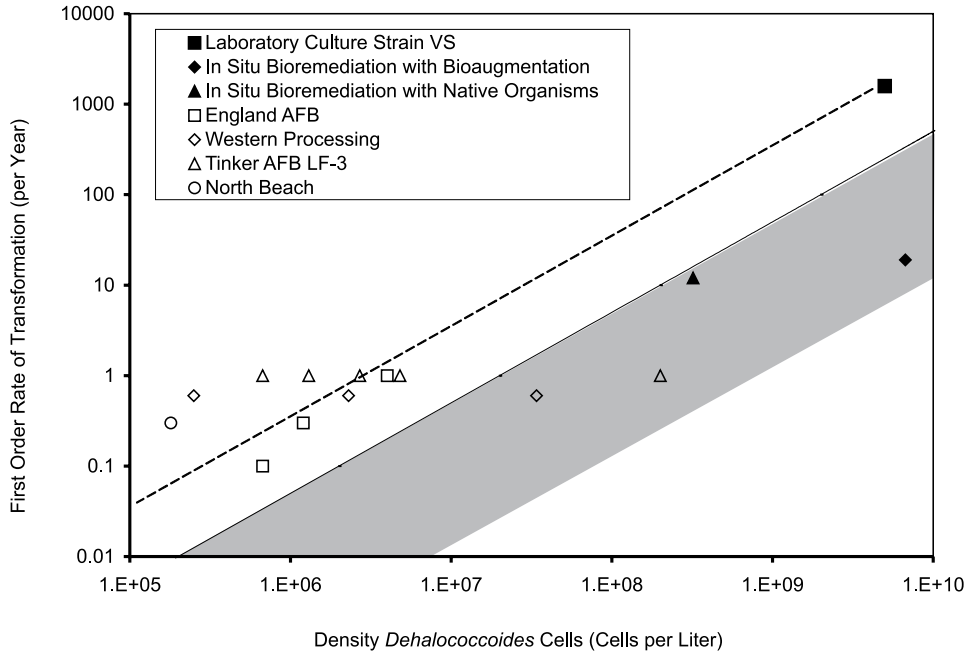

Figure 4.7. *Relationship between the density of Dehalococcoides cells as determined by quantitative PCR and the first order rate of attenuation of cis-DCE in ground water. The data points with an open symbol are from ground water samples collected at natural attenuation sites as presented in Tables 4.3 and 4.6. The data points with a solid diamond symbol or a solid triangle symbol are from sediment samples from a site where biological reductive dechlorination was used to clean up a PCE spill (Lendvay et al., 2003). The data point with a solid square symbol is from a laboratory study of cis-DCE transformation by Dehalococcoides strain VS growing under optimum conditions (Cupples et al., 2004b).*

the bioremediation study of Lendvey et al. (2003) is extrapolated to a "generally useful" rate of bioremediation for natural attenuation of 0.3 per year, then a density of near or greater than 1 x 10^7 *Dehalococcoides* cells per liter is necessary to produce a useful rate of natural attenuation (compare Figure 4.7). Dennis (2005) reported that ethylene as a transformation product was detected in 78% of ground water samples where the density of *Dehalococcoides* cells was greater than 1 x 10^4 cells per liter but less than 9.9 x 10^5 cells per liter. When the density of *Dehalococcoides* cells varied between 1 x 10^6 cells per liter and 9.9 x 10^7 cells per liter, ethylene was detected in 83% of the wells. The detection limit for the quantitative real time PCR assay for *Dehalococcoides* is near 2 x 10^3 cells per liter. The assay can easily detect *Dehalococcoides* cells if they are present at densities that cause the accumulation of ethylene, or that can produce "generally useful" rates of natural attenuation. At its present level of development, the PCR assay has adequate sensitivity. False negative reports from the assay are most likely caused by a failure of a ground water sample to adequately represent the true density of *Dehalococcoides* cells in the aquifer.

In addition to problems associated with sampling bias with water from monitoring wells, the interpretation of the semi-quantitative PCR assay based on gel electrophoresis is further complicated by variability in the estimate of the amplified DNA. Figure 4.8 compares the actual density of *Dehalococcoides* gene copies to the intensity score reported by the semi-quantitative test in Tables 4.4 and 4.6. For a given intensity score, the number of gene copies varied by as much as two orders of magnitude. When the intensity scores were greater than (++), there was no observed increase in the number of *Dehalococcoides* gene copies with an increase in the intensity score. When an intensity score was assigned, the density of *Dehalococcoides* gene copies was uniformly greater than 1 x 10^5 cells per liter. However, there was no quantitative relationship between the different intensity scores.

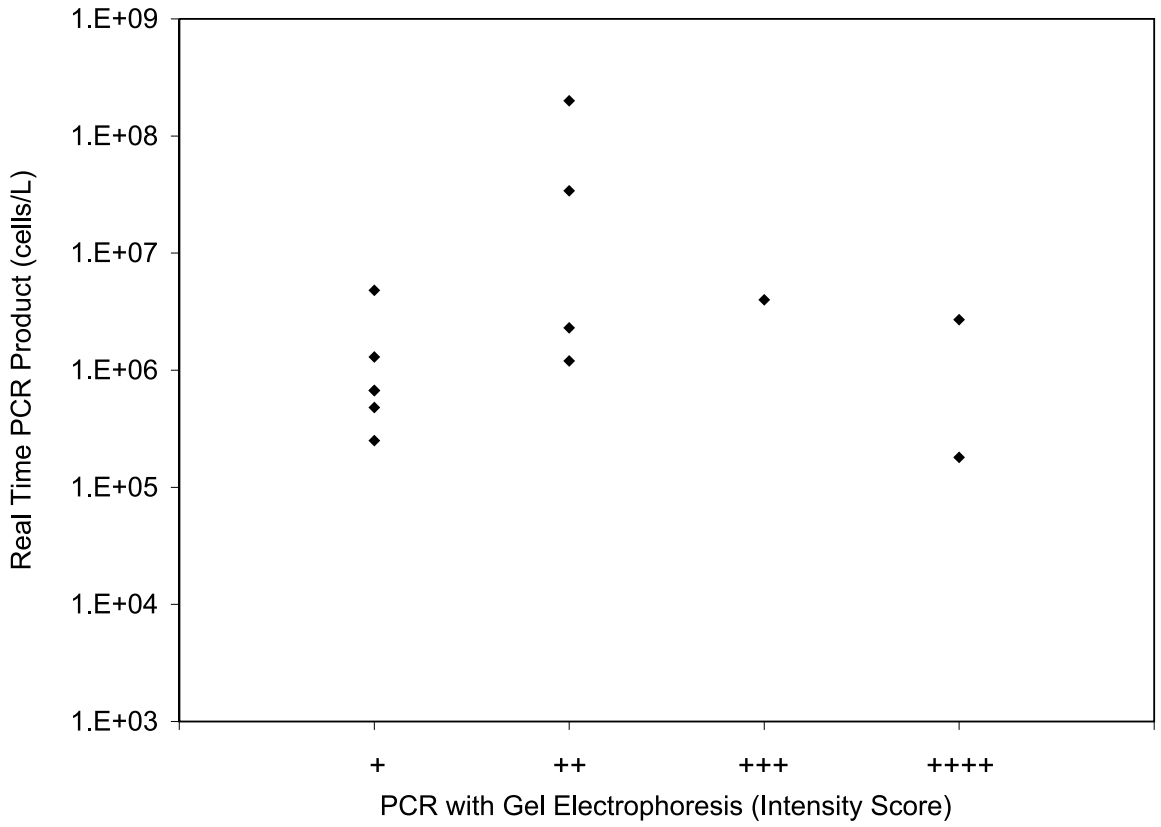

Figure 4.8. *Comparison of the density of Dehalococcoides cells in ground water as determined by quantitative PCR (real time PCR) to the density of Dehalococcoides DNA as determined by the semi-quantitative technique that uses the density of the band produced by Gel Electrophoresis as an estimate of the concentration of amplified DNA.*

Biotransformation and Dominant Terminal Electron Accepting Processes

Table 4.9 compares the geochemistry of the ground water at the studied sites. Based on the concentration of soluble electron acceptors and reduced metabolic products, the ground water at each site was classified and assigned to categories based on the inferred dominant electron accepting process. Most of the water samples fell into more than one category. This may reflect spatial heterogeneity in the aquifer with water from different geochemical environments contributing to the water sampled from the well. It may also reflect concurrent geochemical processes occurring in the aquifer.

The SS-17 site at Altus AFB and the Area 800 site at the former England AFB were included in the survey as controls where reductive dechlorination and the presence of *Dehalococcoides* DNA were not expected. With the exception of well A39L009PZ at the Area 800 site, the water at these two sites is oxygenated, and the concentration of *cis*-DCE and vinyl chloride are a

Table 4.9. Comparison of Rates on Attenuation to the Overall Geochemical Environment of the Sites in the Survey

Site	Rate	Geochemistry[a]	O_2	NO_3+NO_2-N	Fe(II)	SO_4^{-2}	CH_4	H_2
	per year		mg/L					nM
Well SS45L001MW at Area 800	*cis*-DCE 1	oxic, iron reducing	3.8	0.22	2.5	35.7	0.505	-
Well 2-259B at LF3	*cis*-DCE 1	iron reducing	0.2	0.03	9	<0.1	3.1	-
Well T4 at Western Processing	*cis*-DCE 0.6	iron reducing and methanogenic	0.2	0.04	7.5	<0.1	20.7	2.38
Well GM3-30 at North Beach	*cis*-DCE 0.3	sulfate reducing	0.2	<0.1	0.5	59.7	0.060	<0.4
Well MW-5 at North Beach	*cis*-DCE 0.3	sulfate reducing	0.3	<0.1	0.2	5.72	0.055	7.97
Well 2-62B at FTA2 at Tinker AFB	TCE 0.3	nitrate reducing, sulfate reducing, methanogenic	0.1	5.32	-	138	0.938	7.69
Well MW212D at Target Area 1	*cis*-DCE 0.1	nitrate reducing, methanogenic	0.4	2.17	<0.1	0.71	3.26	1.19
Well DM353D at Target Area 1	*cis*-DCE 0.1	no dominant process	0.7	0.53	<0.1	0.36	0.247	10.47
Well A39L009PZ at Area 800	*cis*-DCE 0.1	iron reducing, sulfate reducing	0.1	0.08	7	18.6	0.19	6.54
Well WL080 at SS17 site, Altus AFB	TCE 0.01	oxic or nitrate reducing	0.7	1.58	<0.1	1670	0.003	<0.4
Well #23 at Area 2500	No NA	iron reducing, methanogenic	0.1	0.08	12	<0.1	3.11	2.27

[a] *oxic: dissolved oxygen was greater than 0.5 mg/L; iron-reducing: iron II was greater than 0.5 mg/L; methanogenic: methane was near or greater than 1 mg/L; nitrate-reducing: nitrate plus nitrite –N was greater than 0.5 mg/L, and oxygen was not available; sulfate reducing: oxygen was not available, and the concentration of sulfate was greater than 20 mg/L.*

small fraction of the concentration of TCE. Water in the lower plume at Target Area 1 is reducing in wells near the source (MW212D in Table 4.9), but oxygenated in a down gradient well (DM353D in Table 4.9). Water in the other plumes is iron-reducing, sulfate-reducing, or methanogenic. The rate of attenuation of chlorinated ethylenes in the ground water plumes in this survey could not be correlated with a unique dominant terminal electron accepting process (Table 4.9).

Example of Calibration of BIOCHLOR with Distance along a Flow Path

The plume of PCE at the North Beach Landfill Site at the U.S. Coast Guard Support Center in Elizabeth City, North Carolina, will be used to illustrate the process of extracting rate constants for natural attenuation along a flow path in the aquifer. Figure 4.5 is a map showing the location of monitoring wells in the plume. The plume of contamination is contained in a shallow confined aquifer extending from depths of six to ten meters below land surface. The plume is crossed by a shallow agricultural drainage ditch that does not seem to communicate with the plume.

Monitoring wells (GM3-30, GP23D, GM6-30, MW1, MW6, MW10, and MW7) along the centerline of the plume were used to extract the rate constants (some of the wells including GM3-30, GP23D, MW1, and MW6 were sampled for analysis of *Dehalococcoides* DNA). To calculate the rate constants, site-specific estimates of the hydrological parameters of the aquifer (Wilson et al., 1997) and field data on contaminant concentrations in 2000 were entered into the BIOCHLOR software. Figure 4.9 shows the BIOCHLOR input screen.

The first order rate constants were estimated by calibrating BIOCHLOR to field data following a forward, trial-and-error process until all the predicted concentrations of all the chlorinated ethylenes best matched their field data.

The output of BIOCHLOR compares the measured concentrations of chlorinated ethylenes along a flow path to the expected concentrations along the centerline of the plume. At a real field site, the distribution of contaminants in monitoring wells may not match

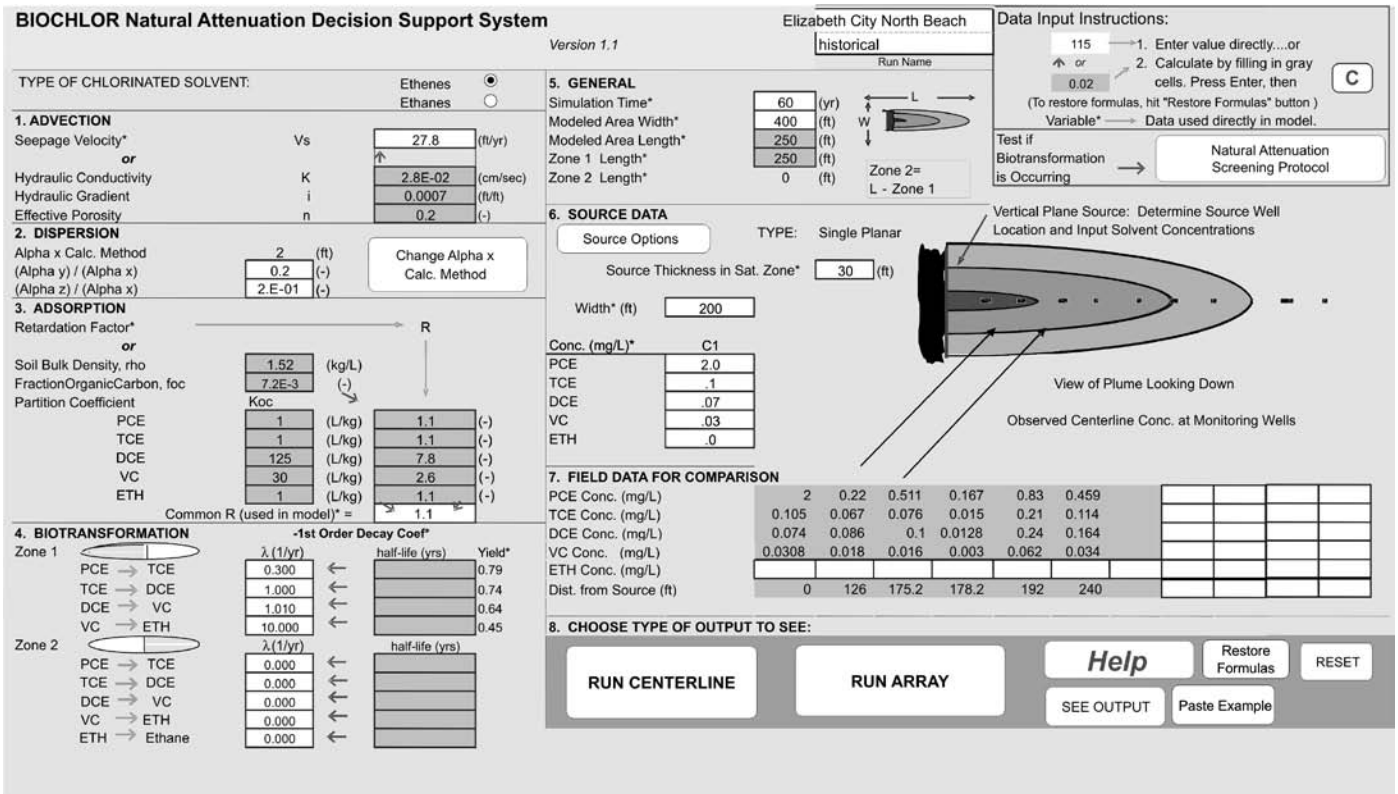

Figure 4.9. *Input screen to BIOCHLOR with calibration parameters for the North Beach Site.*

the assumptions of the mathematical model. The available wells often do not lie along the centerline. The screened intervals of the monitoring wells may not match the vertical distribution of the contaminant in the aquifer. The flow direction of ground water can vary over time, moving a well into or out of a flow path from the source. The concentrations of contaminants in different regions of the source area are usually variable, and different monitoring wells may sample water that originated from different regions in the source area. The actual rates of biotransformation probably vary from one location to the next in an aquifer.

As would be expected, there was scatter in the field data when concentrations were plotted against distance along the inferred flow path (Figure 4.10 and Figure 4.11). A sensitivity analysis revealed that if the rates of transformation varied by a factor of three, it was possible to clearly identify a rate that was the best fit to the data in the calibrations. Calibrations were examined at rate constants of 0.01 per day, 0.03 per day, 0.1 per day, 0.3 per day, 1 per day, 3 per day, and 10 per day. The rate constant producing the best match to the field data for each chlorinated ethylene was selected as the best estimate of the rate.

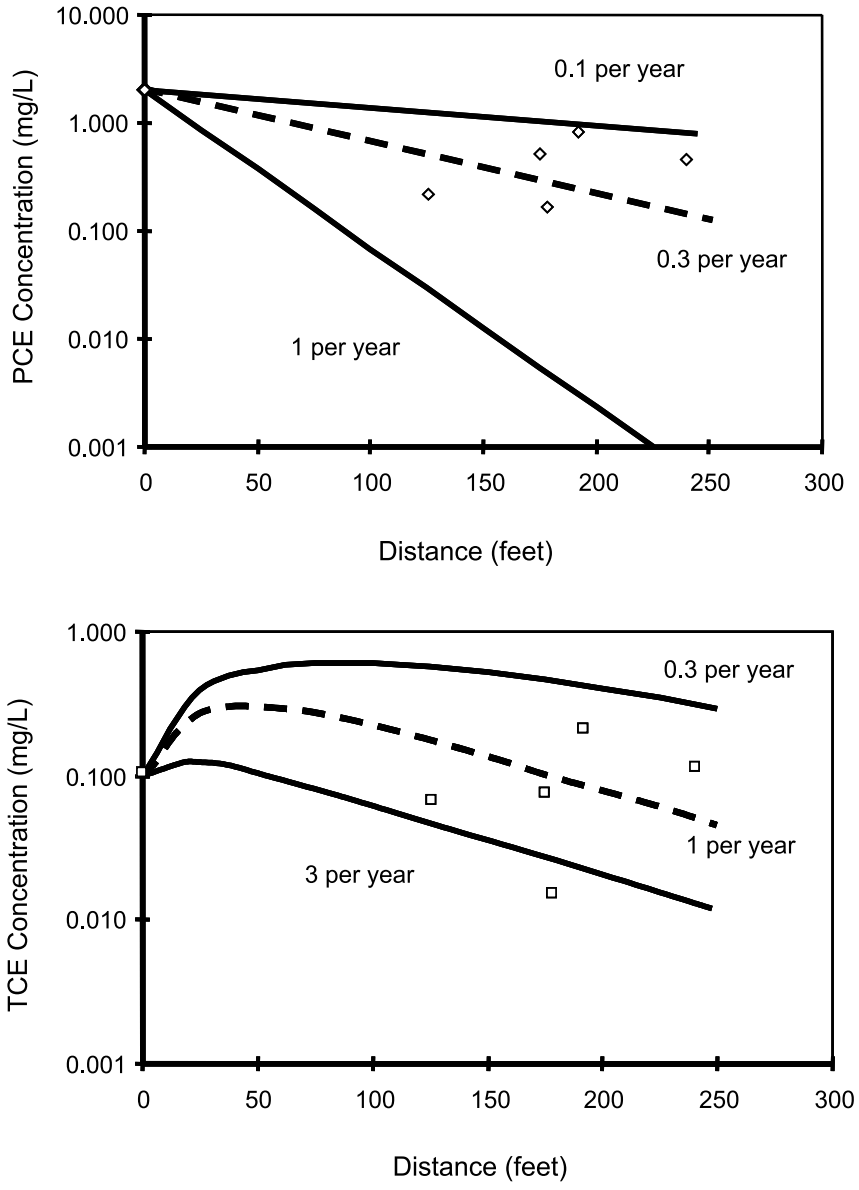

Figure 4.10. *Correspondence between the measured values for PCE and TCE at the North Beach Site in 2002 and the concentrations that were predicted by calibrating BIOCHLOR using three different values for the first order rate constant for biotransformation. The dotted line was considered the best calibration to the field data.*

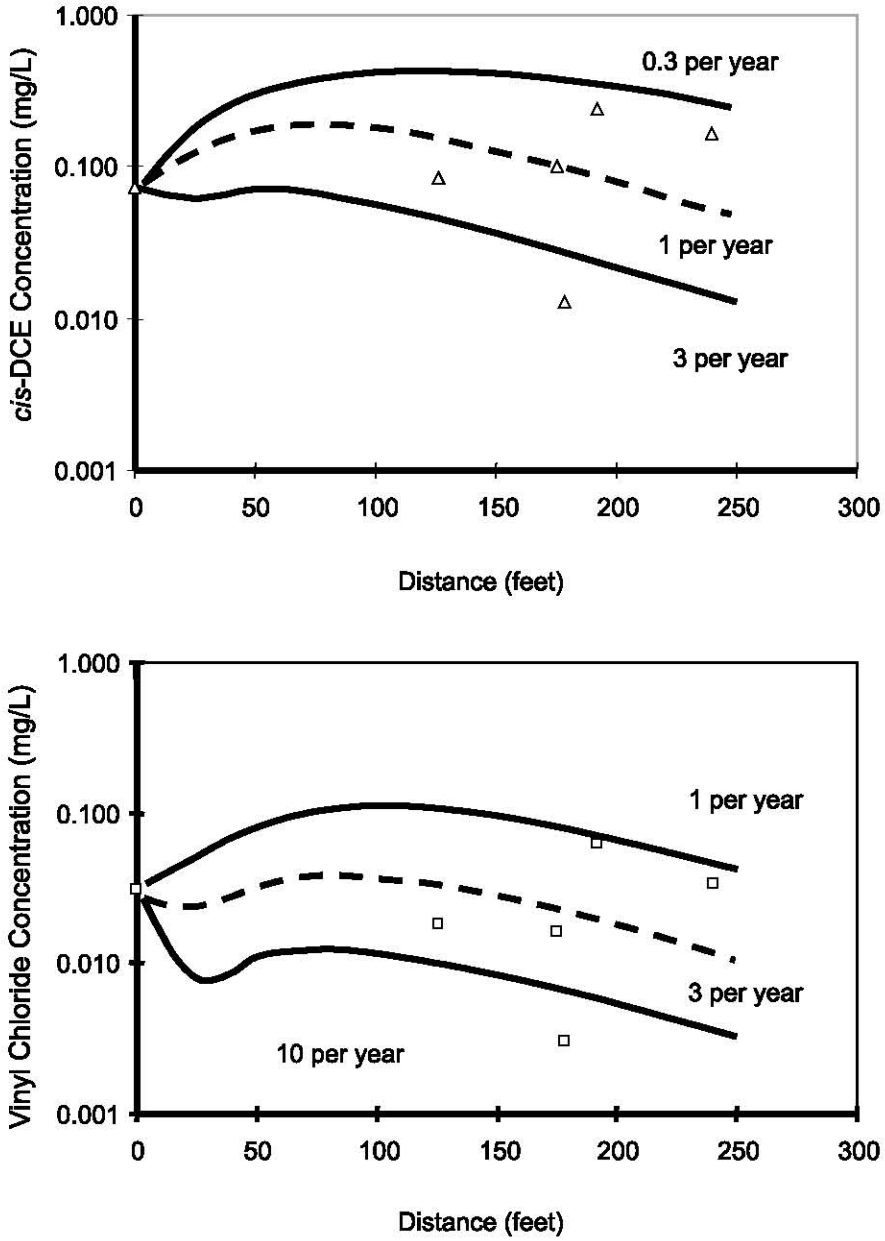

Figure 4.11. *Correspondence between the measured values for cis-DCE and vinyl chloride at the North Beach Site in 2002 and the concentrations that were predicted by calibrating BIOCHLOR using three different values for the first order rate constant for biotransformation. The dotted line was considered the best calibration to the field data.*

Calibration started with PCE, then proceeded to TCE, then to *cis*-DCE, and then to vinyl chloride. Then each calibration was checked again to see if calibration of the down stream transformation products had affected the calibration of the parent chlorinated ethylenes.

Figure 4.10 and Figure 4.11 display the correspondence between the measured concentrations and concentrations predicted by the BIOCHLOR software. The obtained rate constants for reductive dechlorination of PCE, TCE, *cis*-DCE, and vinyl chloride were 0.1, 1, 0.3, and 1 per year, respectively.

Example of Calibration of BIOCHLOR with Time in a Single Monitoring Well

To extract rate constants for natural attenuation over time in a particular well, data from a series of sampling events were used. Data from the first sampling event were used as the source data. Data from other sampling events were arranged in an order according to their time interval to the first sampling date, similar to the way data were arranged according to their distance to the source along a flow path in a plume. Dummy variables for hydrologic properties were inserted into BIOCHLOR to extract the rate constants of dechlorination over time in the particular well, as opposed to over distance along a flow path. Well A39L010PZ at Area 2500 at the former England AFB in Alexandria, Louisiana was used to illustrate the process. Figure 4.12 shows the BIOCHLOR input screen for this well.

The rate constants were estimated by calibrating BIOCHLOR to field data from a series of sampling events following a forward, trial-and-error process until all the predicted concentrations best matched their field data. Figure 4.13 displays the correspondence between the predicted and actual values performed by the BIOCHLOR software. The obtained rate constants for reductive dechlorination of *cis*-DCE and vinyl chloride were both about 0.3 per year.

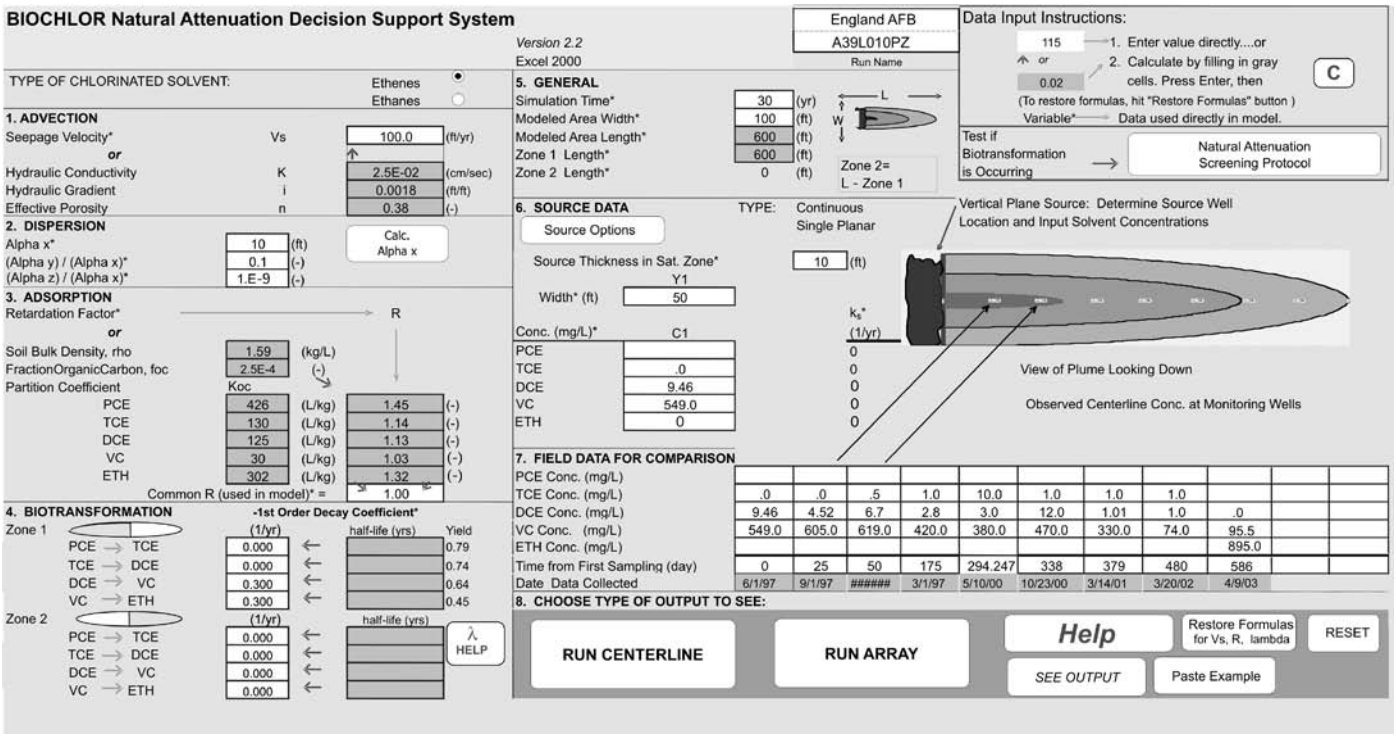

Figure 4.12. *Input screen to BIOCHLOR with calibration parameters for the well A39L010PZ at Area 2500 at England Air Force Base, Alexandria, Louisiana.*

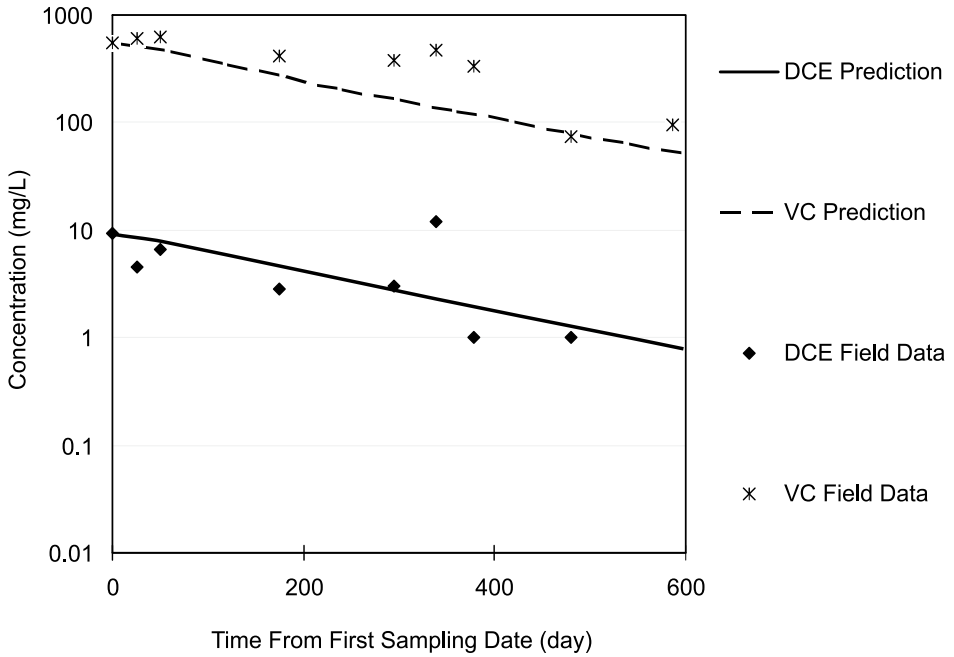

Figure 4.13. *Correspondence between predicted and actual values for the chlorinated ethylenes performed by calibrating BIOCHLOR to field data sampled in different events at the well. A39L010PZ at England Air Force Base, Alexandria, Louisiana.*

Conclusions

The commercially available assay for *Dehalococcoides* DNA had adequate sensitivity to detect concentrations of *Dehalococcoides* DNA that could sustain "generally useful" rates on natural attenuation of *cis*-DCE and vinyl chloride.

The assay is not appropriate in the source area of plumes where the concentrations of chlorinated solvents are controlled by dissolution and desorption of chlorinated solvents from residual contamination. The assay is more appropriate for those areas of a plume outside the source area where the concentrations of chlorinated solvents are controlled by biotransformation of chlorinated solvents already in solution.

The field scale rates of natural attenuation were faster than would be expected from the density of *Dehalococcoides* DNA in ground water samples from monitoring wells, suggesting that monitoring wells failed to adequately sample the *Dehalococcoides* cells in the aquifer. If assays for *Dehalococcoides* DNA are to be used to make quantitative predictions of the rates of natural attenuation, it will generally be necessary to acquire and analyze samples of the aquifer sediment.

Section 5.
Geochemistry of Ground Water and Occurrence of *Dehalococcoides*

Synopsis

The *Technical Protocol for Evaluating Natural Attenuation of Chlorinated Solvents in Ground Water* (Wiedemeier et al., 1998) used a number of geochemical parameters in a scoring system to predict whether ground water could reasonably be expected to contain microorganisms that could cause biotransformation of chlorinated solvents. The scoring system was based on professional judgment. It was not validated against any data set that compared selected geochemical parameters against the presence or absence of microorganisms that might be capable of transformation of chlorinated solvents. The scoring system was criticized by the Committee on Intrinsic Remediation of the National Research Council (NRC, 2000). The Committee recommended that the scoring system should not be used to evaluate prospects for MNA.

Because the *Dehalococcoides* group is the only known group of organisms that can grow by carrying out the reductive dechlorination of DCE or vinyl chloride, it plays a critical role in the evaluation of monitored natural attenuation of chlorinated ethylenes in anaerobic ground water. To determine the association between the presence of *Dehalococcoides* and the critical biogeochemical parameters that define the habitat of these organisms, various contaminated sites across the United States were sampled. This Section compares the distribution of selected geochemical parameters to the distribution of *Dehalococcoides* DNA in ground water at the sites. A commercially available polymerase chain reaction (PCR) assay was used to detect *Dehalococcoides* organisms in the water samples. The presence or absence of *Dehalococcoides* DNA was compared to the values for selected geochemical parameters.

Not every strain of *Dehalococcoides* can dechlorinate every chlorinated ethylene. As discussed in Section 3, many strains cannot dechlorinate the dichloroethylenes or vinyl chloride to ethylene. However, every organism that has been identified that can dechlorinate the dichloroethylenes and vinyl chloride to ethylene is a member of the *Dehalococcoides* group. An assay for the presence of *Dehalococcoides* DNA provides direct evidence for the presence of a strain of bacteria that might be capable of completely dechlorinating chlorinated ethylenes.

Knowledge of the growth of *Dehalococcoides* species is derived primarily from studies in the laboratory. *Dehalococcoides* are strict anaerobes. To date, they are known to use only chlorinated organic compounds as electron acceptors and molecular hydrogen (H_2) as an electron donor. They do not use nitrate, nitrite, fumarate, ferric iron, sulfate, sulfite, thiosulfate, sulfur, or oxygen as electron acceptors (Maymó-Gatell et al., 1997; He et al., 2003a; Adrian et al., 2000). In a contaminated aquifer, multiple terminal electron accepting processes may occur at the same time. In contaminated aquifers, *Dehalococcoides* organisms may have to compete with other H_2-consuming bacteria for the shared electron donors (Lee et al., 1998). Obviously, the growth of *Dehalococcoides* can be influenced by the geochemical environment.

Concentrations of nitrate, methane, and the oxidation/reduction potential were associated with the presence or absence of *Dehalococcoides* DNA with a statistical confidence of 95%. The association of the other geochemical parameters with the presence or absence of *Dehalococcoides* DNA was not significant at 95% confidence. Logistical regression was applied to the data set to develop a formula (equation 5.1) that uses the concentrations of nitrate, methane, and the oxidation/reduction potential to calculate the probability that *Dehalococcoides* is present in ground water.

The formula has several potential applications. A calculated probability would be useful to screen wells at a site in order to decide which wells should be sampled for a PCR assay. As discussed below, it can be used to improve the calibration of computer models of natural attenuation in ground water.

The formula provides a simple and rapid way to calculate the probability that a specific dechlorinating organism (*Dehalococcoides*) is present at the site, as opposed to the scoring system in the *Technical Protocol* (Wiedemeier et al., 1998). However, a calculated

probability that an organism exists in ground water is not equivalent to a PCR assay for its presence, and probabilities calculated from geochemical parameters should not be used to replace PCR assays.

Calibration of Computer Models to Evaluate MNA

Often the computer models that are used to evaluate monitored natural attenuation are distributed parameter models. In these models, different rates of biotransformation can be assigned to each cell of the model, based on the conditions pertaining to that cell. BIOCHLOR (Aziz et al., 2000), a simple screening model for monitored natural attenuation of chlorinated solvents, allows the user to assign two rates of natural biotransformation, depending on the local conditions. To properly calibrate these computer models, it is necessary to know the distribution of the capacity to transform chlorinated ethylenes throughout the aquifer. Data from the PCR assay may not be available from every well at a site. The calculated probability of *Dehalococcoides* organisms based on the geochemistry of ground water in a well could be used to assign rate constants to cells in a model. If *Dehalococcoides* organisms are expected, a rate constant characteristic of reductive dechlorination at that site would be assigned. If *Dehalococcoides* organisms are not expected, the rate constant for reductive dechlorination would be set to zero in that particular region of the aquifer.

Sampling Sites

Samples were collected from fifteen plumes at ten locations across the United States as shown in Figure 5.1 and listed in Table 5.1. The contamination occurred in a variety of geological matrices including consolidated sandstone and siltstone; unconsolidated sandy clay; silty to fine sand; fine to course sand; and sands and gravels. The contamination sources included industrial landfills, airbase fire training areas,

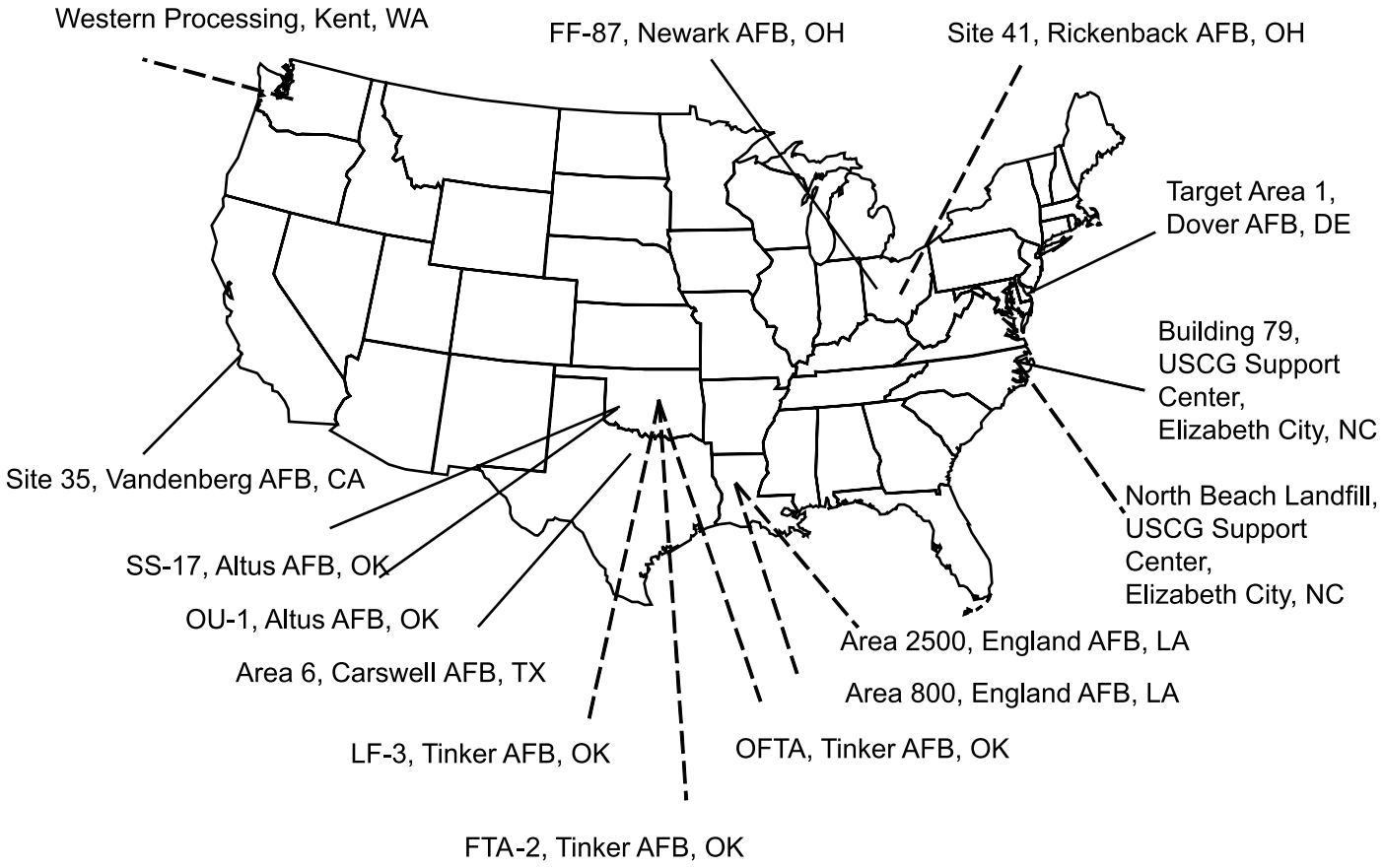

Figure 5.1. *Location of contaminated sites used to compare presence or absence of Dehalococcoides DNA to the geochemistry of the ground water. The dashed lines identify sites under monitored natural attenuation. The solid lines identify sites under active remediation.*

Table 5.1. Location of Sites Included in the Survey Comparing the Presence or Absence of *Dehalococcoides* DNA to the Geochemistry of the Ground Water

Location	Site	Geochemistry[a] Treatment Area	Background Geochemistry	Remedial Technology	No. Wells Sampled
Altus AFB, Oklahoma	SS-17	I,S,M	O,N	Soybean oil, surfactant, yeast extract and lactate	6
	OU-1	I,S,M	O,N	Plant mulch permeable reactive barrier	5
Carswell AFB, TX	Area 6	I,S,M	O,N	Iron permeable reactive barrier	5
Dover AFB, DE	Target Area 1	I,S,M	O,N	Soybean oil injection	8
Vandenberg AFB, CA	Site 35	I,S,M	O,N	Molasses injection	3
USCG Elizabeth City, NC	Building 79	I,S,M	O,N	Iron permeable reactive barrier	11
	North Beach Site	I,S	O,N	MNA	6
England AFB, LA	Area 800	O, N	O,N	MNA	4
	Area 2500	I, M	O	MNA	6
Rickenbacker AFB, OH	Site 41	S, M	S,M	MNA	4
Newark AFB, OH	FF-87	I,S,M	I	Soybean oil injection	3
Tinker AFB, OK	LF-3	O,N,S,M	O	MNA	5
	FTA-2	N,S,M	O,N	MNA	2
	OFTA	O,N	O,N	Chemical oxidation	6
Kent, WA	Western Processing	I,M	I,M	MNA attenuation	6

[a] *O=oxic: dissolved oxygen was greater than 0.5 mg/L; N=nitrate-reducing: nitrate plus nitrite –N was greater than 0.5 mg/L, and oxygen was not available; I=iron-reducing: iron II was greater than 0.5 mg/L; S=sulfate reducing: oxygen was not available, and the concentration of sulfate was greater than 20 mg/L; M=methanogenic: methane was near or greater than 1 mg/L*

surface impoundments for industrial wastes, and accidental spills of solvents. At most sites, the major contaminants were TCE, *cis*-DCE, and vinyl chloride. In three sites (Target Area 1, FF-87, and North Beach), PCE was one of the major contaminants.

A variety of remediation technologies were applied at these sites. An emulsion of soybean oil, surfactant, yeast extract, and lactate was injected into a barrier in the source zone at one site. A permeable reactive barrier composed of shredded tree mulch, cotton gin compost, and sand was installed across a plume of TCE in ground water at another site. Permeable reactive barriers composed of zero-valent iron and sand were constructed across the plumes at two sites. Soybean oils were injected into the plume at two sites. Molasses was injected into a barrier in the source zone at one site. An oxidizing reagent (a mixture of potassium permanganate and Fenton's reagent) was injected into the plume at one site. Seven other sites were managed through monitored natural attenuation. At the LF-3 site at Tinker AFB, Oklahoma, ground water has been extracted from the down gradient portion of the plume in recent years.

Ground Water Sampling, Assay for *Dehalococcoides* DNA, and Chemical Analyses

These procedures and analyses were conducted as described in Section 4.

Detection of *Dehalococcoides* DNA

A total of 81 monitoring wells were sampled from the 15 sites. Most of these wells were contaminated with chlorinated ethylenes at concentrations above their maximum contaminant levels (MCLs) in drinking water. In 26 wells, *Dehalococcoides* DNA was unequivocally detected. These 26 wells were distributed over 12 sites as shown in Table 5.2.

Table 5.2 compares the concentrations of chlorinated ethylenes and ethylene to the intensity score of *Dehalococcoides* DNA in the wells where *Dehalococcoides* DNA was detected. The extent of reductive dechlorination was generally associated with the intensity score of *Dehalococcoides* DNA. That is, in 10 of the 12 wells that had higher intensity scores of *Dehalococcoides* DNA (scores of ++ or more), complete dechlorination to ethylene was observed. In contrast, in 9 of the 14 wells that had lower intensity scores of *Dehalococcoides* DNA (scores of +), dechlorination only proceeded to *cis*-DCE or vinyl chloride. However, it should be noted that complete dechlorination to ethylene was also observed in four wells that had lower intensity scores of *Dehalococcoides* DNA (scores of +).

A monitoring well is not an ideal instrument to sample bacteria in aquifers. In many aquifers, the bacteria are primarily associated with surfaces; they are not planktonic. If the water produced by a well has slight turbidity from silt or clay-sized particles, bacteria associated with these particles will be sampled with the ground water. If the screen of the well has a sand pack and if the well has been properly developed, turbidity will be low, and bacteria in the aquifer may not be sampled effectively. Detectable concentrations of bacterial DNA were recovered from 86% of the wells sampled, and detectable concentrations of *Dehalococcoides* DNA were recovered from 32% of the wells sampled.

In 53 samples, *Dehalococcoides* DNA was not detected unequivocally and in two ground water samples, the assay for *Dehalococcoides* DNA was inconclusive (intensity of the band in the electrophoresis gel was less than 3% of the positive control, intensity scores of +/-). For the 53 negative samples, the control using a universal bacterial PCR primer was performed to determine whether bacterial DNA of any type could be extracted from the water sample. The absence of bacterial DNA may indicate that sampling of the biomass from the aquifer was ineffective, as opposed to indicating that *Dehalococcoides* was not present in the aquifer. Most of the samples contained bacterial DNA; only 11 of the 53 samples of ground water did not contain enough bacterial DNA to allow the amplified DNA to be detected in the electrophoresis gel.

Biogeochemistry of Ground Water with Detectable *Dehalococcoides* DNA

The geochemistry of the water in the 26 wells where *Dehalococcoides* DNA was detected was evaluated to determine the geochemical environment that was associated with the presence of *Dehalococcoides* DNA. It is important to remember that a monitoring well can produce ground waters from different portions of an aquifer and may mix and blend ground waters that have different geochemical properties. The presence or absence of a particular biogeochemical parameter, such as molecular oxygen, in well water containing *Dehalococcoides* DNA does not necessarily mean that the *Dehalococcoides* cells in the aquifer occupied ground water with the average biogeochemical characteristics of the water produced from the monitoring well.

Table 5.3 presents data on concentrations of nitrate plus nitrite, concentrations of methane, and the meter reading for oxidation/reduction potential for the 26 wells. As will be discussed later in this section, these were the parameters with the strongest association with the presence of *Dehalococcoides* DNA. In general, the concentrations of nitrate plus nitrite were low; most were below the detection limit. Concentrations of methane were high, usually above 2 mg/L, and the oxidation/reduction electrode potential was low, usually less than -50 mV against the Ag/AgCl reference electrode.

The associations are reasonable based on the current understanding of biological reductive dechlorination. Nitrate can serve as a direct inhibitor of reductive dechlorination (Nelson et al., 2002) and may prevent the colonization of the aquifer by *Dehalococcoides* organisms. More importantly, nitrate may also serve as a competing terminal electron acceptor, and deplete concentrations of H_2 to concentrations that are below the concentration that is necessary to sustain the growth of *Dehalococcoides* bacteria (Fennell and Gossett. 1998; Yang and McCarty, 1998). The primary cause for the association of the absence of nitrate with the presence of *Dehalococcoides* DNA may be

Table 5.2 The Intensity Scores of *Dehalococcoides* DNA and the Concentrations of Chlorinated Ethylenes and Ethylene in the Wells where *Dehalococcoides* DNA was Detected

Site	Wells	Dhc DNA Score*	Concentration (µg/L)				
			PCE	TCE	*cis*-DCE	VC	Ethylene
SS-17, Altus AFB, OK	TSMW-5 WL410	++++ +	<0.3 <0.3	64 41	250 15	1300 <1.0	91 <1.5
OU-1, Altus AFB, OK	PESMP09	+	<0.3	25	227	1.7	<1.5
Area 6, Carswell AFB, TX	WHGLTA071 WHGLTA072	++++ +	<0.3 <0.3	2.4 2.4	1.7 17	1.0 1.2	58 36
Target Area 1, Dover AFB, DE	MW236S	+	<0.3	<0.4	<0.2	11	<1.5
Site 35, Vandenberg AFB, CA	MW20 MW13	++ +	<0.3 <0.3	210 250	140 13	170 <0.2	2.9 <1.5
Building 79, USCG Support Center, Elizabeth City, NC	ML22.5-0 ML22.5-8	++ +	1.3 1.2	1300 36	22 41	5.8 20	9.0 na
Area 800, England AFB, LA	A39L009PZ	+	<0.3	21	53	5.2	<1.5
Area 2500, England AFB, LA	A39L011PZ A39L010PZ Well#23	+++ ++ +	<0.3 <0.3 <0.3	<0.4 <0.4 <0.4	<0.2 <0.2 92	100 96 61	20 90 <1.5
Site 41, Rickenbacker AFB, OH	MW103 MW104 MW105	++ + +	<0.3 <0.3 <0.3	19 180 <0.4	5400 1700 3.0	940 250 10	41000 1500 1700
LF-3, Tinker AFB, OK	83BR 2-259B 2-299B 2-292B 2-304B	++++ ++ + + +	0.5 8.0 0.4 0.4 <0.3	270 28 230 220 0.5	13 28000 0.8 1.0 0.5	4.0 20000 <0.2 0.7 <0.2	<1.5 5.3 <1.5 <1.5 <1.5
Western Processing, Kent, WA	6M6B T2 T4	++ ++ +	<0.3 <0.3 <0.3	<0.4 <0.4 <0.4	<0.2 <0.2 0.3	<0.2 <0.2 1.6	3.0 8.0 6.0
North Beach, USCG Support Center, Elizabeth City, NC	MW1	++++	380	81	79	12	<1.5

na: not analyzed

* *maximum score detected by any of the Dehalococcoides primers. 0% of positive control = (-), <3% = (+/-), 4% to 33% = (+), 34% to 66% = (++), 67% to 100% = (+++), >100% of positive control = (++++).*

Table 5.3. The Concentrations of Nitrate plus Nitrite Nitrogen, Methane, and the ORP Meter Reading in the Wells where *Dehalococcoides* DNA was Detected

Site/Location	Well	*Dhc* DNA Score*	NO_3+NO_2-N (mg/L)	CH_4 (mg/L)	Meter ORP (mV)
SS-17, Altus AFB, OK	TSMW-5	++++	<0.1	1.7	-210
	WL 410	+	<0.1	0.7	-82
OU-1, Altus AFB, OK	PESMP 09	+	<0.1	4.3	-52
Area 6, Carswell AFB, TX	WHGLTA071	++++	<0.1	4.5	-180
	WHGLTA072	+	<0.1	3.6	-200
Target Area 1, Dover AFB, DE	MW236S	+	1.3	0.001	270
Site 35, Vandenberg AFB, CA	MW 20	++	0.4	4.5	-150
	MW 13	+	8.6	8.3	-61
Building 79, USCG Support Center, Elizabeth City, NC	ML22.5-0	++	0.1	1.7	-19
	ML22.5-8	+	<0.1	na	-64
Area 800, England AFB, LA	A39L009PZ	+	<0.1	0.2	-77
Area 2500, England AFB, LA	A39L011PZ	+++	0.1	2.0	-94
	A39L010PZ	++	<0.1	3.8	-140
	WELL # 23	+	<0.1	3.1	-110
Site 41, Rickenbacker AFB, OH	MW 103	+	<0.1	1.2	na
	MW 104	+	<0.1	0.019	na
	MW 105	+	<0.1	0.97	na
LF-3, Tinker AFB, OK	83BR	++++	0.2	0.03	150
	2-259B	++	<0.1	3.1	-160
	2-299B	+	2.6	<0.001	330
	2-292B	+	2.9	<0.001	210
	2-304B	+	0.2	<0.001	280
Western Processing, Kent, WA	6M6B	++	<0.1	13	180
	T2	++	<0.1	19	-98
	T4	+	<0.1	21	-100
North Beach, USCG Support Center, Elizabeth City, NC	MW1	++++	0.1	0.1	na

na: not analyzed

* *maximum score detected by any of the Dehalococcoides primers. 0% of positive control = (-), <3% = (+/-), 4% to 33% = (+), 34% to 66% = (++), 67% to 100% = (+++), >100% of positive control = (++++).*

an indirect association with dissolved oxygen. The absence of nitrate or nitrite is generally associated with the absence of dissolved oxygen. The presence of *Dehalococcoides* DNA in oxygenated ground water is not expected based on laboratory studies that show that *Dehalococcoides* organisms are strict anaerobes (Maymó-Gatell et al., 1997; He et al., 2003a, He et al., 2003b).

The accumulation of methane would be expected in anoxic ground water with high concentrations of dissolved hydrogen. The accumulation of methane would suggest that supplies of dissolved hydrogen are also adequate to allow proliferation of *Dehalococcoides* organisms. The low electrode potentials (ORP) reflect reducing conditions that would favor growth of strict anaerobic bacteria such as *Dehalococcoides* organisms.

Data on the association of *Dehalococcoides* DNA and concentrations of oxygen, iron (II), and sulfate are presented in Table 5.4. As mentioned above, the presence of *Dehalococcoides* DNA is not expected in oxic conditions. In general, ground water that contained *Dehalococcoides* DNA had low concentrations of dissolved oxygen. The four oxic wells that contained *Dehalococcoides* DNA (83BR, 2-299B, 2-292B, and 2-304B) were all located in site LF-3 at Tinker AFB. For several years, this plume has been managed by extracting ground water from the down gradient portion of the plume. Monitoring wells 83BR, 2-299B, 2-292B, and 2-304B were located adjacent to the pumped wells. The source area of the plume (represented by well 2-259B) was strongly anaerobic and had a high intensity score of *Dehalococcoides* DNA. The influence of the pumped wells may have mixed oxygenated ground water with the anaerobic plume that contained *Dehalococcoides* DNA. The PCR assay can detect DNA from non-viable *Dehalococcoides* cells.

At three sites, *Dehalococcoides* DNA was not detected in any of the wells that were sampled (site FF-87 in Newark AFB, Ohio, and sites FTA-2 and OFTA in Tinker AFB, Oklahoma). At these three sites, the dissolved oxygen level was generally high (data not shown) with the median concentrations ranging from 0.8 mg/L to 7.7 mg/L.

In most cases, the presence of *Dehalococcoides* DNA was associated with detectable concentrations of iron (II) (Table 5.4). The scoring system presented in the *Technical Protocol for Evaluating Natural Attenuation of Chlorinated Solvents in Ground Water* (Wiedemeier et al., 1998) suggested that reductive dechlorination should not be expected in ground water with sulfate concentrations above 20 mg/L. In this survey, *Dehalococcoides* DNA was found in several ground waters with concentrations of sulfate above 100 mg/L. In this survey, there is no evidence that sulfate inhibits growth of *Dehalococcoides* organisms or prevents reductive dechlorination in contaminated aquifers (Table 5.4).

Biological reductive dechlorination requires a source of reducing power either as dissolved hydrogen or as an organic substrate. Table 5.5 compares the presence of *Dehalococcoides* DNA to the concentrations of dissolved hydrogen and total organic carbon (TOC). Yang and McCarty (1998) found that a mixed culture growing on benzoate with *cis*-DCE available as an electron acceptor (at 28° C) poised the hydrogen concentration at 2 nanomolar (nM). They interpreted this concentration as the minimum concentration of hydrogen that would support utilization by organisms carrying out reductive dechlorination. Fennell and Gossett (1998) reported that the lowest hydrogen concentration that would support dechlorination (at 35° C) was as low as 1.5 nM. Following their approach, the *Technical Protocol for Evaluating Natural Attenuation of Chlorinated Solvents in Ground Water* (Wiedemeier et al., 1998) predicts that reductive dechlorination of solvents will occur if the concentration of hydrogen exceeds 1 nM. The concentration of dissolved hydrogen was measured in 18 of the 26 wells that contained detectable concentrations of *Dehalococcoides* DNA. Of the 18 wells, 14 had dissolved hydrogen equal to or greater than 1.0 nM (Table 5.5).

The *Technical Protocol for Evaluating Natural Attenuation of Chlorinated Solvents in Ground Water* (Wiedemeier et al., 1998) suggests that biological reductive dechlorination is favored when concentrations of TOC are greater than 20 mg/L. Detectable concentrations of *Dehalococcoides* DNA were found in ground water with much lower concentrations of TOC, in the range of 1 to 2 mg/L.

In Table 5.5, wells were assigned to redox conditions based on the concentrations of methane, sulfate, ferrous iron, and oxygen as follows: conditions were considered to be methanogenic when methane was greater than 1.0 mg/L; sulfate and iron reducing when methane was less than 1.0 mg/L, sulfate was greater than 20 mg/L, and ferrous iron was detected; and oxic when dissolved oxygen was greater than 1.0 mg/L, and ferrous iron and methane were not detected. This assignment was done to facilitate comparisons between general redox conditions and the presence or absence of *Dehalococcoides* DNA. Of the 26 wells where *Dehalococcoides* DNA was detected, 17 had

Table 5.4. The Concentrations of Oxygen, Ferrous Iron, and Sulfate in the Wells where *Dehalococcoides* DNA was Detected

Site/Location	Well	*Dhc* DNA Score*	O_2 (mg/L)	Fe(II) (mg/L)	SO_4^{-2} (mg/L)
SS-17, Altus AFB, OK	TSMW-5	++++	0.2	3.0	880
	WL 410	+	0.2	0.2	520
OU-1, Altus AFB, OK	PESMP 09	+	0.3	0.7	1100
Area 6, Carswell AFB, TX	WHGLTA071	++++	0.1	1.5	<5
	WHGLTA072	+	0.1	0.4	<5
Target Area 1, Dover AFB, DE	MW236S	+	2.3	0.8	56
Site 35, Vandenberg AFB, CA	MW 20	++	<0.1	30	23
	MW 13	+	0.1	0.3	740
Building 79, USCG Support Center, Elizabeth City, NC	ML22.5-0	++	0.2	3.0	280
	ML22.5-8	+	0.1	<0.1	21
Area 800, England AFB, LA	A39L009PZ	+	0.1	7.0	19
Area 2500, England AFB, LA	A39L011PZ	+++	0.5	14	<5
	A39L010PZ	++	0.2	16	<5
	WELL # 23	+	0.1	12	<5
Site 41, Rickenbacker AFB, OH	MW 103	+	na	na	470
	MW 104	+	na	na	110
	MW 105	+	na	na	260
LF-3, Tinker AFB, OK	83BR	++++	1.5	<0.1	42
	2-259B	++	0.2	9.0	<5
	2-299B	+	2.9	<0.1	64
	2-292B	+	4.9	<0.1	52
	2-304B	+	3.8	<0.1	21
Western Processing, Kent, WA	6M6B	++	0.5	0.5	<5
	T2	++	0.1	2.3	<5
	T4	+	0.2	7.5	<5
North Beach, USCG Support Center, Elizabeth City, NC	MW1	++++	0.1	0.9	77

na: not analyzed

* *maximum score detected by any of the Dehalococcoides primers. 0% of positive control = (-), <3% = (+/-), 4% to 33% = (+), 34% to 66% = (++), 67% to 100% = (+++), >100% of positive control = (++++).*

Table 5.5. The Concentration of Dissolved Molecular Hydrogen and Total Organic Carbon, and the Oxidation/Reduction Potential in the Wells where *Dehalococcoides* DNA was Detected

Site/Location	Well	*Dhc* DNA Score*	Redox Condition	H_2 (nM)	TOC (mg/L)
SS-17, Altus AFB, OK	TSMW-5	++++	Methanogenic	4.6	8.7
	WL 410	+	Sulfate and iron reducing	1.0	9.9
OU-1, Altus AFB, OK	PESMP 09	+	Methanogenic	1.0	15
Area 6, Carswell AFB, TX	WHGLTA071	++++	Methanogenic	>10	0.8
	WHGLTA072	+	Methanogenic	0.4	9.6
Target Area 1, Dover AFB, DE	MW236S	+	Oxic	6.7	4.6
Site 35, Vandenberg AFB, CA	MW 20	++	Methanogenic	>10	910
	MW 13	+	Methanogenic	>10	4.8
Building 79, USCG Support Center, Elizabeth City, NC	ML22.5-0	++	Methanogenic	3.7	2.1
	ML22.5-8	+		0.9	5.6
Area 800, England AFB, LA	A39L009PZ	+	Sulfate and iron reducing	6.5	1.1
Area 2500, England AFB, LA	A39L011PZ	+++	Methanogenic	>10	1.9
	A39L010PZ	++	Methanogenic	0.8	2.1
	WELL # 23	+	Methanogenic	2.3	1.4
Site 41, Rickenbacker AFB, OH	MW 103	+	Methanogenic	na	na
	MW 104	+	Methanogenic	na	na
	MW 105	+	Methanogenic	na	na
LF-3, Tinker AFB, OK	83BR	++++	Sulfate and iron reducing	na	8.8
	2-259B	++	Methanogenic	na	92
	2-299B	+	Oxic	na	<0.5
	2-292B	+	Oxic	na	<0.5
	2-304B	+	Oxic	na	<0.5
Western Processing, Kent, WA	6M6B	++	Methanogenic	6.4	13
	T2	++	Methanogenic	2.0	21
	T4	+	Methanogenic	2.4	16
North Beach, USCG Support Center, Elizabeth City, NC	MW1	++++	Sulfate and iron reducing	0.2	na

na: not analyzed

* *maximum score detected by any of the Dehalococcoides primers. 0% of positive control = (-), <3% = (+/-), 4% to 33% = (+), 34% to 66% = (++), 67% to 100% = (+++), >100% of positive control = (++++).*

methanogenic conditions, 4 had sulfate and iron reducing conditions, and 4 had oxic conditions. There was a strong association between a high intensity score for *Dehalococcoides* DNA and low redox potential. With two exceptions, all the wells with higher intensity scores of *Dehalococcoides* DNA (scores of ++ or more) had methanogenic conditions.

In general, the geochemical habitat of *Dehalococcoides*, as defined by the parameters measured, was wide. In the 26 wells where *Dehalococcoides* DNA was detected, the pH ranged from 4.7 to 7.4, the temperature ranged from 13.4° C to 33.4° C, the concentration of BTEX ranged from not detected to more than 800 mg/L, the alkalinity ranged from 60 mg/L to 680 mg/L, the electrical conductivity was in the range of 200 µs/cm to 5430 µs/cm, and the concentration of chloride was in the range of 5 to 1270 mg/L.

Comparison of Geochemistry where *Dehalococcoides* DNA is Present or Absent

The geochemical parameters discovered as significant by the multiple testing were used as the explanatory variables in a statistical model using logistic regression. The prediction accuracy of the model was evaluated by comparing the observed response and the predicted probability for the presence of *Dehalococcoides* DNA.

To determine whether a particular biogeochemical parameter could predict the presence or absence of *Dehalococcoides* DNA in ground water, the distribution of values for that parameter in water where *Dehalococcoides* DNA was detected was compared to the distribution in water where *Dehalococcoides* DNA was not detected. The samples that did not have enough DNA to be amplified by the PCR assay and the samples with inconclusive test results for *Dehalococcoides* DNA were excluded from the comparison. A two-sample Kolmogorov-Smirnov test was used to compare the data distributions for each geochemical parameter.

Fourteen geochemical parameters were compared. To deal with the problem of multiple comparisons, the False Discovery Rate (FDR) developed by Benjamini and Hochberg (1995) was used to control the proportion of significant results that were in fact type I errors (false positive). The two-sample Kolmogorov-Smirnov test was performed for each biogeochemical parameter to generate a probability (p) that there was no difference between the distribution of the parameter in wells where *Dehalococcoides* DNA was detected and the distribution in wells where *Dehalococcoides* DNA was not detected. This is the probability of a false positive, the probability that the distributions appear to be different when in fact they are not different.

A False Discovery Rate Ø of 0.05 (that is, in every 20 significant results for different parameters, the expected number of false positives is one) was used in this study. The fourteen separate p-values were sorted in ascending order $p_{(1)}$, $p_{(2)}$, …$p_{(14)}$. All geochemical parameters with p-values $p \le p_{(j)}$ were rejected where j was the largest index for which

$$p_{(j)} \le \frac{0.05 \bullet j}{14}$$

Table 5.6 shows the probability (p) that the distributions of each biogeochemical parameter are not different between water with and water without *Dehalococcoides* DNA as predicted by the Kolmogorov-Smirnov test. Table 5.6 also shows the FDR threshold for the values of p. To be protected from false positives, the value of p must be less than the FDR threshold. When the FDR threshold was set at a probability Ø of 0.05, significant test results were obtained for three of the biogeochemical parameters presented in Table 5.5. The distribution of the values of the concentration of NO_3^{-1} plus NO_2^{-1}-N, of ORP, and of the concentration of CH_4 was significantly different between the samples of ground water where *Dehalococcoides* DNA was detected and samples where *Dehalococcoides* DNA was not detected. As for the other parameters, (O_2, H_2, Fe (II), SO_4^{-2}, TOC, Cl^{-1}, BTEX compounds, alkalinity, electrical conductivity, pH and temperature) there were no significant differences in the distribution of the values of the parameters at the specified FDR controlling level (Table 5.6).

A series of Chi-square tests were performed on each of these three parameters. It was found that the proportion of NO_3^{-1} plus NO_2^{-1}-N below 0.5 mg/L, the proportion of ORP below 0 mV, and the proportion of CH_4 over 0.5 mg/L were significantly different between the water samples where *Dehalococcoides* DNA was either present or absent at 95% confidence level.

The two-sample Kolmogorov-Smirnov test was performed using SPSS software. The Chi-square test was performed using the Data Analysis Tool in Microsoft Excel. The logistic regression was performed using SAS software.

Table 5.6. The Probability (*p*) that the Distribution of the Measured Values for Selected Geochemical Parameters between Ground Water where *Dehalococcoides* DNA was Present and Ground Water where *Dehalococcoides* DNA was not Present is not Statistically Different. To be Accepted as Statistically Significant, the Value of (*p)* for a Parameter must be Less than the Value for the Threshold for the False Discover Rate (FDR)

Parameter	Sample Size (No. Wells where *Dehalococcoides* DNA)		Z value [a]	*p*-value (2-tailed)	Index Number *j*	Threshold for FDR [b]
	Detected	Not Detected				
Parameters found to be statistically significant at 90% confidence or greater						
NO_3^{-1}+NO_2^{-1} -N	26	39	1.874	**0.002**	1	**0.004**
ORP	20	30	1.848	**0.002**	2	**0.007**
CH_4	25	40	1.687	**0.007**	3	**0.011**
Parameters not found to be statistically significant at 90% confidence						
Fe(II)	23	39	1.544	0.017	4	0.014
TOC	22	32	1.457	0.029	5	0.018
SO_4^{-2}	26	42	1.196	0.114	6	0.021
O_2	23	40	1.080	0.194	7	0.025
Alkalinity	15	18	1.017	0.252	8	0.029
Temperature.	17	24	0.943	0.336	9	0.032
BTEX	19	26	0.919	0.367	10	0.036
pH	23	37	0.761	0.608	11	0.039
Electrical Conductivity	17	24	0.704	0.705	12	0.043
Cl^{-1}	18	32	0.636	0.813	13	0.046

[a] *Z value in the Kolmogorov-Smirnov test.*

[b] *Threshold for False Discovery Rate, defined as* $\frac{Ø \bullet j}{n}$*, where j is the index number of each parameter, and n is the total number of parameters compared. In this study, the Ø was 0.05, and n was 14.*

A Predictive Model for the Presence of *Dehalococcoides* DNA

It would be useful to have a predictive model for the presence of *Dehalococcoides* DNA based on the three simple geochemical parameters (methane, nitrate plus nitrite nitrogen, and oxidation reduction potential) that were found to be statistically correlated with the presence or absence of *Dehalococcoides* DNA. For this purpose, a logistic regression with a binary response for the presence or absence of *Dehalococcoides* DNA was used to model the probability of the presence of *Dehalococcoides* DNA as a function of the biogeochemical parameters expressed as continuous variables. In the model, the response variable (Y) had two values: Y=1 for the presence of *Dehalococcoides* DNA and Y=0 for the absence of *Dehalococcoides* DNA. The three biogeochemical parameters (ORP, CH_4, and NO_3^{-1} plus NO_2^{-1}-N) were the explanatory variables. The model was as follows (Equation 5.1):

$$\text{logit}(p) = \log\left(\frac{p}{1-p}\right) =$$

$$-0.1370 - 0.0050 \times ORP + 0.1328 \times CH_4 - 0.0468 \times NO_3^{-1}\ plus\ NO_2^{-1} - N$$

where *p* is the probability of the presence of *Dehalococcoides* DNA, *ORP* is the value of oxidation reduction potential against a silver/silver chloride reference electrode in millivolts, CH_4 is the concentration of methane in mg/L, and NO_3^{-1} plus NO_2^{-1}-*N* is the concentration of nitrate plus nitrite nitrogen in mg/L.

A total of 45 cases (wells) from ten sites were used to develop the model. Of the 45 cases, 20 had response value of 1, and 25 had response value of 0. The generalized coefficient of determination (R^2) of the model was 0.2497, and the Max-rescaled R^2 was 0.3343. The parameter estimates indicated that the probability for the presence of *Dehalococcoides* DNA increased with the increase of CH_4 or the decrease of ORP or NO_3^{-1} plus NO_2^{-1}-N. Table 5.7 compares the

geochemical data from the wells used to develop the model to the presence or absence of *Dehalococcoides* DNA. Table 5.8 compares the presence or absence of *Dehalococcoides* DNA to the predicted probability for the presence of *Dehalococcoides* DNA and the lower and upper 95% confidence limits for the predicted probability. Wells are listed in Table 5.8 from the well with the highest probability that *Dehalococcoides* DNA is present to the well with the lowest probability that *Dehalococcoides* DNA is present.

The model accurately predicted the observed presence or absence of *Dehalococcoides* DNA in most of the wells. In 15 of the 20 wells that had observed response of 1, the predicted probability for the presence of *Dehalococcoides* DNA was greater than 0.50. In 23 of the 25 wells that had observed response of 0, the predicted probability for the presence of *Dehalococcoides* DNA was less than 0.50.

There were seven "outlier" wells where the predicted probabilities for the presence of *Dehalococcoides* DNA were greatly different from the observed responses. They were well PESMP07 at site OU-1 on Altus AFB, OK; well MW236S at the Target Area 1 site at Dover AFB, DE; well 15M45B at the Western Processing site in Kent, WA; and wells 83BR, 2-292B, 2-299B, and 2-304B at the LF-3 site at Tinker AFB, OK. The behavior of the wells at the LF-3 site at Tinker AFB, OK, may be a result of the ground water extraction system, which may have blended anaerobic water from the plume bearing the *Dehalococcoides* DNA with clean aerobic ground water. The discrepancy of the other sites cannot be explained.

It is possible that the high concentration of methane, low concentrations of nitrate, and low values for redox potential are better predictors of biological reductive dechlorination than the presence or absence of *Dehalococcoides* DNA. However, we did not attempt a statistical comparison of the achieved rates of attenuation at field scale to the values of the geochemical parameters. Fitted rates of attenuation of *cis*-DCE and vinyl chloride were available from only three sites (Table 4.5). These are too few data for a robust comparison.

Summary and Conclusions

For the evaluation of natural attenuation of chlorinated solvents, particularly in an anoxic aquifer, it's important to know if *Dehalococcoides* organisms are present. The presence of *Dehalococcoides* DNA may be used to directly demonstrate the occurrence of biotransformation at the site. The predictive model established in this report provides a simple and rapid way to estimate the presence of *Dehalococcoides* organisms based on geochemical condition. This is very useful considering the cost associated with the analysis of *Dehalococcoides* DNA.

The predictive model can be used to screen samples to be submitted for the biochemical assay. Fennell and her coworkers discovered that *Dehalococcoides* organisms are heterogeneously distributed in the aquifer (Fennell et al., 2001). To obtain useful DNA data from the field, it is a good practice to calculate the probability that *Dehalococcoides* is present based on the more easily available geochemical information. The water samples that most likely have *Dehalococcoides* can be submitted to the biochemical assay.

It should be noted that the predictive model is not intended to replace the biochemical assay. A calculated probability that *Dehalococcoides* exists in ground water is not equivalent to a direct biochemical assay for its presence. However, under conditions where data from a direct biochemical assay are not available, a calculated probability could be used to properly calibrate computer models of natural attenuation. In particular, a rate constant for biotransformation of lower chlorinated ethylenes such as *cis*-DCE and vinyl chloride is not appropriate to be applied to regions of the aquifer where the geochemical parameters suggest that *Dehalococcoides* organisms should not be expected.

Any well that blends ground water from different geochemical environments will produce confusing data. The predictions based on Equation 5.1 will work best from monitoring wells with relatively short screens. The short screens minimize the blending of ground water from different geochemical environments. The predictions of Equation 5.1 should not be applied to pumped treatment wells if the wells produce water that is obviously not in geochemical equilibrium.

Table 5.7. Comparisons between the Observed Presence or Absence of *Dehalococcoides* DNA and the Concentrations of Nitrate plus Nitrite Nitrogen, Methane, and the ORP Meter Reading in the Wells

Site/Location	Well	Observed Response	Nitrate+ Nitrite N (mg/L)	CH_4 (mg/L)	ORP (mV)
Western Processing, Kent, WA	T4	present	0.04	21	-100
Western Processing, Kent, WA	T2	present	0.04	19	-98
Western Processing, Kent, WA	15M45B	absent	0.04	12	-93
OU-1, Altus AFB, OK	PESMP 07	absent	0.07	4.6	-200
Area 6, Carswell AFB, TX	WHGLTA071	present	0.04	4.5	-180
Area 6, Carswell AFB, TX	WHGLTA072	present	0.01	3.6	-196
Site 35, Vandenberg AFB, CA	MW 20	present	0.35	4.5	-150
SS-17, Altus AFB, OK	TSMW-5	present	0.07	1.7	-210
LF-3, Tinker AFB, OK	2-259B	present	0.03	3.1	-160
Area 2500, England AFB, LA	A39L010PZ	present	0.07	3.8	-140
Site 35, Vandenberg AFB, CA	MW 13	present	8.6	8.3	-61
Area 2500, England AFB, LA	WELL # 23	present	0.08	3.1	-110
OU-1, Altus AFB, OK	PESMP 09	present	0.04	4.3	-52
Western Processing, Kent, WA	6M6B	present	0.04	13	180
Area 2500, England AFB, LA	A39L011PZ	present	0.12	2.0	-94
SS-17, Altus AFB, OK	WL 410	present	0.05	0.7	-82
Area 800, England AFB, LA	A39L009PZ	present	0.08	0.2	-77
Area 800, England AFB, LA	WELL # 19	absent	0.8	0.002	-24
Western Processing, Kent, WA	15M39B	absent	0.03	1.8	73
Target Area 1, Dover AFB, DE	MW236D	absent	0.04	0.2	51
Area 6, Carswell AFB, TX	WHGLFE002	absent	1.2	0.3	54
Area 800, England AFB, LA	WELL # 17	absent	0.1	0.02	82
Western Processing, Kent, WA	15M17B	absent	0.04	2.1	170
Area 6, Carswell AFB, TX	LF04-4E	absent	0.23	2.4	180
Area 800, England AFB, LA	SS45L001MW	absent	0.22	0.5	150
FTA-2, Tinker AFB, OK	2-62B	absent	5.3	0.9	110
OU-1, Altus AFB, OK	WL 019	absent	1.6	0.09	130
OU-1, Altus AFB, OK	WL 250	absent	0.7	0.005	140
LF-3, Tinker AFB, OK	83BR	present	0.2	0.03	150
FTA-2, Tinker AFB, OK	2-393B	absent	0.5	<0.001	170
OFTA, Tinker AFB, OK	2-440B	absent	3.10	<0.001	150
OFTA, Tinker AFB, OK	2-144B	absent	0.61	0.9	220
OFTA, Tinker AFB, OK	2-394B	absent	2.0	0.06	210
LF-3, Tinker AFB, OK	2-292B	present	2.9	0	210
SS-17, Altus AFB, OK	WL 082	absent	2.0	0	230
SS-17, Altus AFB, OK	WL 094	absent	2.6	0.001	230
Target Area 1, Dover AFB, DE	DM353D	absent	0.5	0.3	270
SS-17, Altus AFB, OK	WL 090	absent	3.7	<0.001	240
Target Area 1, Dover AFB, DE	MW236S	present	1.3	0.001	270
LF-3, Tinker AFB, OK	2-304B	present	0.2	<0.001	280
OFTA, Tinker AFB, OK	2-143B	absent	3.4	<0.001	260
Target Area 1, Dover AFB, DE	MW101S	absent	1.1	0.003	290
OFTA, Tinker AFB, OK	2-329B	absent	7.3	0.007	240
SS-17, Altus AFB, OK	WL 080	absent	1.6	0.003	330
LF-3, Tinker AFB, OK	2-299B	present	2.6	<0.001	330

[a] LCL: lower 95% confidence limit for the predicted probability; [b] UCL: upper 95% confidence limit for the predicted probability.

Table 5.8. Comparisons between the Observed Presence or Absence of *Dehalococcoides* DNA and the Predicted Probabilities for the Presence of *Dehalococcoides* DNA

Site/Location	Well	Observed Response	Predicted Probability	LCL[a]	UCL[b]
Western Processing, Kent, WA	T4	present	0.958	0.315	0.999
Western Processing, Kent, WA	T2	present	0.949	0.334	0.999
Western Processing, Kent, WA	15M45B	absent	0.867	0.449	0.981
OU-1, Altus AFB, OK	PESMP 07	absent	0.811	0.526	0.943
Area 6, Carswell AFB, TX	WHGLTA071	present	0.799	0.519	0.936
Area 6, Carswell AFB, TX	WHGLTA072	present	0.789	0.491	0.936
Site 35, Vandenberg AFB, CA	MW 20	present	0.765	0.500	0.914
SS-17, Altus AFB, OK	TSMW-5	present	0.756	0.407	0.934
LF-3, Tinker AFB, OK	2-259B	present	0.746	0.461	0.909
Area 2500, England AFB, LA	A39L010PZ	present	0.743	0.477	0.901
Site 35, Vandenberg AFB, CA	MW 13	present	0.705	0.087	0.984
Area 2500, England AFB, LA	WELL # 23	present	0.692	0.440	0.865
OU-1, Altus AFB, OK	PESMP 09	present	0.668	0.436	0.84
Western Processing, Kent, WA	6M6B	present	0.651	0.108	0.966
Area 2500, England AFB, LA	A39L011PZ	present	0.643	0.387	0.837
SS-17, Altus AFB, OK	WL 410	present	0.591	0.320	0.815
Area 800, England AFB, LA	A39L009PZ	present	0.567	0.292	0.807
Area 800, England AFB, LA	WELL # 19	absent	0.487	0.259	0.721
Western Processing, Kent, WA	15M39B	absent	0.435	0.254	0.634
Target Area 1, Dover AFB, DE	MW236D	absent	0.407	0.219	0.628
Area 6, Carswell AFB, TX	WHGLFE002	absent	0.394	0.226	0.592
Area 800, England AFB, LA	WELL # 17	absent	0.366	0.191	0.584
Western Processing, Kent, WA	15M17B	absent	0.329	0.150	0.576
Area 6, Carswell AFB, TX	LF04-4E	absent	0.327	0.149	0.574
Area 800, England AFB, LA	SS45L001MW	absent	0.306	0.151	0.522
FTA-2, Tinker AFB, OK	2-62B	absent	0.306	0.075	0.707
OU-1, Altus AFB, OK	WL 019	absent	0.301	0.162	0.491
OU-1, Altus AFB, OK	WL 250	absent	0.296	0.152	0.496
LF-3, Tinker AFB, OK	83BR	present	0.287	0.135	0.508
FTA-2, Tinker AFB, OK	2-393B	absent	0.268	0.126	0.482
OFTA, Tinker AFB, OK	2-440B	absent	0.260	0.109	0.501
OFTA, Tinker AFB, OK	2-144B	absent	0.246	0.105	0.475
OFTA, Tinker AFB, OK	2-394B	absent	0.220	0.099	0.422
LF-3, Tinker AFB, OK	2-292B	present	0.207	0.083	0.432
SS-17, Altus AFB, OK	WL 082	absent	0.203	0.086	0.408
SS-17, Altus AFB, OK	WL 094	absent	0.194	0.077	0.410
Target Area 1, Dover AFB, DE	DM353D	absent	0.183	0.062	0.434
SS-17, Altus AFB, OK	WL 090	absent	0.182	0.059	0.441
Target Area 1, Dover AFB, DE	MW236S	present	0.174	0.063	0.399
LF-3, Tinker AFB, OK	2-304B	present	0.174	0.053	0.442
OFTA, Tinker AFB, OK	2-143B	absent	0.171	0.057	0.416
Target Area 1, Dover AFB, DE	MW101S	absent	0.162	0.054	0.398
OFTA, Tinker AFB, OK	2-329B	absent	0.154	0.017	0.655
SS-17, Altus AFB, OK	WL 080	absent	0.132	0.037	0.372
LF-3, Tinker AFB, OK	2-299B	present	0.128	0.037	0.363

[a] LCL: lower 95% confidence limit for the predicted probability; [b] UCL: upper 95% confidence limit for the predicted probability

Section 6.
Presence of *Dehalococcoides* DNA and the Extent of Biodegradation

Presence of *Dehalococcoides* DNA in ground water may not indicate that the bacteria are active and degrading the chlorinated solvents. On the other hand, the failure to detect *Dehalococcoides* using a particular PCR primer does not prove that the aquifer does not contain *Dehalococcoides* organisms or other bacteria that can degrade chlorinated solvents to ethylene. Laboratory microcosm studies conducted with sediment or enrichment cultures conducted with ground water from a contaminated site play a key role in determining if the bacteria at a site have the potential for complete dechlorination (David Ellis, DuPont Company, Wilmington, Delaware, Personal Communication, September 16, 2005). To determine if dechlorination of chlorinated ethylenes could be carried out by indigenous microorganisms in the samples of ground water, ground water was used to establish enrichment cultures. The results of laboratory studies were compared to the distribution of *Dehalococcoides* DNA in the contaminated ground water at field scale. These experiments were intended to establish a connection between the extent of observed activity and the presence of *Dehalococcoides* as detected by PCR.

Enrichment Culture Preparation

Ground Water samples for inoculation were collected from PCE and TCE contaminated wells at the SS-17 Site at Altus AFB, OK; the OU-1 Site at Altus AFB, OK; Area 6 at Carswell AFB, TX; Target Area 1 at Dover AFB, DE; Site 35 at Vandenberg AFB, CA; Building 79 at the USCG Support Center, Elizabeth City, NC; the North Beach Site at the USCG Support Center, Elizabeth City, NC; Area 800 at England AFB, LA; Area 2500 at England AFB, LA; the LF-3 Site at Tinker AFB, OK; the OFTA Site at Tinker AFB, OK; and the Western Processing Site at Kent, WA (described in Table 5.1). A total of 76 ground water samples were collected for constructing the enrichment cultures. Ground water samples were collected in sterile 40 ml VOA vials with no preservatives. The samples were collected with no headspace in the vials.

Enrichment cultures were prepared in 160 ml serum bottles in an anaerobic glove box. For each ground water sample, two enrichment cultures were constructed in the same manner except that propionate (5 mM) was added as an additional elector donor in one enrichment culture but not in the other. Each enrichment culture contained 100 ml autoclaved basal medium and 25 ml ground water (as an inoculum) and was amended with 10 ml of a PCE stock solution in water or 1.5 ml of a TCE stock solution in water to produce a nominal concentration of 100 µM in the enrichment culture (nominal concentration corrects for headspace-liquid partitioning equilibrium within a bottle). The enrichment culture was sealed with a Teflon-lined butyl rubber septum and an aluminum crimp cap. The composition of the basal medium is shown in Table 6.1. The enrichments contained 0.1 g/L yeast extract, which is adequate to completely dechlorinate 100 µM of PCE.

Five rounds of enrichment cultures were constructed depending on the date the ground water samples were collected. In each round of enrichment cultures, two controls (with and without propionate) were prepared in the same manner except that an additional 25 ml of basal medium were added to each vial instead of ground water. The controls were preserved with 1% trisodium phosphate and autoclaved for 45 minutes at 121°C. All the enrichment cultures were incubated statically on their sides in an anaerobic glove box at room temperature (roughly 20°C).

The enrichment cultures are similar to the microcosm studies described in the RABITT protocol (Battelle Memorial Institute et al., 2002; Morse et al., 1998), and to microcosm studies conducted commercially (Findlay and Fogel, 2000). The concentrations of chlorinated solvents, of yeast extract, and of primary substrate are similar. An important exception is the absence of aquifer sediment in the enrichment cultures in this study. The RABITT protocol suggests 50 g sediment and 50 ml of ground water. At many hazardous waste

sites, the site owner cannot afford to collect sediment for the laboratory studies, and the studies are often done with ground water from established monitoring wells (Margaret Findlay, Bioremediation Consulting Incorporated, Watertown, MA, Personal Communication, November 3, 2005).

Table 6.1. Composition of the Basal Medium (pH 7)

Composition	Concentration	Unit
NH_4Cl	1.0	g/L
$MgSO_4 \cdot 7H_2O$	0.1	g/L
$CaCl_2 \cdot 2H_2O$	0.05	g/L
yeast extract	0.1	g/L
resazurin	1.0	mg/L
KH_2PO_4	0.0066	Molar
Na_2HPO_4	0.013	Molar
EDTA	1.0	µg/L
$FeSO_4 \cdot 7H_2O$	2.0	µg/L
$CaCl_2 \cdot 6H_2O$	0.2	µg/L
$MnCl_2 \cdot 4H_2O$	0.03	µg/L
$NiCl_2 \cdot 6H_2O$	0.02	µg/L
$ZnSO_4 \cdot 7H_2O$	0.1	µg/L
H_2SeO_3	0.02	µg/L
H_3BO_3	0.3	µg/L
$CuCl_2 \cdot 2H_2O$	0.01	µg/L
$NaMoO_4 \cdot 2H_2O$	0.033	µg/L
$CoCl_2 \cdot 6H_2O$	0.2	µg/L
p-aminobenzoic acid	100	µg/L
folic acid	50	µg/L
lipoic acid	100	µg/L
nicotinic acid	200	µg/L
riboflavine	100	µg/L
thiamine	200	µg/L
panthotenic acid	100	µg/L
pyridoxamine	500	µg/L
vitamin B12	100	µg/L
biotine	20	µg/L

Sampling and Analysis of Enrichment Cultures

Resazurin was used as a visual indicator of redox condition in the enrichment cultures (purple indicating an oxidizing condition, and the absence of color indicating a reduced condition). Enrichment cultures were sampled periodically (nondestructively) using small samples of liquid or headspace. The samples were analyzed for chlorinated ethylenes (PCE, TCE, DCE isomers, and vinyl chloride), dissolved gases (methane, ethylene, ethane, and hydrogen), and organic acids (propionate and acetate).

For analysis of chlorinated ethylenes, as well as for analysis of fatty acids, 1 ml of liquid was taken from each enrichment culture using a 1 ml glass syringe and injected to the bottom of a 40 ml VOA vial containing 40 ml RO (reverse osmosis) water and 2 drops of concentrated hydrochloric acid. The vial was immediately sealed after sample collection. Analysis of chlorinated ethylenes was performed using automated purge and trap gas chromatography. Organic acids were analyzed using high performance liquid chromatography.

For analysis of methane, ethylene, and ethane, 0.2 ml of gas was taken from the headspace of each enrichment culture into a 2.0 ml gas tight syringe; the sample was diluted to 2.0 ml with nitrogen and then injected into a GC for analysis. The high purity nitrogen used to dilute the sample was free of detectable concentrations of methane, ethylene, and ethane. Henry's Law was used to calculate the original concentrations of methane, ethylene, and ethane in the water in the enrichment cultures.

For analysis of hydrogen, a gas sample of 0.2 ml was taken from the headspace of each enrichment culture into a 2.0 ml gas tight syringe; the sample was diluted to 2.0 ml with nitrogen and then injected into a RGA3 Reduction Gas Analyzer. The high purity nitrogen used to dilute the sample was free of detectable concentrations of molecular hydrogen. The original concentrations of dissolved molecular hydrogen in the water in the enrichment cultures were calculated using Henry's Law.

Biodegradation of Chlorinated Ethylenes in the Enrichment Cultures

All the enrichment cultures were incubated for at least 20 months. Some enrichment cultures were incubated for more than 30 months. Out of 152 enrichment cultures from ground water, ethylene as

a dechlorination end product was observed in 28 enrichments. These 28 enrichments originated from 17 ground water samples (15 had been spiked with propionate and 13 had not). Of the 28 enrichments, ethylene as the only end product (100% recovery of the total chlorinated ethylenes on a molar basis) was observed in eight cases; ethylene as the major end product (68%-99% recovery of the total chlorinated ethylenes) was observed in four cases; and a smaller amount of ethylene (1%-16% recovery of the total chlorinated ethylenes) was observed in 16 cases.

Vinyl chloride as a dechlorination end product was observed in nine enrichments. These nine enrichments originated from six ground water samples (2 had been spiked with propionate and 7 had not). The observed molar recovery of total chlorinated ethylenes as vinyl chloride ranged from 1% to 24%.

Dichloroethylenes (mainly *cis*-DCE) as a dechlorination end product, were observed in 28 enrichments. These 28 enrichments originated from 15 ground water samples (14 had been spiked with propionate and 14 had not). The observed molar recovery of total chlorinated ethylenes as total DCE (sum of three isomers) ranged from 1% to 74%.

In three enrichment cultures amended with PCE, a trace of TCE was detected as a transformation product. No transformation of the added PCE or TCE was observed in the other 84 ground water amended enrichment cultures. No transformation of the added PCE or TCE was observed in the ten sterile controls.

For most ground water samples, the spike of propionate as an additional electron donor did not seem to greatly increase the rate and extent of dechlorination in the enrichments. Complete dechlorination to ethylene or partial dechlorination to DCE or vinyl chloride frequently occurred in the enrichments without the spike of propionate. This observation was not surprising because a significant amount of yeast extract (100 mg/L) was contained in the basal medium in the enrichment cultures. Acetate in the range of 0.02 to 3.7 mM (1.2 to 220 mg/L) was detected in the enrichments without the spike of propionate, probably due to fermentation of yeast extract in the basal medium and organic compounds in the ground water.

The measurement of dissolved molecular hydrogen also confirmed the availability of molecular hydrogen as an electron donor. In nearly all of the enrichment cultures (96%), the dissolved hydrogen concentrations were greater than 1 nM, a condition favorable for reductive dechlorination. The concentration of molecular hydrogen was greater than 9 nM in 75% of the enrichments, greater than 130 nM in 50% of the enrichments, and greater than 3000 nM in 25% of the enrichments. There was no statistically significant difference in the distribution of dissolved hydrogen concentration between the enrichment cultures spiked with propionate and the enrichment cultures without the spike of propionate (significance level α=0.05).

Association of Dechlorination in Enrichment Cultures with *Dehalococcoides* DNA

Prior to constructing the enrichment cultures, ground water samples collected from the same wells were analyzed for the presence or absence of *Dehalococcoides* DNA. Table 6.2 compares the extent of biotransformation of the chlorinated ethylenes obtained in the enrichment cultures to the presence or absence of *Dehalococcoides* DNA in the field samples used to establish the enrichments.

Dehalococcoides DNA was detected in water samples from 22 wells out of a total of 72 sampled. The water was used to establish 44 enrichment cultures (one with propionate, one without for each of the 22 wells). Of the 44 enrichment cultures, 20 showed complete biotransformation to ethylene. The 20 microcosms corresponded to 11 of the 22 ground water samples where *Dehalococcoides* DNA was detected. In two ground water samples where *Dehalococcoides* DNA was detected, dechlorination proceeded only as far as DCE in the four corresponding enrichment cultures. Vinyl chloride as an end product was not detected in enrichment cultures that were inoculated with ground water that contained amplifiable *Dehalococcoides* DNA. If dechlorination proceeded past dichloroethylene, it went all the way to ethylene.

Dehalococcoides DNA was not detected in water samples from 50 wells out of the 72 wells that were used to establish the enrichment cultures. Complete dechlorination to ethylene was detected in only 7 of the 100 enrichment cultures that were constructed from the ground water samples that did not have amplifiable *Dehalococcoides* DNA. Dechlorination to vinyl chloride was detected in 8 enrichment cultures and dechlorination to DCE was detected in 20 of the 100 enrichment cultures. If *Dehalococcoides* DNA was not detected, the majority of the cultures that showed activity stopped at the production of DCE.

The capacity to transform PCE or TCE to *cis*-DCE is common in anaerobic bacteria. The PCR assay for *Dehalococcoides* DNA is conventionally interpreted as an assay for organisms that might have the capabil-

Table 6.2. Comparisons of Biotransformation of Chlorinated Ethylenes in Enrichment Cultures to the Corresponding Presence or Absence of Amplifiable *Dehalococcoides* DNA in the Ground Water Sample Used to Inoculate the Enrichment Cultures

Biotransformation Product Detected on the Last Sampling Date	Number of Enrichment Cultures where the Presence of *Dehalococcoides* DNA was detected in the Field	Number of Enrichment Cultures where the Presence of *Dehalococcoides* DNA was not detected in the Field
No Product	20 of 44	65 of 100
Dichloroethylene	4 of 44	20 of 100
Vinyl Chloride	0 of 44	8 of 100
Ethylene	20 of 44	7 of 100

The test results for the presence of Dehalococcoides DNA in two ground water samples were inconclusive (score of +/-); therefore, the enrichment cultures constructed from these two samples were not included in the comparison. Twelve enrichment cultures prepared using material from the North Beach Site were amended with PCE as the source contaminant. Of the 12 enrichment cultures, three had TCE as the biotransformation end product on the last sampling date (not shown in Table 6.2).

ity to completely transform chlorinated ethylenes to ethylene. Out of 44 enrichment cultures established with ground water with detectable concentrations of *Dehalococcoides* DNA, 24 cultures failed to produce ethylene. If false positives for the assay are defined as water samples where *Dehalococcoides* DNA was detected, but ethylene was not detected in the enrichment culture, then 55% of the cultures were false positives. As will be discussed later, it is possible that the community of dechlorinating organisms was damaged during collection, transport, or storage of the sample prior to construction of the enrichment cultures. It is also possible that conditions in the enrichment cultures failed to support growth of organisms that could and did grow in the aquifer, and could dechlorinate PCE or TCE to ethylene. If the proportion of false positives is calculated on the basis of cultures that produced any transformation product, then 4 out of 24 enrichment cultures failed to produce ethylene. On this basis, the proportion of false positives is 17%.

It is also possible that the *Dehalococcoides* DNA detected by the PCR assay belonged to strains that could not transform chlorinated ethylenes to ethylene.

Although the number of false positives in our survey is high, the PCR assay can still be useful to evaluate monitored natural attenuation. This is particularly true when the overall determination is based on a variety of tests and conditions. The interpretation of the PCR assay is more straightforward if it is supported by other information that would be consistent with on-going dechlorination to ethylene in the aquifer. Additional information might include geochemical conditions that are conducive to the growth of *Dehalococcoides* species, a reduction in the concentration of vinyl chloride in ground water over time, and the accumulation of significant concentrations of ethylene.

An absence of *Dehalococcoides* DNA would suggest that *Dehalococcoides* organisms were absent from the ground water and that dechlorination would not proceed to vinyl chloride or ethylene. If false negatives for the assay are defined as water samples where *Dehalococcoides* DNA was not detected, but vinyl chloride or ethylene was detected in the enrichment culture, then the proportion of false negatives is 15 out of 100 enrichments, or 15%. When *Dehalococcoides* DNA was not detected in the ground water used to establish the cultures, 35 out of 100 enrichments showed production of some transformation product (dichloroethylene, vinyl chloride, or ethylene). The cultures transformed PCE or TCE to ethylene in 7 out of the 35 enrichments, to vinyl chloride in 8 of the 35 enrichments, and stopped at the level of DCE in 20 of the 35 enrichments. The proportion of false negative predictions, as evaluated against the number of enrichment cultures that showed production of at least one of the transformation products, was 15 out of 35 cultures, or 43%. A failure to detect *Dehalococcoides* DNA in a sample of ground water using the PCR assay should not be taken to mean that dechlorination in the aquifer will stop at the level of DCE.

Several interactions might account for the complete lack of dechlorination activity in roughly half of the microcosms. First, the activity of dechlorinating bacteria might be suppressed by competition from other hydrogen utilizing bacteria such as the methanogens. Yang and McCarty (1998) reported that dechlorinators competed best against methanogens and acetogens when the hydrogen level was maintained between 2

and 11 nM. The basal medium in the microcosms is a much richer nutritional environment than the contaminated ground water at the sites. In the enrichment cultures, the hydrogen level was greater than 11 nM in 72% of the enrichment cultures. This may have posed a competitive advantage to other bacteria over dechlorinating bacteria.

Another possibility is that the living dechlorinating bacteria in the ground water samples that were returned to the laboratory for the enrichment study were killed by oxygen before the enrichment cultures were constructed. Many of the water samples had detectable concentrations of dissolved oxygen. The oxygen may have entered the well water in the monitoring well when oxygenated ground water in uncontaminated portions of the aquifer was mixed with the contaminant plume. The oxygen may also have entered the well water from the atmosphere when the well was sampled.

A plot of the relationship between the production of ethylene in the enrichment cultures against the dissolved oxygen concentration measured in the field in the corresponding ground water used to inoculate the culture indicated that the dechlorination activity was strongly influenced by the dissolved oxygen concentration (Figure 6.1). The production of ethylene was expressed as the molar ratio of the final concentration of ethylene to the initial concentration of PCE or TCE supplied to the enrichments. In all 20 enrichment cultures where *Dehalococcoides* DNA was detected in the ground water used to establish the enrichment cultures, and the enrichment cultures had accumulated ethylene, the corresponding dissolved oxygen concentration in the well water sample used to establish the enrichment culture was below 0.5 mg/L. This observation agrees with reports that *Dehalococcoides* organisms are strict anaerobes. The same relationship held for the enrichments where *Dehalococcoides* DNA was not detected in the ground water used to establish the enrichment culture. With two exceptions, no ethylene was produced in any enrichment that was inoculated with ground water that had dissolved oxygen concentrations higher than 0.5 mg/L. In the two exceptional enrichments, only trace concentrations of ethylene were detected (the molar ratio of the final concentration of ethylene to the initial concentration of TCE was less than 0.01).

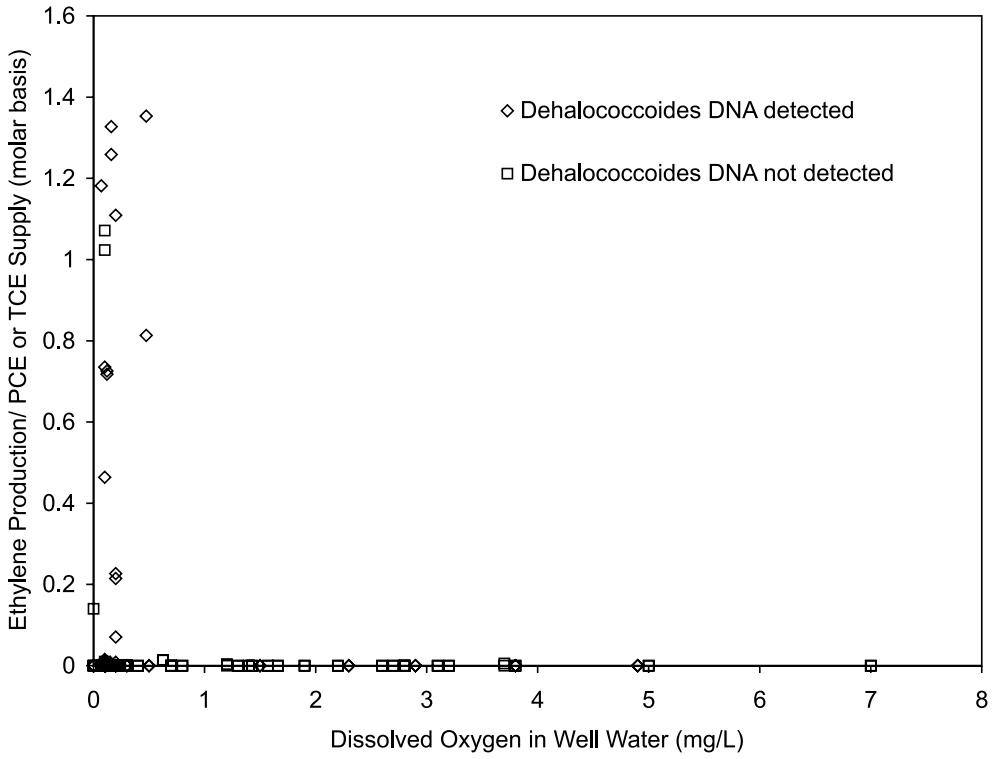

Figure 6.1. *Relationship between the production of ethylene in the enrichment cultures and the concentration of dissolved oxygen in the corresponding ground water used for the inoculums of the enrichment cultures. Diamond symbols are ground water samples where Dehalococcoides DNA was detected, and square symbols are ground water samples where Dehalococcoides DNA was not detected.*

Table 6.3 compares the extent of dechlorination in the enrichment cultures with the geochemical character of the ground water used to inoculate the culture. (See Section 5 for the definition of the geochemical categories.) Dechlorination of PCE and TCE to ethylene or vinyl chloride occurred most frequently and most extensively in the enrichment cultures inoculated with methanogenic ground water. Dechlorination to vinyl chloride or ethylene was limited in cultures inoculated with water that was sulfate-reducing, iron-reducing, or oxic. However, dechlorination to DCE was frequent and extensive when the cultures were inoculated with ground water that was sulfate-reducing or iron-reducing.

Table 6.3. Comparison of Biotransformation of Chlorinated Ethylenes in Enrichment Cultures to the Corresponding Geochemistry of the Ground Water used for Inoculation of the Enrichment Cultures

Geochemistry	Number of Enrichment Cultures in Category			
	Mole Percent of Final Ethylene to Initial PCE or TCE			
	No Ethylene	<1%	1%-50%	>50%
Methanogenic	29	5	9	9
Sulfate and/or iron reducing	52	0	0	2
Oxic	44	2	0	0
	Mole Percent of Final VC to Initial PCE or TCE			
	No VC	<1%	1-50%	50%
Methanogenic	39	1	8	4
Sulfate and/or iron reducing	51	3	0	0
Oxic	44	1	1	0
	Mole Percent of Final DCE to Initial PCE or TCE			
	No DCE	<1%	1-50%	50%
Methanogenic	25	18	9	0
Sulfate and/or iron reducing	29	18	7	0
Oxic	38	7	1	0

Section 7.
Recommendations to Evaluate Biotransformation of Chlorinated Solvents

Based on the performance of PCR assays at the sites in this study that were undergoing natural attenuation of chlorinated solvents, the authors offer a number of recommendations for collecting samples for PCR assays and interpreting the data provided. The recommendations are summarized in Table 7.1 and are discussed in detail in the remainder of this section.

Recommendations for Interpreting Data on Density of DNA in Ground Water

The OSWER Directive on MNA (U.S. EPA 1999) identifies three lines of evidence that can be used to support the selection of MNA as a remedy. The first line of evidence is *historical monitoring data that provide a clear and meaningful trend of decreasing concentrations or contaminant mass over time*. The second line of evidence is *hydrogeologic and geochemical data that can be used to demonstrate indirectly the types of natural attenuation processes active at the site, and the rate at which such processes will reduce contaminant concentrations to required levels*. The third line of evidence is *data from the field or microcosm studies which directly demonstrate the occurrence of a particular natural attenuation process at the site and its ability to degrade the contaminant of concern*. The presence of *Dehalococcoides* DNA in an aquifer can contribute to the third line of evidence.

As specified in the OSWER Directive (U.S. EPA 1999), *unless EPA or the overseeing regulatory authority determines that historical data* (the first line of evidence) *are of sufficient quality and duration to support a decision to use MNA, data characterizing the nature and rates of natural attenuation processes at the site* (the second line of evidence) *should be provided. Where the latter are inadequate or inconclusive, data from microcosm studies* (or genetic analysis, the third line of evidence) *may also be necessary*.

Data provided from analysis of DNA in water samples from wells are a semi-quantitative lower boundary on the density of organisms in the aquifer. The microorganisms may be attached to aquifer solids, and as a consequence, not adequately sampled by ground water from a monitoring well. As a practical matter, many evaluations of the distribution of *Dehalococcoides* DNA will be done with ground water samples from permanent wells. These evaluations done with samples of ground water will be subject to false negatives.

As a consequence, the density of *Dehalococcoides* cells in ground water does not provide direct evidence for a particular rate of biotransformation of chlorinated solvents. An assay for *Dehalococcoides* DNA in ground water does not provide the second line of evidence for natural attenuation. However, an assay for *Dehalococcoides* DNA in ground water can readily provide the third line of evidence for natural attenuation.

Although the PCR assay for *Dehalococcoides* DNA in ground water can only provide the third line evidence, the assay has two desirable features. The assays are relatively inexpensive, and they can be preformed in a short period of time. The PCR assay can reduce uncertainty in the role and contribution of biological reductive dechlorination to monitored natural attenuation in plumes of chlorinated ethylenes in ground water.

The strong possibility of false negatives for the presence of *Dehalococcoides* DNA makes it important that no interpretation be put on a failure to recover *Dehalococcoides* DNA from a water sample. The fact that *Dehalococcoides* DNA is not detected in a sample of well water does not mean that *Dehalococcoides* organisms are absent in the aquifer. This is particularly true if DNA corresponding to a universal bacterial primer is absent from the water sample. If the assay fails to detect *Dehalococcoides* DNA, it simply fails to contribute to the third line of evidence.

If the third line of evidence is critical to accepting MNA as a remedy, then other means to provide the third line of evidence are necessary, or MNA should be rejected. If the third line of evidence is not critical,

Table 7.1. Recommendations for Use of PCR Assays to Evaluate Biotransformation of Chlorinated Solvents

Concern	Recommendation
Does PCR for *Dehalococcoides* DNA provide the Second Line of Evidence for MNA?	PCR should not be expected to provide the Second Line of Evidence when applied to water samples from monitoring wells.
Does PCR for *Dehalococcoides* DNA provide the Third Line of Evidence for MNA?	The presence of DNA from *Dehalococcoides* provides the Third Line of Evidence. However, the absence of DNA from *Dehalococcoides* should not be interpreted as the absence of biological natural attenuation.
Can PCR for *Dehalococcoides* DNA provide the Second Line of Evidence in the future?	It will be necessary to extract DNA from sediment samples, and compare data on density of *Dehalococcoides* cells to rates attained in benchmark field studies.
How should sites be "scored" to determine whether site characterization of biological processes is justified?	The scoring system in the EPA *Technical Protocol to Evaluate Natural Attenuation at Chlorinated Solvents in Ground Water* should be replaced with Equation 5.1 in Section 5 of this report.
How can limited PCR data from a few wells be extrapolated to other wells in a contaminated aquifer?	Rather than assume that PCR data from a few wells apply to an entire aquifer, use Equation 5.1 to estimate whether it is likely that *Dehalococcoides* is present in ground water from a particular well.
Which wells should have the highest priority for a PCR assay for *Dehalococcoides*?	Sample wells screened in material with high hydraulic conductivity compared to the rest of the aquifer, and wells with high concentrations of transformation products. Sample an equal number of wells in the source area, in the region with intermediate concentrations, and at the toe of the plume.
What precautions are needed to sample monitoring wells for a PCR assay for *Dehalococcoides*?	Avoid cross contamination. Use dedicated sampling tubing. The EPA low flow procedure is not optimal for sampling for *Dehalococcoides.* Pump as rapidly as possible.
What precautions are needed to collect water samples for a PCR assay for *Dehalococcoides*?	Collect one liter samples into plastic bottles that have never been used for another purpose. Collect spare samples from each well. Store on ice in the field before transportation to the laboratory. Protect samples from cross contamination during storage and transportation. Prepare shipping container to keep samples cool during transportation to the laboratory.
What controls are needed to document data quality?	Prepare field blanks and field duplicates as specified in the Quality Assurance Plan for the site. Include positive and negative controls for amplification of DNA in the PCR assay, and include a control for extraction of bacterial DNA from the samples.

then the presence of *Dehalococcoides* DNA merely strengthens a decision that was based on the first two lines of evidence. The absence of *Dehalococcoides* DNA fails to strengthen a decision that was made on the first two lines of evidence.

Whenever possible, aquifer solids should be sampled and assayed for *Dehalococcoides* DNA. This will reduce the chance of false negative results for the presence of *Dehalococcoides* DNA as determined by the qualitative assay using electrophoresis, or the density of *Dehalococcoides* cells as determined by the quantitative real time PCR assay. If aquifer solids are extracted and analyzed, there is at least a possibility that at some time in the future it will be possible to relate the density of active organisms as revealed by a PCR assay to the achieved rate of reductive dechlorination at field scale. At many sites, it is possible to recover core samples quickly and at low cost with push technology (e.g. GeoProbe® tools). At other sites, the cost of acquiring core samples may be prohibitively expensive.

Recommendations for Interpreting Geochemistry of Ground Water

The scoring system in the Technical Protocol for Evaluating Natural Attenuation of Chlorinated Solvents in Ground Water (Wiedemeier et al., 1998) was offered as a screening mechanism to identify ground water where biological reductive dechlorination is likely to occur; however, the scoring system has been criticized. Equation 5.2 as discussed in Section 5 provides a simple and rapid way to calculate the probability that a specific dechlorinating organism, *Dehalococcoides*, is present at the site. The probability that *Dehalococcoides* DNA occurs in the ground water, as calculated by Equation 5.2, should replace the scoring system. However, a calculated probability that an organism exists in ground water is not equivalent to a PCR assay for its presence, and probabilities calculated from geochemical parameters should not be used to replace PCR assays.

Often the computer models that are used to evaluate monitored natural attenuation are distributed parameter models. To properly calibrate these computer models, it is necessary to know the distribution of the capacity to transform chlorinated ethylenes throughout the aquifer. Data from the PCR assay may not be available from every well at a site. The calculated probability of *Dehalococcoides* organisms based on the geochemistry of ground water in a well could be used to assign rate constants to cells in a model. If *Dehalococcoides* organisms are expected, a rate constant characteristic of reductive dechlorination at that site would be assigned. If *Dehalococcoides* organisms are not expected, the rate constant for reductive dechlorination would be set to zero in that particular region of the aquifer.

Recommendations for Selecting Wells for Sampling

At many chlorinated solvent sites, computer models have been used to describe the previous behavior of the plume and make future projections of its natural attenuation over time. Often these computer projections are an important part of the conceptual model of a site. Frequently, the calibration of the computer models will assume a uniform rate constant for biotransformation across the entire plume or major portions of the plume. The calibration of the model frequently assumes that the rate constant will be sustained into the future. If biotransformation carried out by *Dehalococcoides* organisms is the primary process for natural attenuation, an assay for *Dehalococcoides* DNA can be used to test these assumptions about uniform distribution and sustainability of biotransformation. *Dehalococcoides* organisms should be uniformly present in those portions of the aquifer where the computer model projects a rate constant for biotransformation, and the populations of *Dehalococcoides* organisms should be sustained during long-term monitoring.

At most sites, there is little value in sampling every well for analysis of *Dehalococcoides* DNA. Wells with relatively high hydraulic conductivity should be sampled from the more permeable portions of the aquifer. These are the portions of the aquifer with the greatest capacity to transport contaminated water and may be the portions that provide the most risk of impacting a receptor. Wells with higher hydraulic conductivity should be selected for assays for *Dehalococcoides* DNA.

Wells with higher relative concentrations of transformation products provide circumstantial evidence that biotransformation has occurred at some point along the flow path from the source to the well. However, the high concentrations of transformation products do not prove that the transformation occurred in the portion of the aquifer immediately proximate to the monitoring well. Nonetheless, the wells with higher relative concentrations of vinyl chloride and ethylene should be sampled.

Wells should be sampled at various positions along the flow path from the source to the most down gradient wells containing detectable concentrations of chlorinated solvents. For the specific purpose of evaluating *in situ* biotransformation of chlorinated solvents, there

is little value in sampling background wells. Equation 5.2 as discussed in Section 5 can be used to identify wells where the geochemical environment is favorable for *Dehalococcoides* species, and to select wells for a PCR assay for *Dehalococcoides* species. Sample an equal number of wells in the source area, in the region with intermediate concentrations, and in the distal portion of the plume where concentrations are within a factor of ten to one hundred of the Maximum Contaminant Level (MCL) or other relevant clean-up goals.

There is little value in sampling for *Dehalococcoides* DNA in every round of sampling. A better strategy is to perform a comprehensive baseline assessment across a plume as part of the selection of MNA as a remedy, and perform a second comprehensive assessment before the performance of the remedy goes for review. The biogeochemical parameters, particularly concentrations of nitrate, methane, and ORP should be determined at the same time ground water is collected for determination of *Dehalococcoides* DNA.

Recommendations for Sampling and Shipping of Samples

To avoid any cross-contamination, equipment to purge and sample ground water should not be moved from one well to another even if an attempt is made to sterilize or decontaminate the equipment. The PCR assay can amplify DNA, even though the organisms have been killed. Water samples should be obtained using a dedicated pump or pump tubing, freshly installed pump tubing, a disposable pump that has not been used on another well, or a new clean bailer and line. Neither the bailer nor the line should have been used on another well. If possible, avoid using bailers. If water is produced with a peristaltic pump at the well head, the peristaltic pump tubing and effluent tubing should be replaced immediately before a well is purged and sampled. A minimum of one casing volume should be purged before the sample is taken. It is better to purge the casing volume plus a volume equal to the porosity of the sand pack around the well screen, if one is present.

The EPA low-flow sampling protocol is designed to produce a sample that is free of turbidity and suspended solids. If *Dehalococcoides* cells are sorbed to sediment particles in the aquifer, they may not be sampled efficiently. Sediment and turbidity in the water sample do not interfere with the extraction of bacterial DNA. To increase the turbidity and suspended solids in the water sample, purge and sample the well at the fastest rate the pump will allow. Set the pump or the end of the sampling tube at the center of the screened interval of the monitoring well.

Water samples for analysis of *Dehalococcoides* DNA should be collected in a one liter plastic bottle constructed of polypropylene (e.g. Nalgene®) or high density polyethylene. The bottles should be new and should never have been used for another purpose. Use the bottles as supplied by the manufacturer. Do not attempt to clean the bottles before use. If the original shipping container has been opened, the empty bottles should be shipped to the field site with the lid firmly attached to the bottle.

Fill the bottle in the field and screw the lid back on the bottle to make a tight seal. As far as possible, avoid exposing the water sample to the atmosphere. Fill the bottle with the fill tube at the bottom of the bottle. Do not allow the water sample to flow down the inside of the bottle.

Label the sample with a permanent marker on a strip of labeling tape that entirely circles the bottle. Provide, at a minimum, the complete name of the well being sampled, the name of the facility or location where the well is installed, the date and time the sample was collected, and the name or initials of the person collecting the samples. If a chain of custody form is required, fill in the required information at the same time. Seal the sample bottle in a plastic bag with a ZipLock® closure or equivalent. Remove excess air from the bag before the closure is sealed. The purpose of the bag is to prevent cross contamination of the samples in case one of the sample bottles leaks during shipment. Place the bottles on water ice immediately after they are collected, labeled, and sealed in a plastic bag. Keep the bottles on ice until they are packaged for shipment for analysis.

Collect a sample and a spare sample from each well sampled. Ship both samples for analysis. Identify which is the sample and which is the spare. The spare will be available to the laboratory analyst if there is any problem with preparation or analysis of the sample. If a field duplicate is desired, collect a third sample from the well and label the third sample as the field duplicate.

Prepare water to make a field blank by filtering distilled water or deionized water through a 0.22 micron filter into a sterile container. This is best done in the laboratory before going to the field. To prepare a trip blank, take the filtered water to the well head of the well at the site that is most likely to contain *Dehalococcoides* and pour the previously prepared water

into a one liter plastic bottle just as would be done for any other sample. Seal the lid, label the bottle, seal in a plastic bag, store on water ice, and ship to the laboratory along with the other samples.

Collect and ship the number of trip blanks and field duplicates that are specified in the quality assurance project plan for the site.

Package the samples in a plastic cooler with an adequate number of bricks of frozen brine sealed in a plastic cover (Blue Ice® or equivalent) to keep the samples at or below 10° C for two days. Do not ship the samples with water ice. The samples should arrive at the laboratory within five days after they were originally collected in the field. If the field site is remote from the analytical laboratory, ship the samples by air freight for overnight delivery. Avoid shipping on a Friday or the day before a holiday if the receiving laboratory will not be open for business and not available to accept and properly store the samples.

The laboratory should provide information in their report on the procedures used to prepare the water samples for the polymerase chain assay, on the number and types of primers used, and the results with each primer. The laboratory should also provide information on the number of gene copies that would be required to provide the minimal density of *Dehalococcoides* DNA that can be detected by the assay. The minimum number of gene copies should be expressed in gene copies per liter of well water or gene copies per kilogram sediment, whichever is appropriate.

Dehalococcoides DNA may be absent in a water sample because bacteria of any kind were absent in the sample. To be able to determine if *Dehalococcoides* organisms are not present in the microbial community of the aquifer being sampled, it is necessary to include a primer for a gene that is essentially universal in bacteria in the polymerase chain reaction assay. The absence of bacterial DNA suggests that the microbial community in the aquifer was not effectively sampled, and that *Dehalococcoides* organisms may have been present but were not sampled. Positive and negative controls should be included in the PCR assay to ensure that the reaction is working properly and that contamination of reagents has not occurred. Amplicons (DNA amplified by the PCR assay using the *Dehalococcoides* primers) should be cloned and sequenced periodically to ensure that the assay is working as intended.

Section 8.
Data Quality

Analysis of Chemical Concentrations

Laboratory analyses for data presented in Table 4.2, Table 4.4, Table 4.6, Table 5.2, Table 5.3, Table 5.4, Table 5.5, Table 5.7, and Table 6.4 were conducted at the R.S. Kerr Environmental Research Center in accordance with a Quality Assurance Project Plan prepared for in-house task 3674 (Monitored Natural Attenuation of Chlorinated Solvents). Concentrations of chlorinated solvents, dissolved gases (methane, ethylene, ethane, and hydrogen), inorganic compounds (nitrate plus nitrite, sulfate, and chloride), fatty acids (propionate and acetate), and total organic carbon (TOC) were determined following in-house Standard Operating Procedures (SOPs). Chlorinated solvents were analyzed by automated headspace gas chromatography and mass spectrometry. Methane, ethylene, and ethane were analyzed by gas chromatography with a thermal conductivity detector. Hydrogen was analyzed by RGA3 Reduction Gas Analyzer with a reduction gas detector. Nitrate plus nitrite was analyzed by Lachat flow injection analysis. Sulfate and chloride were analyzed by waters capillary electrophoresis. Total Organic Carbon (TOC) was analyzed by Dohrman DC-80 Carbon Analyzer. Propionate and acetate were analyzed by high performance liquid chromatography.

Major quality assurance and quality control (QA/QC) evaluations for the analyses included method blank (MB), continuing calibration check (CCC), second source check (QC) using a sample obtained from the second source as identified by their designated names, laboratory duplicates (LD), and matrix spike (MS). Method blank was analyzed in the beginning and end of sample set. Calibration check standards were analyzed every ten samples as well as in the beginning and end of sample set. QC checks were analyzed every ten samples. Lab duplicates were analyzed every ten samples. Matrix spikes were analyzed every twenty samples. The data quality objectives were as follows: The target analyte in the method blank would be below method detection limit. The reported concentration of continuing calibration check standard, QC check standard, and matrix spike would agree with the expected concentration plus or minus 20% of the known concentration (i.e., recovery of the expected value would be in the range of 80-120%). Laboratory duplicates would agree with each other plus or minus 20%. All the samples were held less than thirty days before analysis.

Table 8.1 summarizes typical data quality for TCE in the ground water samples and in the water samples from the enrichment cultures. Two out of 38 of the calibration check standards did not meet the goal of ± 20% of the nominal values (One calibration check analyzed on 4/2/03 was reported as 122% of the nominal value, and one calibration check analyzed on 6/26/03 was reported as 69% of the nominal value.) One out of 43 of the QC check standards did not meet the goal of ± 20% of the nominal values (One QC check analyzed on 4/2/03 was reported as 126% of the nominal value.) One out of 36 laboratory duplicates did not agree within 20% (The relative percent difference for the duplicates analyzed on 4/2/03 was 21%.) Four out of 50 method blanks had TCE concentration above method detection limit (The values were 0.99 and 0.675 on 4/2/03, and 1.03 and 0.89 on 4/30/03.) All 25 matrix spikes met the goal of ± 20% of the expected values.

Several quality controls did not meet the criteria on date 4/2/03 in the beginning of the analysis. The instrument was recalibrated, and the subsequent checks met the criteria. All the data for TCE were determined to be of acceptable quality, and the data were used in the report.

Table 8.2 summarizes typical data quality for *cis*-DCE in the ground water samples and in the water samples from the enrichment cultures. Five out of 38 of the calibration check standards did not meet the goal of ± 20% of the nominal values. (Two calibration checks analyzed on 3/9/03 were reported as 127% and 129% of the nominal values, one calibration check on 4/30/03 was reported as 136% the nominal value, one calibration check analyzed on 6/26/03 was reported as 78% of the nominal value, and one

calibration check analyzed on 1/20/04 was 123% of the nominal value.)

Four out of 43 of the QC check standards did not meet the goal of ± 20% of the nominal values (One QA check analyzed on 4/10/03 was reported as 129% of the nominal value, one QA check analyzed on 4/30/03 was reported as 153% of the nominal value, and two QA checks analyzed on 2/20/04 were reported as 169% and 145% of the nominal values.) All other quality controls met the objectives, including all 36 duplicates, which agreed with each other within 20% difference, all 50 method blanks did not have detectable *cis*-DCE, and all 25 matrix spikes met the goal of ± 20% of the expected values.

The chromatographic response for *cis*-DCE in the two QA checks analyzed on 2/20/04 showed evidence of peak asymmetry suggesting coelution of another compound. The previous and subsequent recoveries for *cis*-DCE in the continuing calibration checks met the data quality objectives. On 4/30/03, the reported values for *cis*-DCE might be slightly elevated over the true values, as shown in the continuing calibration checks and QC checks. Not all of the calibration checks met the goal of ± 20% of the nominal values. When the calibration checks did not meet the goal, they were still within 36% of the nominal values. An error of 36% would not change the interpretation placed on the data. Therefore, all the data for *cis*-DCE were determined to be of acceptable quality, and the data were used in the report.

Table 8.3 summarizes typical data quality for vinyl chloride in the ground water samples and in the water samples from the enrichment cultures. Compared to TCE and *cis*-DCE, vinyl chloride is more volatile and more easily lost, resulting in more missed data quality objectives. Three out of 38 of the calibration check standards did not meet the goal of ± 20% of the nominal values (One calibration check analyzed on 3/9/03 was reported as 78% of the nominal value, one calibration check analyzed on 3/30/03 was reported as 76% of the nominal value, and one calibration check analyzed on 6/5/03 was reported as 131% of the nominal value.) Four out of 43 of the QC check standards did not meet the goal of ± 20% of the nominal values (One QC check analyzed on 4/10/03 was reported as 77% of the nominal value, one QC check analyzed on 6/26/03 was reported as 49% of the nominal value, and two QC checks analyzed on 10/28/03 were reported as 51% and 62% of the nominal values.) Three out of 36 laboratory duplicates did not agree within 20% (The relative percent differences were 43.5% for the duplicates analyzed on 3/21/03, 84.1% for duplicates analyzed on 6/26/03, and 42.9% for duplicates analyzed on 6/26/03.) One out of 50 method blanks had vinyl chloride concentration above method detection limit. Five out of 25 matrix spikes did not meet the goal of ± 20% of the expected values (See Table 8.3.).

Most of the missed data quality objectives for vinyl chloride were due to the problem of preparing the check standards, not the problem of the instrument. Subsequent samples using the analysis applied a modified standard preparation technique to minimize vinyl chloride losses. All the data for vinyl chloride were determined to be of acceptable quality, and the data were used in the report.

If there is no data provided for calibration check controls (CCCs) in Tables 8.1, 8.2, and 8.3, that information was not included in the report provided by the analyst.

Table 8.4 summarizes typical data quality for ethylene in the ground water samples and in the gas phase of the enrichment cultures. All 98 calibration checks met the goal of ± 20% of the nominal values. All 24 method blanks did not have detectable ethylene. All 16 laboratory duplicates agreed with each other within 20% difference. All seven matrix spikes met the goal of ± 20% of the expected values.

All the data for ethylene were determined to be of acceptable quality, and the data were used in the report.

Table 8.5 summarizes typical data quality for methane in the ground water samples and in the gas phase of the enrichment cultures. All 119 calibration checks met the goal of ± 20% of the nominal values. All 24 method blanks did not have detectable methane. All 16 laboratory duplicates agreed within 20% except the duplicates analyzed on 8/25/03 where the relative percent difference was 23.0%. All seven matrix spikes met the goal of ± 20% of the expected values.

All the data for methane were determined to be of acceptable quality, and the data were used in the report.

Table 8.6 summarizes typical data quality for hydrogen in the gas samples stripped from ground water and in the gas phase of the enrichment cultures. All 106 calibration checks met the goal of ± 20% of the nominal values. Two out of 22 method blanks had

hydrogen concentration above detection limit. Three out of 12 laboratory duplicates did not agree within 20% (See Table 8.6.).

All the data for hydrogen were determined to be of acceptable quality, and the data were used in the report.

Table 8.7 summarizes typical data quality for nitrate plus nitrite nitrogen in the ground water samples. All 26 calibration checks met the goal of 20% of the nominal values. All seven QC checks met the goal of ± 20% of the nominal values. All ten laboratory duplicates agreed with each other within 20% difference. All 16 method blanks had nitrate plus nitrite nitrogen concentration below quantification limit. All ten matrix spikes met the goal of ± 20% of the expected values.

All the data for nitrate plus nitrite nitrogen were determined to be of acceptable quality, and the data were used in the report.

Analysis of DNA Concentrations

The analyses of *Dehalococcoides* DNA were performed by SiREM (Guelph, Ontario) using their Gene-Trac® Test. The QA/QC was maintained by implementing clean techniques and control PCR reactions.

DNA was extracted using a single-use sterile filter unit and single-use DNA extraction kit. Prior to PCR, all micropipettes and other equipments used in setting up reactions were swabbed with DNA AWAY™, and 10% bleach or 70% ethanol to ensure cleanliness and sterility. PCR reaction mixtures were assembled in a Forma HEPA flow cabinet to prevent the introduction of particles and bacteria/DNA that might produce false positives.

Three types of control reactions were used in the Gene-Trac procedure: a negative control, a positive control, and a DNA extraction control. The controls were conducted and interpreted by SiREM, the vendor for the PCR assays. The negative control involved processing sterile water through the same DNA extraction procedure as the sample. It ensured that contamination of samples did not occur via the DNA extraction process, PCR setup, or performance of the reactions. In the data reported to EPA, a sample was flagged when DNA was amplified in the negative control. There was no DNA amplified in the negative control in the data reported in Tables 4.5, 4.8, or 5.4. The positive control consisted of a PCR assay containing a cloned *Dehalococcoides* 16S rRNA gene. It ensured that all reagents and equipment were performing properly. If the PCR procedure failed to amplify the *Dehalococcoides* 16S rRNA gene, the data were discarded by SiREM and were not reported to U.S. EPA. The problem with the PCR procedure was corrected by SiREM, and the DNA extracted from the ground water sample was assayed and reported. The DNA extraction control was performed when the *Dehalococcoides* test was negative. In the DNA extraction control, PCR was performed on the sample using a universal bacterial PCR primer set. It was used to determine whether bacterial DNA was present and extracted from the sample. The results of the DNA extraction control are provided in Tables 4.5 and 4.8, and are interpreted in Section 4. PCR data from the *Dehalococcoides* primers from three wells sampled at the Area 2500 Site at the former England AFB, Louisiana, were discarded because the bacterial DNA primer was not detected.

Table 8.1. Typical Quality Performance Data for Analysis of TCE in Water. All Values are µg/L Unless Otherwise Indicated

Date Collected				1/24/03	2/4/03
Date Analyzed	11/7/02		1/7/03	2/11/03	3/6/03
CCC Standard Nominal			10		
CCC Standard Measured			10.1		
Percent of Check Standard			101%		
CCC Standard Nominal			100		
CCC Standard Measured			96		
Percent of Check Standard			96%		
CCC Standard Nominal			250		
CCC Standard Measured			235		
Percent of Check Standard			94%		
QC Standard Nominal	20	20	50	20	20
QC Standard Measured	19.3	22.1	51.0	21.1	21.3
Percent of Check Standard	96.4%	111%	107%	106%	107%
QC Standard Nominal	200	200	50	200	200
QC Standard Measured	189	219	48.1	225	194
Percent of Check Standard	94.4%	110%	96.2%	113%	97%
Blank 1	<0.5	<0.23	<0.39	<0.3	<0.23
Blank 2	<0.5	<0.23	<0.39	<0.3	<0.23
Sample Analysis 1	80.5	2180	227	263	<0.23
Laboratory Duplicate 1	75.2	2130	225	235	<0.23
Relative Percent Difference	6.8%	2.3%	0.9%	11.2%	-
Sample Analysis 2				231	
Laboratory Duplicate 2				240	
Relative Percent Difference				3.8%	
Spike Concentration 1	200	200	50	200	200
Sample Concentration 1	<0.5	28.4	<0.39	215	10.4
Spike Recovery (Percent)	97%	108%	83.6%	100%	105%
Spike Concentration 2			50		
Sample Concentration 2			232		
Spike Recovery (Percent)			87.2%		
Date Collected	2/24/03	3/6/03	3/11/03	3/26/03	4/8/03
Date Analyzed	3/9/03	4/2/03	3/21/03	4/10/03	4/30/03
CCC Standard Nominal	50	10		10	10
CCC Standard Measured	44.2	12.2		10.6	11.2
Percent of Check Standard	88%	122%		106%	112%
CCC Standard Nominal	200	100		250	50
CCC Standard Measured	168	113		259	45
Percent of Check Standard	84%	113%		104%	90%

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.1. Typical Quality Performance Data for Analysis of TCE in Water. All Values are µg/L Unless Otherwise Indicated *continued*

CCC Standard Nominal	50	10		250	100
CCC Standard Measured	42	8.44		253	108
Percent of Check Standard	84%	84.4%		101%	108%
QC Standard Nominal	20	25	10	100	50
QC Standard Measured	23.4	26.8	9.7	111	58.1
Percent of Check Standard	117%	107%	97%	111%	116%
QC Standard Nominal	200	50	200		
QC Standard Measured	211	63.0	202		
Percent of Check Standard	105%	126%	101%		
Blank 1	<0.39	0.99	<0.07	<0.39	1.03
Blank 2	<0.39	0.675	<0.07	<0.39	0.89
Sample Analysis 1	204	63.9	96.7	158	<0.39
Laboratory Duplicate 1	203	51.7	102	157	<0.39
Relative Percent Difference	0.5%	21.1%	5.3%	0.6%	-
Sample Analysis 2	212			160	
Laboratory Duplicate 2	192			156	
Relative Percent Difference	9.9%			2.5%	
Spike Concentration 1	200	100	100		
Sample Concentration 1	234	36.4	37.2		
Spike Recovery (Percent)	100%	81%	95%		
Spike Concentration 2					
Sample Concentration 2					
Spike Recovery (Percent)					
Date Collected	4/21/03	4/29/03	5/16/03	6/23/03	7/23/03
Date Analyzed	5/20/03	5/5/03	6/5/03	6/26/03	8/5/03
CCC Standard Nominal	10			25	
CCC Standard Measured	11.4			24	
Percent of Check Standard	114%			96%	
CCC Standard Nominal	50			100	
CCC Standard Measured	51.2			93.9	
Percent of Check Standard	102%			93.9%	
CCC Standard Nominal	100			250	
CCC Standard Measured	98			173	
Percent of Check Standard	98%			69.2%	
QC Standard Nominal	50	20	20	50	20
QC Standard Measured	52.3	21.3	20.3	44.8	22.3
Percent of Check Standard	105%	106%	102%	89.6%	112%
QC Standard Nominal		200	50		200

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.1. Typical Quality Performance Data for Analysis of TCE in Water. All Values are µg/L Unless Otherwise Indicated *continued*

QC Standard Measured		206	48.4		218
Percent of Check Standard		103%	97%		112%
Blank 1	<0.39	<0.23	<0.07	<0.39	<0.23
Blank 2	<0.39	<0.23	<0.07	<0.39	<0.23
Sample Analysis 1	344	<0.23	185	241	<0.23
Laboratory Duplicate 1	348	<0.23	182	231	<0.23
Relative Percent Difference	1.2%	-	1.6%	4.2%	-
Sample Analysis 2			182	188	
Laboratory Duplicate 2			179	202	
Relative Percent Difference			1.7%	7.2%	
Spike Concentration 1		200	200		100
Sample Concentration 1		<0.23	190		247
Spike Recovery (Percent)		102%	91%		84%
Date Collected	8/7/03	8/20/03	10/3/03	10/7/03	10/24/03
Date Analyzed	8/12/03	8/22/03	10/22/03	11/3/03	10/28/03
Spike Concentration 2			200		
Sample Concentration 2			168		
Spike Recovery (Percent)			84%		
CCC Standard Nominal			500	100	25
CCC Standard Measured			547	98.6	24.5
Percent of Check Standard			109%	98.6%	98%
CCC Standard Nominal					50
CCC Standard Measured					49.9
Percent of Check Standard					99.8%
CCC Standard Nominal					100
CCC Standard Measured					97.6
Percent of Check Standard					97.6%
QC Standard Nominal	20	20	20	100	5
QC Standard Measured	19.3	20.5	21	99.6	5.48
Percent of Check Standard	96%	103%	105%	100%	110%
QC Standard Nominal	200	200	200		50
QC Standard Measured	214	210	178		46.4
Percent of Check Standard	107%	105%	89%		92.8%
Blank 1	<0.23	<0.23	<0.07	<0.07	<0.39
Blank 2	<0.23	<0.23	<0.07	<0.07	<0.39
Sample Analysis 1	127	1410	155	60.0	147
Laboratory Duplicate 1	119	1370	155	57.0	143
Relative Percent Difference	6.5%	2.9%	0.0%	5.1%	2.8%
Sample Analysis 2	106		186		150
Laboratory Duplicate 2	105		176		151

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.1. Typical Quality Performance Data for Analysis of TCE in Water. All Values are µg/L Unless Otherwise Indicated *continued*

Relative Percent Difference	0.9%		5.5%		0.7%
Spike Concentration 1	200	200	100	100	
Sample Concentration 1	111	9.3	163	59.3	
Spike Recovery (Percent)	102%	106%	91%	94.5%	
Spike Concentration 2	200		200		
Sample Concentration 2	108		3.3		
Spike Recovery (Percent)	105%		93%		
Date Collected	1/14/04	2/19/04	6/4/04	9/7/04	7/14/05
Date Analyzed	1/20/04	2/20/04	6/17/04	9/17/04	7/15/05
CCC Standard Nominal	10	10	20	20	20
CCC Standard Measured	8.03	10.8	19.9	20.6	17.8
Percent of Check Standard	80%	108%	100%	103%	89%
CCC Standard Nominal	100	50	20		200
CCC Standard Measured	92.5	50.4	20		193
Percent of Check Standard	93%	101%	100%		96%
CCC Standard Nominal	100	100			20
CCC Standard Measured	92.8	103			21
Percent of Check Standard	93%	103%			105%
QC Standard Nominal	50	10	20		20
QC Standard Measured	46.3	11.0	22.7		21.4
Percent of Check Standard	93%	110%	114%		107%
QC Standard Nominal	100	100	200		
QC Standard Measured	96.0	99.1	200		
Percent of Check Standard	96%	99%	100%		
Blank 1	<0.39	<0.39	<0.23	<0.23	<0.23
Blank 2	<0.39	<0.39	<0.23	<0.23	<0.23
Sample Analysis 1		1.20	80.8	149	148
Laboratory Duplicate 1		1.17	83.1	148	139
Relative Percent Difference		2.5%	2.8%	0.7%	6.3%
Sample Analysis 2		91.5	110	152	72.6
Laboratory Duplicate 2		93.2	108	154	63.8
Relative Percent Difference		1.8%	1.8%	1.3%	12.9%
Spike Concentration 1			100	100	100
Sample Concentration 1			<0.23	159	62.9
Spike Recovery (Percent)			105%	105%	95%
Spike Concentration 2			100	100	100
Sample Concentration 2			134	132	117
Spike Recovery (Percent)			99%	103%	89%

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.2. Typical Quality Performance Data for Analysis of *cis*-DCE in Water. All Values are µg/L Unless Otherwise Indicated

Date Collected	10/23/02	11/25/02	12/23/02	1/24/03	2/4/03
Date Analyzed	11/7/02	12/19/02	1/7/03	2/11/03	3/6/03
CCC Standard Nominal			10		
CCC Standard Measured			8.71		
Percent of Check Standard			87.1%		
CCC Standard Nominal			100		
CCC Standard Measured			100		
Percent of Check Standard			100%		
CCC Standard Nominal			250		
CCC Standard Measured			233		
Percent of Check Standard			93.2%		
QC Standard Nominal	20	20	50	20	20
QC Standard Measured	21.1	21.9	54.8	21.3	21.6
Percent of Check Standard	106%	110%	110%	107%	108%
QC Standard Nominal	200	200	50	200	200
QC Standard Measured	187	216	51.1	219	206
Percent of Check Standard	93.6%	108%	102%	110%	103%
Blank 1	<0.2	<0.2	<0.36	<0.2	<0.2
Blank 2	<0.2	<0.2	<0.36	<0.2	<0.2
Sample Analysis 1	79.3	57.1	111	0.48	<0.2
Laboratory Duplicate 1	75.7	54.8	110	0.48	<0.2
Relative Percent Difference	4.6%	4.1%	0.9%	0.0%	-
Sample Analysis 2				<0.2	
Laboratory Duplicate 2				<0.2	
Relative Percent Difference				-	
Spike Concentration 1	200	200	50	200	200
Sample Concentration 1	<0.2	28400	<0.36	0.48	72
Spike Recovery (Percent)	101%	108%	95%	105%	104%
Spike Concentration 2			50		
Sample Concentration 2			<0.36		
Spike Recovery (Percent)			106%		
Date Collected	2/24/03	3/6/03	3/11/03	3/26/03	4/8/03
Date Analyzed	3/9/03	4/2/03	3/21/03	4/10/03	4/30/03
CCC Standard Nominal	50	10		10	10
CCC Standard Measured	48	9.58		10.6	11.0
Percent of Check Standard	95%	95.8%		106%	110%
CCC Standard Nominal	200	100		250	50
CCC Standard Measured	254	105		258	68.2
Percent of Check Standard	127%	105%		103%	136%

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.2. Typical Quality Performance Data for Analysis of *cis*-DCE in Water. All Values are µg/L Unless Otherwise Indicated *continued*

CCC Standard Nominal	50	100		250	100
CCC Standard Measured	64	109		260	117
Percent of Check Standard	129%	109%		104%	117%
QC Standard Nominal	20	25	10	100	50
QC Standard Measured	22.1	24.4	9.4	129	76.3
Percent of Check Standard	110%	97.6%	94%	129%	153%
QC Standard Nominal	200	50	200		
QC Standard Measured	211	57.7	185		
Percent of Check Standard	105%	115%	92%		
Blank 1	<0.36	<0.14	<0.06	<0.14	<0.14
Blank 2	<0.36	<0.14	<0.06	<0.14	<0.14
Sample Analysis 1	<0.36	249	56.7	<0.14	<0.14
Laboratory Duplicate 1	<0.36	<0.14	57.7	<0.14	<0.14
Relative Percent Difference	-	-	1.7%	-	-
Sample Analysis 2	<0.36			0.42	
Laboratory Duplicate 2	<0.36			0.46	
Relative Percent Difference	-			9.1%	
Spike Concentration 1	200	100	100		
Sample Concentration 1	<0.36	40.7	18.8		
Spike Recovery (Percent)	109%	88%	95%		
Spike Concentration 2					
Sample Concentration 2					
Spike Recovery (Percent)					
Date Collected	4/21/03	4/29/03	5/16/03	6/23/03	7/23/03
Date Analyzed	5/20/03	5/5/03	6/5/03	6/26/03	8/5/03
CCC Standard Nominal	10			25	
CCC Standard Measured	10.3			19.5	
Percent of Check Standard	103%			78%	
CCC Standard Nominal	50			100	
CCC Standard Measured	51.1			91.8	
Percent of Check Standard	102%			91.8%	
CCC Standard Nominal	100			250	
CCC Standard Measured	102			264	
Percent of Check Standard	102%			106%	
QC Standard Nominal	50	20	20	50	20
QC Standard Measured	51.8	18.7	19.7	46.6	21
Percent of Check Standard	104%	93%	99%	93.2%	105%
QC Standard Nominal		200	50		200
QC Standard Measured		195	46.6		206

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.2. Typical Quality Performance Data for Analysis of *cis*-DCE in Water. All Values are µg/L Unless Otherwise Indicated *continued*

Percent of Check Standard		98%	93%		103%
Blank 1	<0.14	<0.2	<0.06	<0.14	<0.2
Blank 2	<0.14	<0.2	<0.06	<0.14	<0.2
Sample Analysis 1	17.5	<0.2	0.2	<0.14	<0.2
Laboratory Duplicate 1	19.4	<0.2	0.2	<0.14	<0.2
Relative Percent Difference	10.3%	-	0.0%	-	-
Sample Analysis 2			1.2	140	
Laboratory Duplicate 2			1.2	105	
Relative Percent Difference			0.0%	28.6%	
Spike Concentration 1		200	200		100
Sample Concentration 1		0.34	<0.06		12.9
Spike Recovery (Percent)		104%	94%		103%
Spike Concentration 2			200		
Sample Concentration 2			<0.06		
Spike Recovery (Percent)			87%		
Date Collected	8/7/03	8/20/03	10/3/03	10/7/03	10/24/03
Date Analyzed	8/12/03	8/22/03	10/22/03	11/3/03	10/28/03
CCC Standard Nominal			500	100	25
CCC Standard Measured			518	93.7	21.8
Percent of Check Standard			104%	93.7%	87.2%
CCC Standard Nominal					50
CCC Standard Measured					51
Percent of Check Standard					102%
CCC Standard Nominal					100
CCC Standard Measured					95.6
Percent of Check Standard					95.6%
QC Standard Nominal	20	20	20	100	5
QC Standard Measured	20.4	20.8	20.2	96.2	5.59
Percent of Check Standard	102%	104%	101%	96%	112%
QC Standard Nominal	200	200	200		50
QC Standard Measured	204	200	191		46.3
Percent of Check Standard	102%	100%	95%		92.6%
Blank 1	<0.2	<0.2	<0.06	<0.06	<0.14
Blank 2	<0.2	<0.2	<0.06	<0.06	<0.14
Sample Analysis 1	<0.2	99.7	0.18	127	<0.14
Laboratory Duplicate 1	<0.2	99.0	0.18	122	<0.14
Relative Percent Difference	-	0.7%	0.0%	4.0%	-
Sample Analysis 2	<0.2		16.6		1.62

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.2. Typical Quality Performance Data for Analysis of *cis*-DCE in Water. All Values are µg/L Unless Otherwise Indicated *continued*

Laboratory Duplicate 2	<0.2		15.7		1.58
Relative Percent Difference	-		5.6%		2.5%
Spike Concentration 1	200	200	100	100	
Sample Concentration 1	<0.2	250	<0.06	0.2	
Spike Recovery (Percent)	103%	97%	94%	88%	
Spike Concentration 2	200		200		
Sample Concentration 2	<0.2		1.2		
Spike Recovery (Percent)	100%		91%		
Date Collected	1/14/04	2/19/04	6/4/04	9/7/04	7/14/05
Date Analyzed	1/20/04	2/20/04	6/17/04	9/17/04	7/15/05
CCC Standard Nominal	10	10	20	20	20
CCC Standard Measured	12.3	11.5	20.7	19.4	20.2
Percent of Check Standard	123%	115%	103%	97%	101%
CCC Standard Nominal	100	50	20		200
CCC Standard Measured	104	57.7	19.8		210
Percent of Check Standard	104%	115%	99%		105%
CCC Standard Nominal	100	100			20
CCC Standard Measured	113	112			19.3
Percent of Check Standard	113%	112%			97%
QC Standard Nominal	50	10	20		20
QC Standard Measured	46.9	16.9	20.8		21.1
Percent of Check Standard	94%	169%	104%		105%
QC Standard Nominal	100	100	200		200
QC Standard Measured	95.0	145	217		207
Percent of Check Standard	95%	145%	109%		103%
Blank 1	<0.14	<0.14	<0.2	<0.2	<0.2
Blank 2	<0.14	<0.14	<0.2	<0.2	<0.2
Sample Analysis 1		<0.14	<0.2	<0.2	0.73
Laboratory Duplicate 1		<0.14	<0.2	<0.2	0.73
Relative Percent Difference		-	-	-	0.0%
Sample Analysis 2		<0.14	<0.2	<0.2	<0.2
Laboratory Duplicate 2		<0.14	<0.2	<0.2	<0.2
Relative Percent Difference		-	-	-	-
Spike Concentration 1			100	100	100
Sample Concentration 1			<0.2	<0.2	1.31
Spike Recovery (Percent)			107%	105%	94%
Spike Concentration 2			100	100	100
Sample Concentration 2			<0.2	<0.2	<0.2
Spike Recovery (Percent)			105%	103%	91%

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.3. Typical Quality Performance Data for Analysis of Vinyl Chloride in Water. All Values are µg/L Unless Otherwise Indicated

Date Collected	10/23/02	11/25/02	12/23/02	1/24/03	2/4/03
Date Analyzed	11/7/02	12/19/02	1/7/03	2/11/03	3/6/03
CCC Standard Nominal			10		
CCC Standard Measured			8.78		
Percent of Check Standard			88%		
CCC Standard Nominal			100		
CCC Standard Measured			90.5		
Percent of Check Standard			91%		
CCC Standard Nominal			250		
CCC Standard Measured			219		
Percent of Check Standard			88%		
QC Standard Nominal	20	20	50	20	20
QC Standard Measured	20.1	21.6	50.8	21.3	20.8
Percent of Check Standard	101%	108%	102%	107%	104%
QC Standard Nominal	200	200	50	200	200
QC Standard Measured	180	208	44.3	211	196
Percent of Check Standard	90.1%	104%	88.6%	106%	98%
Blank 1	<0.5	<0.3	<0.22	<0.3	<0.3
Blank 2	<0.5	<0.3	<0.22	<0.3	<0.3
Sample Analysis 1	12	0.47	78.9	<0.3	<0.3
Laboratory Duplicate 1	9.93	<0.3	92.0	<0.3	<0.3
Relative Percent Difference	18.9%	-	15.3%	-	-
Sample Analysis 2				<0.3	
Laboratory Duplicate 2				<0.3	
Relative Percent Difference				-	
Spike Concentration 1	200	200	50	200	200
Sample Concentration 1	<0.5	20400	<0.22	<0.3	3.69
Spike Recovery (Percent)	98%	87%	50%	101%	103%
Spike Concentration 2			50		
Sample Concentration 2			<0.22		
Spike Recovery (Percent)			56%		
Date Collected	2/24/03	3/6/03	3/11/03	3/26/03	4/8/03
Date Analyzed	3/9/03	4/2/03	3/21/03	4/10/03	4/30/03
CCC Standard Nominal	50	10		10	10
CCC Standard Measured	41	10.9		8.89	7.62
Percent of Check Standard	82%	109%		89%	76.2%
CCC Standard Nominal	200	100		250	50
CCC Standard Measured	156	113		288	42.3
Percent of Check Standard	78%	113%		115%	84.6%

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.3. Typical Quality Performance Data for Analysis of Vinyl Chloride in Water. All Values are µg/L Unless Otherwise Indicated *continued*

CCC Standard Nominal	50	100		250	100
CCC Standard Measured	40	113		236	87.9
Percent of Check Standard	80%	113%		94%	87.9%
QC Standard Nominal	20	25	10	100	50
QC Standard Measured	23.3	25.5	9.0	76.7	55.6
Percent of Check Standard	117%	102%	90%	77%	111%
QC Standard Nominal	200	50	200		
QC Standard Measured	192	58.4	180		
Percent of Check Standard	96%	117%	90%		
Blank 1	<0.22	<0.22	<0.49	<0.22	<0.22
Blank 2	<0.22	<0.22	<0.49	<0.22	<0.22
Sample Analysis 1	<0.22	1290	8.4	0.26	0.41
Laboratory Duplicate 1	<0.22	1290	5.4	0.26	0.38
Relative Percent Difference	-	0.0%	43.5%	0.0%	7.6%
Sample Analysis 2	<0.22			<0.22	
Laboratory Duplicate 2	<0.22			<0.22	
Relative Percent Difference	-			-	
Spike Concentration 1	200		100		
Sample Concentration 1	<0.22		3.3		
Spike Recovery (Percent)	103%		125%		
Spike Concentration 2			100		
Sample Concentration 2			20.3		
Spike Recovery (Percent)			81%		
Date Collected	4/21/03	4/29/03	5/16/03	6/23/03	7/23/03
Date Analyzed	5/20/03	5/5/03	6/5/03	6/26/03	8/5/03
CCC Standard Nominal	10		20	25	
CCC Standard Measured	10		23.5	20.4	
Percent of Check Standard	100%		118%	81.6%	
CCC Standard Nominal	50		50	100	
CCC Standard Measured	44.4		65.9	93.5	
Percent of Check Standard	88%		131%	93.5%	
CCC Standard Nominal	100			250	
CCC Standard Measured	98.0			252	
Percent of Check Standard	98%			101%	
QC Standard Nominal	50	20		50	20
QC Standard Measured	56.7	22.3		24.4	21.2
Percent of Check Standard	113%	111%		49%	106%
QC Standard Nominal		200			200

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.3. Typical Quality Performance Data for Analysis of Vinyl Chloride in Water. All Values are µg/L Unless Otherwise Indicated *continued*

QC Standard Measured		220			227
Percent of Check Standard		110%			113%
Blank 1	<0.22	<0.3	<0.49	<0.22	<0.3
Blank 2	1.37	<0.3	<0.49	<0.22	<0.3
Sample Analysis 1	5.28	<0.3	<0.49	0.22	<0.3
Laboratory Duplicate 1	4.56	<0.3	<0.49	0.34	<0.3
Relative Percent Difference	14.6%	-	-	42.9%	-
Sample Analysis 2			<0.49	32.1	
Laboratory Duplicate 2			<0.49	78.7	
Relative Percent Difference			-	84.1%	
Spike Concentration 1		200	200		100
Sample Concentration 1		1.59	<0.49		<0.3
Spike Recovery (Percent)		101%	144%		113%
Spike Concentration 2			200		
Sample Concentration 2			<0.49		
Spike Recovery (Percent)			141%		
Date Collected	8/7/03	8/20/03	10/3/03	10/7/03	10/24/03
Date Analyzed	8/12/03	8/22/03	10/22/03	11/3/03	10/28/03
CCC Standard Nominal			500	100	50
CCC Standard Measured			470	89.4	41.7
Percent of Check Standard			94%	89.4%	83.4%
CCC Standard Nominal					
CCC Standard Measured					
Percent of Check Standard					
CCC Standard Nominal					
CCC Standard Measured					
Percent of Check Standard					
QC Standard Nominal	20	20	20	100	5
QC Standard Measured	22.1	22.9	22.7	117	2.57
Percent of Check Standard	110%	115%	113%	117%	51%
QC Standard Nominal	200	200	200		50
QC Standard Measured	212	233	183		30.3
Percent of Check Standard	106%	116%	92%		62%
Blank 1	<0.3	<0.3	<0.49	<0.49	<0.22
Blank 2	<0.3	<0.3	<0.49	<0.49	<0.22
Sample Analysis 1	<0.3	0.42	<0.49	97	<0.22
Laboratory Duplicate 1	<0.3	0.35	<0.49	91.2	<0.22
Relative Percent Difference	-	18.2%	-	6.2%	-
Sample Analysis 2	<0.3		<0.49		<0.22

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.3. Typical Quality Performance Data for Analysis of Vinyl Chloride in Water. All Values are µg/L Unless Otherwise Indicated *continued*

Laboratory Duplicate 2	<0.3		<0.49		<0.22
Relative Percent Difference	-		-		-
Spike Concentration 1	200	200	100	100	
Sample Concentration 1	<0.3	2.62	<0.49	4.6	
Spike Recovery (Percent)	113%	111%	93%	89.5%	
Spike Concentration 2	200		200		
Sample Concentration 2	<0.3		3.4		
Spike Recovery (Percent)	116%		87%		
Date Collected	1/14/04	2/19/04	6/4/04	9/7/04	7/14/05
Date Analyzed	1/20/04	2/20/04	6/17/04	9/17/04	7/15/05
CCC Standard Nominal	10	10	20	20	20
CCC Standard Measured	9.97	10.7	19.8	20.8	19.9
Percent of Check Standard	100%	107%	99%	104%	100%
CCC Standard Nominal	100	50	20		200
CCC Standard Measured	105	48.9	20.8		169
Percent of Check Standard	105%	97.8%	104%		84%
CCC Standard Nominal	100	100			20
CCC Standard Measured	108	103			19.4
Percent of Check Standard	108%	103%			97%
QC Standard Nominal	50	10	20		20
QC Standard Measured	57.2	10.9	18.0		20.6
Percent of Check Standard	114%	109%	90%		103%
QC Standard Nominal	100	100	200		200
QC Standard Measured	113	101	192		188
Percent of Check Standard	113%	101%	96%		94%
Blank 1	<0.22	<0.22	<0.3	<0.3	<0.3
Blank 2	<0.22	<0.22	<0.3	<0.3	<0.3
Sample Analysis 1		<0.22	<0.3	<0.3	<0.3
Laboratory Duplicate 1		<0.22	<0.3	<0.3	<0.3
Relative Percent Difference		-	-	-	-
Sample Analysis 2		<0.22	<0.3	<0.3	<0.3
Laboratory Duplicate 2		<0.22	<0.3	<0.3	<0.3
Relative Percent Difference		-	-	-	-
Spike Concentration 1			100	100	100
Sample Concentration 1			<0.3	<0.3	<0.3
Spike Recovery (Percent)			101%	105%	100%
Spike Concentration 2			100	100	100
Sample Concentration 2			<0.3	<0.3	<0.3
Spike Recovery (Percent)			91%	105%	83%

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.4. Typical Quality Performance Data for Analysis of Ethylene in Water or in Gas. The Values for Check Standard Nominal and Check Standard Measured are ppm (v/v). All Other Values are mg/L Unless Otherwise Indicated

Date Collected	10/23/02	11/25/02	12/23/02	1/22/03	2/4/03
Date Analyzed	11/13/02	12/4/02	12/23/02	1/22/03	2/7/03
Check Standard Nominal	10	10	10	10	10
Check Standard Measured	11	10.5	10.9	10.1	10.9
Percent of Check Standard	110%	105%	109%	101%	109%
Check Standard Nominal	10	100	10	10	100
Check Standard Measured	10.7	108	11.1	10.4	106
Percent of Check Standard	107%	108%	111%	104%	106%
Check Standard Nominal	100	100	10	100	100
Check Standard Measured	104	106	10.9	109	108
Percent of Check Standard	104%	106%	109%	109%	108%
Check Standard Nominal		10000	10	100	
Check Standard Measured		10200	11.1	107	
Percent of Check Standard		102%	111%	107%	
Check Standard Nominal			100	1000	
Check Standard Measured			106	1010	
Percent of Check Standard			106%	101%	
Blank 1	<0.00028	<0.00028	<0.28*	<0.28*	<0.00028
Blank 2				<0.28*	
Sample Analysis 1	<0.00028	<0.00028			<0.00028
Laboratory Duplicate 1	<0.00028	<0.00028			<0.00028
Relative Percent Difference	-	-			-
Sample Analysis 2	<0.00028				
Laboratory Duplicate 2	<0.00028				
Relative Percent Difference	-				
Spike Concentration	0.0275	0.275			2.71
Sample Concentration	<0.00028	<0.00028			<0.00028
Spike Recovery (Percent)	104%	113%			99%
Date Collected	2/20/03	3/6/03	3/11/03	3/25/03	4/8/03
Date Analyzed	2/20/03	3/11/03	3/19/03	3/25/03	4/14/03
Check Standard Nominal	10	10	10	10	10
Check Standard Measured	10.5	10.1	10.4	10.7	10.3
Percent of Check Standard	105%	101%	104%	107%	103%
Check Standard Nominal	10	100	100	10	100
Check Standard Measured	10.2	107	106	10.4	98
Percent of Check Standard	102%	107%	106%	104%	98%
Check Standard Nominal	10	100		10	100
Check Standard Measured	10.3	102		10.5	106

**The values are ppm (v/v).*

Table 8.4. Typical Quality Performance Data for Analysis of Ethylene in Water or in Gas. The Values for Check Standard Nominal and Check Standard Measured are ppm (v/v). All Other Values are mg/L Unless Otherwise Indicated *continued*

Percent of Check Standard	103%	102%		105%	106%
Check Standard Nominal	100			10	1000
Check Standard Measured	106			10.4	1030
Percent of Check Standard	106%			104%	103%
Check Standard Nominal	1000				10000
Check Standard Measured	1080				10800
Percent of Check Standard	108%				108%
Blank 1		<0.00057	<0.00028		<0.00057
Blank 2					
Sample Analysis 1		<0.00057	<0.00028		<0.00057
Laboratory Duplicate 1		<0.00057	<0.00028		<0.00057
Relative Percent Difference		-	-		-
Sample Analysis 2		<0.00057			<0.00057
Laboratory Duplicate 2		<0.00057			<0.00057
Relative Percent Difference		-			-
Spike Concentration		0.964	2.68		1.94
Sample Concentration		<0.00057	<0.00028		<0.00057
Spike Recovery (Percent)		116%	99%		100%
Date Collected	4/21/03	4/29/03	5/15/03	6/20/03	7/23/03
Date Analyzed	5/1/03	5/6/03	5/15/03	6/20/03	7/31/03
Check Standard Nominal	10	10	10	10	10
Check Standard Measured	11	10.2	10.3	10.3	9.92
Percent of Check Standard	110%	102%	103%	103%	99%
Check Standard Nominal	100	100	10	10	100
Check Standard Measured	109	108	10.4	10.4	107
Percent of Check Standard	109%	108%	104%	104%	107%
Check Standard Nominal			10	10	
Check Standard Measured			10.2	10.6	
Percent of Check Standard			102%	106%	
Check Standard Nominal			10	10	
Check Standard Measured			10.5	10.3	
Percent of Check Standard			105%	103%	
Check Standard Nominal			100	10	
Check Standard Measured			107	10.4	
Percent of Check Standard			107%	104%	
Blank 1	<0.00057	<0.00057			<0.00057
Blank 2					<0.00057

**The values are ppm (v/v).*

Table 8.4. Typical Quality Performance Data for Analysis of Ethylene in Water or in Gas. The Values for Check Standard Nominal and Check Standard Measured are ppm (v/v). All Other Values are mg/L Unless Otherwise Indicated *continued*

Sample Analysis 1	<0.00057	0.003			<0.00057
Laboratory Duplicate 1	<0.00057	<0.00057			<0.00057
Relative Percent Difference	-	-			-
Sample Analysis 2					
Laboratory Duplicate 2					
Relative Percent Difference					
Spike Concentration	1.96				
Sample Concentration	<0.00057				
Spike Recovery (Percent)	92%				
Date Collected	8/6/03	8/20/03	10/2/03	10/7/03	10/22/03
Date Analyzed	8/6/03	8/25/03	10/2/03	10/16/03	10/22/03
Check Standard Nominal	10	10	10	100	10
Check Standard Measured	10.9	9.44	10.4	101	10.1
Percent of Check Standard	109%	94.4%	104%	101%	101%
Check Standard Nominal	10	100	10	1000	100
Check Standard Measured	10.3	106	9.9	1030	106
Percent of Check Standard	103%	106%	99%	103%	106%
Check Standard Nominal	100		100	10000	1000
Check Standard Measured	106		105	10100	1080
Percent of Check Standard	106%		105%	101%	108%
Check Standard Nominal	100		100	100000	
Check Standard Measured	108		106	97000	
Percent of Check Standard	108%		106%	97%	
Check Standard Nominal	10000		1000		
Check Standard Measured	10900		1080		
Percent of Check Standard	109%		108%		
Blank 1	<0.57*	<0.00057	<0.57*	<0.0003	<0.57*
Blank 2					
Sample Analysis 1		<0.00057		<0.0003	
Laboratory Duplicate 1		<0.00057		<0.0003	
Relative Percent Difference		-		-	
Sample Analysis 2		<0.00057		0.039	
Laboratory Duplicate 2		<0.00057		0.037	
Relative Percent Difference		-		5.3%	
Spike Concentration					
Sample Concentration					
Spike Recovery (Percent)					

**The values are ppm (v/v).*

Table 8.4. Typical Quality Performance Data for Analysis of Ethylene in Water or in Gas. The Values for Check Standard Nominal and Check Standard Measured are ppm (v/v). All Other Values are mg/L Unless Otherwise Indicated *continued*

Date Collected	1/14/04	2/18/04	6/1/04	9/9/04	7/13/05
Date Analyzed	1/14/04	2/18/04	6/1/04	9/9/04	7/13/05
Check Standard Nominal	10	10	10	10	10
Check Standard Measured	9.98	10.3	10.1	10.3	10.8
Percent of Check Standard	100%	103%	101%	103%	108%
Check Standard Nominal	100	10	10	10	10
Check Standard Measured	104	9.82	10.4	10.4	10.1
Percent of Check Standard	104%	98%	104%	104%	101%
Check Standard Nominal	100	10	10	100	100
Check Standard Measured	106	10.4	10.2	112	105
Percent of Check Standard	106%	104%	102%	112%	105%
Check Standard Nominal	1000	10	100	100	100
Check Standard Measured	1070	10.5	107	110	104
Percent of Check Standard	107%	105%	107%	110%	104%
Check Standard Nominal		100	100	1000	1000
Check Standard Measured		106	108	1100	1020
Percent of Check Standard		106%	108%	110%	102%
Blank 1	<0.57*	<0.57*	<0.57*	<0.57*	<0.57*
Blank 2					<0.57*
Sample Analysis 1					
Laboratory Duplicate 1					
Relative Percent Difference					
Sample Analysis 2					
Laboratory Duplicate 2					
Relative Percent Difference					
Spike Concentration					
Sample Concentration					
Spike Recovery (Percent)					

**The values are ppm (v/v).*

Table 8.5. Typical Quality Performance Data for Analysis of Methane in Water or in Gas. The Values for Check Standard Nominal and Check Standard Measured are ppm (v/v). All Other Values are mg/L Unless Otherwise Indicated

Date Collected	10/23/02	11/25/02	12/23/02	1/22/03	2/4/03
Date Analyzed	11/13/02	12/4/02	12/23/02	1/22/03	2/7/03
Check Standard Nominal	100	10	10	10	10
Check Standard Measured	104	10.9	10.7	10.4	10.6
Percent of Check Standard	104%	109%	107%	104%	106%
Check Standard Nominal	100	100	10	10	100
Check Standard Measured	106	106	10.6	9.95	110
Percent of Check Standard	106%	106%	106%	100%	110%
Check Standard Nominal	1000	100	10	10	100
Check Standard Measured	1056	109	10.8	9.88	106
Percent of Check Standard	105%	109%	108%	99%	106%
Check Standard Nominal	10000	10000	100	100	1000
Check Standard Measured	9340	10000	107	102	1060
Percent of Check Standard	93%	100%	107%	102%	106%
Check Standard Nominal	100000		100	100	
Check Standard Measured	99800		107	106	
Percent of Check Standard	100%		107%	106%	
Blank 1	<0.00003	<0.0003	<0.3*	<0.3*	<0.0003
Blank 2				<0.3*	
Sample Analysis 1	0.2	<0.0003			<0.0003
Laboratory Duplicate 1	0.198	<0.0003			<0.0003
Relative Percent Difference	1.0%	-			-
Sample Analysis 2	0.0551				
Laboratory Duplicate 2	0.0548				
Relative Percent Difference	0.5%				
Spike Concentration	0.0098	0.0978			0.971
Sample Concentration	0.0009	<0.0003			0.0015
Spike Recovery (Percent)	96%	114%			106%
Date Collected	2/20/03	3/6/03	3/11/03	3/25/03	4/8/03
Date Analyzed	2/20/03	3/11/03	3/19/03	3/25/03	4/14/03
Check Standard Nominal	10	10	10	10	10
Check Standard Measured	10.6	10.4	10.5	10.8	9.56
Percent of Check Standard	106%	104%	105%	108%	96%
Check Standard Nominal	10	100	100	10	100
Check Standard Measured	9.99	109	109	9.85	97.9
Percent of Check Standard	100%	109%	109%	99%	98%
Check Standard Nominal	10	1000	1000	100	1000
Check Standard Measured	10.2	1040	1050	105	1020

**The values are ppm (v/v).*

Table 8.5. Typical Quality Performance Data for Analysis of Methane in Water or in Gas. The Values for Check Standard Nominal and Check Standard Measured are ppm (v/v). All Other Values are mg/L Unless Otherwise Indicated *continued*

Percent of Check Standard	102%	104%	105%	105%	102%
Check Standard Nominal	10	10000	10000	1000	10000
Check Standard Measured	10.1	10100	10300	1060	9900
Percent of Check Standard	101%	101%	103%	106%	99%
Check Standard Nominal	100	100000		10000	100000
Check Standard Measured	107	100000		9790	95900
Percent of Check Standard	107%	100%		98%	96%
Blank 1		<0.00042	<0.00003		<0.00042
Blank 2					
Sample Analysis 1		0.086	0.007		0.002
Laboratory Duplicate 1		0.0854	0.007		0.002
Relative Percent Difference		0.7%	0.0%		0.0%
Sample Analysis 2		<0.00042			0.147
Laboratory Duplicate 2		<0.00042			0.145
Relative Percent Difference		-			1.4%
Spike Concentration		0.964	0.964		0.957
Sample Concentration		0.007	<0.00003		0.019
Spike Recovery (Percent)		93%	108%		89%
Date Collected	4/21/03	4/29/03	5/15/03	6/20/03	7/23/03
Date Analyzed	5/1/03	5/6/03	5/15/03	6/20/03	7/31/03
Check Standard Nominal	10	100	10	10	100
Check Standard Measured	11	102	10.1	10.4	101
Percent of Check Standard	110%	102%	101%	104%	101%
Check Standard Nominal	100	10000	10	10	100
Check Standard Measured	104	10200	10.5	10.3	98.8
Percent of Check Standard	104%	102%	105%	103%	99%
Check Standard Nominal	1000	100000	100	100	1000
Check Standard Measured	1070	97300	102	100	1030
Percent of Check Standard	107%	97%	102%	100%	103%
Check Standard Nominal	10000		1000	1000	10000
Check Standard Measured	10300		1070	1060	9850
Percent of Check Standard	103%		107%	106%	99%
Check Standard Nominal	100000		10000	10000	100000
Check Standard Measured	98200		10300	10200	95300
Percent of Check Standard	98%		103%	102%	95%
Blank 1	<0.00042	<0.00042			<0.00042
Blank 2					<0.00042

**The values are ppm (v/v).*

Table 8.5. Typical Quality Performance Data for Analysis of Methane in Water or in Gas. The Values for Check Standard Nominal and Check Standard Measured are ppm (v/v). All Other Values are mg/L Unless Otherwise Indicated *continued*

Sample Analysis 1	0.265	12.3			7.28
Laboratory Duplicate 1	0.229	12.9			7.02
Relative Percent Difference	14.6%	4.8%			3.6%
Sample Analysis 2					
Laboratory Duplicate 2					
Relative Percent Difference					
Spike Concentration	0.964				
Sample Concentration	0.001				
Spike Recovery (Percent)	97%				
Date Collected	8/6/03	8/20/03	10/2/03	10/7/03	10/22/03
Date Analyzed	8/6/03	8/25/03	10/2/03	10/16/03	10/22/03
Check Standard Nominal	100	100	10	100	10
Check Standard Measured	102	101	9.6	107	10.3
Percent of Check Standard	102%	101%	96%	107%	103#
Check Standard Nominal	100	100	100	10	100
Check Standard Measured	99.9	95.8	101	10.1	103
Percent of Check Standard	100%	96%	101%	101%	103%
Check Standard Nominal	1000	1000	100	100	1000
Check Standard Measured	1060	1020	102	103	1070
Percent of Check Standard	106%	102%	102%	103%	107%
Check Standard Nominal	10000	10000	1000	1000	10000
Check Standard Measured	10300	9810	1070	1050	10300
Percent of Check Standard	103%	98%	107%	105%	103%
Check Standard Nominal	100000	100000	10000		100000
Check Standard Measured	97800	94700	10300		98500
Percent of Check Standard	98%	95%	103%		99%
Blank 1	<0.42*	<0.00042	<0.42*	<0.0001	<0.42*
Blank 2					
Sample Analysis 1		1.70		2.15	
Laboratory Duplicate 1		1.35		2.15	
Relative Percent Difference		23.0%		0.0%	
Sample Analysis 2		2.44		3.85	
Laboratory Duplicate 2		2.78		3.36	
Relative Percent Difference		13.0%		13.6%	
Spike Concentration					
Sample Concentration					
Spike Recovery (Percent)					

**The values are ppm (v/v).*

Table 8.5. Typical Quality Performance Data for Analysis of Methane in Water or in Gas. The Values for Check Standard Nominal and Check Standard Measured are ppm (v/v). All Other Values are mg/L Unless Otherwise Indicated *continued*

Date Collected	1/14/04	2/18/04	6/1/04	9/9/04	7/13/05
Date Analyzed	1/14/04	2/18/04	6/1/04	9/9/04	7/13/05
Check Standard Nominal	100	10	10	10	10
Check Standard Measured	98.4	10.7	10.9	10.7	9.88
Percent of Check Standard	98%	107%	109%	107%	98.8%
Check Standard Nominal	100	100	100	100	100
Check Standard Measured	101	102	107	105	101
Percent of Check Standard	101%	102%	107%	105%	101%
Check Standard Nominal	1000	1000	1000	1000	1000
Check Standard Measured	1070	1070	1080	1110	1040
Percent of Check Standard	107%	107%	108%	111%	104%
Check Standard Nominal	10000	10000	10000	10000	10000
Check Standard Measured	10200	10300	10500	11200	10300
Percent of Check Standard	102%	103%	105%	112%	103%
Check Standard Nominal	100000	100000	100000	100000	100000
Check Standard Measured	97700	98000	99200	99800	99800
Percent of Check Standard	98%	98%	99.2%	99.8%	99.8%
Blank 1	<0.42*	<0.42*	<0.42*	<0.42*	<0.42*
Blank 2					<0.42*
Sample Analysis 1					
Laboratory Duplicate 1					
Relative Percent Difference					
Sample Analysis 2					
Laboratory Duplicate 2					
Relative Percent Difference					
Spike Concentration					
Sample Concentration					
Spike Recovery (Percent)					

**The values are ppm (v/v).*

Table 8.6. Typical Quality Performance Data for Analysis of Hydrogen in Gas. All Values are ppm Unless Otherwise Indicated

Date Collected	10/23/02	12/23/02	2/20/03	3/6/03	3/11/03
Date Analyzed	11/5/02	12/23/02	2/20/03	3/11/03	3/19/03
Check Standard Nominal	1	0.5	0.5	0.5	1
Check Standard Measured	1.037	0.59	0.49	0.54	0.98
Percent of Check Standard	103%	118%	99%	108%	98%
Check Standard Nominal	5	1.19	0.5	0.5	0.5
Check Standard Measured	5.479	1.26	0.5	0.486	0.52
Percent of Check Standard	110%	92.4%	100%	97%	104%
Check Standard Nominal	0.5	2.5	1	1	1
Check Standard Measured	0.46	2.45	1.07	1.094	1.06
Percent of Check Standard	96%	98%	107%	109%	106%
Check Standard Nominal	1	10.1	5	5	5
Check Standard Measured	1.03	9.34	5.6	5.489	5.32
Percent of Check Standard	103%	98%	110%	109%	106%
Check Standard Nominal	5	10.1	10		
Check Standard Measured	5.42	11.0	9.96		
Percent of Check Standard	108%	109%	99%		
Blank 1	<0.19	<0.19	<0.19	<0.19	<0.19
Blank 2					
Sample Analysis 1	0.348			0.74	0.564
Laboratory Duplicate 1	0.351			2.67	0.601
Relative Percent Difference	0.9%			113.2%	6.4%
Sample Analysis 2	2.847			0.33	
Laboratory Duplicate 2	2.042			0.36	
Relative Percent Difference	32.9%			8.7%	
Date Collected	3/25/03	4/8/03	4/21/03	4/29/03	5/15/03
Date Analyzed	3/25/03	4/14/03	5/1/03	5/6/03	5/15/03
Check Standard Nominal	0.5	0.5	1	0.5	0.5
Check Standard Measured	0.51	0.473	0.943	0.534	0.533
Percent of Check Standard	102%	95%	94%	107%	94%
Check Standard Nominal	1	0.5	1	1	1
Check Standard Measured	0.99	0.534	0.899	1.04	0.939
Percent of Check Standard	99%	107%	90%	104%	94%
Check Standard Nominal	1	1	5	5	5
Check Standard Measured	1	1.03	5.05	5.06	5.01
Percent of Check Standard	100%	103%	101%	101%	100%
Check Standard Nominal	5	1	10	10	10
Check Standard Measured	4.98	1.05	10.4	10.5	10.6

The values are ppm (v/v).

Table 8.6. Typical Quality Performance Data for Analysis of Hydrogen in Gas. All Values are ppm Unless Otherwise Indicated *continued*

Percent of Check Standard	99%	105%	104%	105%	106%	
Check Standard Nominal	5	5	20	20	20	
Check Standard Measured	4.9	4.56	21.3	19.5	20.5	
Percent of Check Standard	98%	91%	106%	98%	103%	
Blank 1		<0.19	<0.19	<0.19	<0.19	
Blank 2						
Sample Analysis 1		2.08	<0.5	2.5		
Laboratory Duplicate 1		2.59	<0.5	2.1		
Relative Percent Difference		21.8%	-	17.4%		
Sample Analysis 2		0.669				
Laboratory Duplicate 2		0.744				
Relative Percent Difference		10.6%				
Date Collected	6/20/03	7/23/03	8/6/03	8/20/03	10/2/03	10/7/03
Date Analyzed	6/20/03	7/31/03	8/6/03	8/25/03	10/2/03	10/16/03
Check Standard Nominal	0.5	0.5	0.5	0.5	0.5	1
Check Standard Measured	0.5	0.523	0.461	0.51	0.585	1.02
Percent of Check Standard	100%	105%	92%	102%	117%	102%
Check Standard Nominal	1	1	1	1	1	5
Check Standard Measured	1.04	1.17	0.959	0.96	0.964	4.6
Percent of Check Standard	104%	117%	96%	96%	96%	92%
Check Standard Nominal	10	5	5	20	5	10
Check Standard Measured	10.5	4.4	5.45	21.6	5.55	9.52
Percent of Check Standard	105%	88%	109%	108%	111%	95%
Check Standard Nominal		10	10		10	20
Check Standard Measured		11.1	11		8.89	17.6
Percent of Check Standard		111%	110%		89%	88%
Check Standard Nominal		20	20		20	
Check Standard Measured		18.8	18.5		16.9	
Percent of Check Standard		94%	93%		85%	
Blank 1	<0.19	<0.19	<0.19	<0.19	0.232	<0.19
Blank 2						
Sample Analysis 1		13.9		0.48		134
Laboratory Duplicate 1		13.1		0.42		136
Relative Percent Difference		5.9%		13.3%		1.5%
Sample Analysis 2						
Laboratory Duplicate 2						
Relative Percent Difference						

The values are ppm (v/v).

Table 8.6. Typical Quality Performance Data for Analysis of Hydrogen in Gas. All Values are ppm Unless Otherwise Indicated *continued*

Date Collected	10/22/03	1/14/05	2/18/04	6/1/04	9/9/04	7/13/05
Date Analyzed	10/22/03	1/14/05	2/18/04	6/1/04	9/9/04	7/13/05
Check Standard Nominal	0.5	1	1	0.5	0.5	0.5
Check Standard Measured	0.444	1.12	1.07	0.586	0.465	0.459
Percent of Check Standard	113%	112%	107%	117%	93%	92%
Check Standard Nominal	1	5	5	1	1	1
Check Standard Measured	0.991	4.30	5.13	0.931	1.13	0.937
Percent of Check Standard	99%	116%	103%	93.1%	113%	93.7%
Check Standard Nominal	5	10	10	5	5	5
Check Standard Measured	5.23	9.45	9.72	4.42	5.67	4.88
Percent of Check Standard	105%	95%	97.2%	88.4%	113%	97.6%
Check Standard Nominal	10	20	10	10	10	10
Check Standard Measured	8.98	18.4	9.9	11.5	11.1	10.1
Percent of Check Standard	90%	109%	99%	115%	111%	101%
Check Standard Nominal	20		20	20	20	20
Check Standard Measured	19.5		19	18.1	18.6	19.2
Percent of Check Standard	98%		95%	90.5%	93%	96%
Blank 1	0.244	<0.19	<0.19	<0.19	<0.19	<0.19
Blank 2						<0.19
Sample Analysis 1						
Laboratory Duplicate 1						
Relative Percent Difference						
Sample Analysis 2						
Laboratory Duplicate 2						
Relative Percent Difference						

The values are ppm (v/v).

Table 8.7. Typical Quality Performance Data for Analysis of Nitrate Plus Nitrite Nitrogen in Water. All Values are mg/L Unless Otherwise Indicated

Date Collected	10/23/02	2/4/03	3/6/03	3/11/03	4/8/03
Date Analyzed	11/6/02	2/7/03	3/20/03	3/26/03	4/14/03
CCC Standard Nominal	0.5	2.5	2.5	0.5	0.5
CCC Standard Measured	0.49	2.36	2.53	0.51	0.45
Percent of Check Standard	98%	94%	101%	102%	90%
CCC Standard Nominal	1		1	1	1
CCC Standard Measured	0.97		0.97	1	0.93
Percent of Check Standard	97%		97%	100%	93%
CCC Standard Nominal	2.5				
CCC Standard Measured	2.45				
Percent of Check Standard	98%				
QC Standard Nominal					
QC Standard Measured					
Percent of Check Standard					
QC Standard Nominal	7.06		2.99	2.99	2.99
QC Standard Measured	7.06		3.02	3.10	3.05
Percent of Check Standard	100%		101%	104%	102%
Blank 1	<0.1	<0.1	<0.1	<0.1	<0.1
Blank 2	<0.1				
Sample Analysis	<0.1	0.62	1.58	1.25	<0.1
Laboratory Duplicate	<0.1	0.59	1.57	1.29	<0.1
Relative Percent Difference	-	5.0%	0.6%	3.1%	-
Spike Concentration	10	10	10	10	10
Sample Concentration	<0.1	2.04	1.97	0.03	0.06
Spike Recovery (Percent)	107%	103%	110%	106%	116%
Date Collected	4/21/03	4/29/03	7/23/03	8/20/03	10/7/03
Date Analyzed	5/13/03	5/13/03	8/6/03	8/25/03	10/28/03
CCC Standard Nominal	1	1	2.5	0.5	0.5
CCC Standard Measured	0.95	0.95	2.39	0.48	0.5
Percent of Check Standard	95%	95%	95.6%	96%	100%
CCC Standard Nominal	5	1	5	1	1
CCC Standard Measured	4.99	0.96	4.78	0.92	0.93
Percent of Check Standard	100%	96%	96%	92%	93%
CCC Standard Nominal	1		5	5	5
CCC Standard Measured	0.96		4.74	4.91	5
Percent of Check Standard	96%		95%	98.2%	100%
QC Standard Nominal				10	10
QC Standard Measured				9.8	9.87
Percent of Check Standard				98%	98.7%

CCC: Continuing Calibration Check; QC: Second Source Check

Table 8.7. Typical Quality Performance Data for Analysis of Nitrate Plus Nitrite Nitrogen in Water. All Values are mg/L Unless Otherwise Indicated *continued*

QC Standard Nominal	13.3	13.3	21.3		
QC Standard Measured	12.7	12.7	18.9		
Percent of Check Standard	95%	95%	89%		
Blank 1	<0.1	<0.1	<0.1	<0.1	<0.1
Blank 2	<0.1	<0.1	<0.1	<0.1	<0.1
Sample Analysis	3.39	<0.1	11.2	<0.1	<0.1
Laboratory Duplicate	3.49	<0.1	11.1	<0.1	<0.1
Relative Percent Difference	2.9%	-	0.9%	-	-
Spike Concentration	10	10	5	10	5
Sample Concentration	1.26	0.04	<0.004	1.86	0.04
Spike Recovery (Percent)	116%	109%	84.6%	109%	101%

CCC: Continuing Calibration Check; QC: Second Source Check

Section 9.
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