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CHARACTERIZATION OF BACTERIAL PROCESSES IN THE SUBSURFACE AND THE ATMOSPHERE

by

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A dissertation submitted to the
Graduate School – New Brunswick
Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Environmental Sciences

Written under the direction of

Professor Donna E. Fennell

And approved by

New Brunswick, New Jersey

October, 2009

ABSTRACT OF THE DISSERTATION

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This dissertation describes research that seeks to expand understanding of bacterially-mediated biotransformation in subsurface groundwater and the atmosphere. In the first study a highly tetrachloroethene (PCE)-enriched culture, RU11/PCE was developed from contaminated aquifer materials. Denaturing gradient gel electrophoresis (DGGE), 16S rRNA clonal library analysis, and direct sequencing of 16S rRNA, *sod* and *gyrB* genes revealed a single bacterial species, *Dehalococcoides*, in the RU11/PCE culture. However, because the 16S rRNA, *sod* and *gyrB* genes of the *Dehalococcoides* spp. are highly conserved, the possibility that more than one strain of *Dehalococcoides* was present could not be conclusively eliminated. The reductive dehalogenase gene profile of the RU11/PCE culture was different than that of other previously reported *Dehalococcoides* pure cultures and unlike other chloroethene-respiring *Dehalococcoides* spp., RU11/PCE grew on PCE, TCE, cis-1,2-DCE or VC.

The second study addressed the hypothesis that the air contains an active microbial ecosystem. Rotating bioaerosol bioreactors were manufactured to keep bacteria suspended in the presence of a volatile substrate while measuring their activity. A qPCR method was developed and used along with an ATP assay, microscopy, and plate counts to enumerate airborne bacteria. Although the gas-phase reactors could retain bacteria and volatile substrates for days, results using live aerosolized *Xanthobacter autotrophicus* and *Bacillus subtilis* indicated no growth. In tests with *X. autotrophicus*, no culturable cells were recovered under any condition. *B. subtilis* aerosols from dilute substrate yielded higher culturability than aerosols from distilled water with no TSB substrate. Lack of culturability occurred despite presence of airborne bacteria over time, as measured by qPCR and ATP.

Techniques were also developed to characterize microbial communities in atmospheric samples. The bacterial components of a pooled sample of atmospheric water collected in the vicinity of Oklahoma City, OK were analyzed using DGGE and clone library analysis. From DGGE analysis, six out of eight strains detected belong to the phyla of *Actinobacteria*, *Firmicutes*, *Proteobacteria* and *Bacteroidetes*. In clone library analysis, 12 bacterial strains were identified (from 78 total) with dominant occurrence of the genera, *Sphingomonas*, *Pedobacter*, and *Curtobacterium spp.* The bacterial populations detected from the two methods were composed of strains of diverse origins.

ACKNOWLEDGEMENTS

The author is grateful for financial support from the National Science Foundation, Small Grant for Exploratory Research grant no. 0606576; and the Strategic Environmental Research and Development Program of the U.S. Department of Defense, grant ER-1492. The author also acknowledges support via a teaching assistantship from the department of Environmental Sciences, a graduate assistantship from the New Jersey Agricultural Experiment Station and a graduate student grant-in-aid from the New Jersey Water Resources Research Institute.

Any opinions, findings, and conclusions or recommendations expressed in this dissertation are those of the author and do not necessarily reflect the views of the National Science Foundation.

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Chapter 1

Overview

Microorganisms, especially the bacteria, are ubiquitous in all parts of the biosphere including the soil, the ocean, fresh water, hot springs, other living organisms, high in the atmosphere and deep inside rocks within the Earth's crust (Madigan et al., 2003; Stolp, 1988). Bacteria are critical to biogeochemical cycling in Earth's ecosystems through metabolic or cometabolic activities such as oxidation of organic matter, fixation of carbon and nitrogen, oxidation and reduction of metals, and halogenation and dehalogenation of organic compounds (Bowers et al., 2009; Christner et al., 2008; Madigan et al., 2003). Recent studies indicate that airborne bacteria may extend the reach of known microbial processes to the atmosphere where they play a role in cloud formation and precipitation (Bowers et al., 2009; Christner et al., 2008; Madigan et al., 2003).

This dissertation describes research that sought to expand understanding of bacterially-mediated biotransformation in two microbially challenging environments, the subsurface groundwater and the atmosphere. Of the many ecosystems on Earth, oligotrophic groundwater and the atmosphere are among the more recent environments to be recognized as habitats that support diverse microbial communities that have evolved special features for life. These environments are linked by the hydrologic and biogeochemical cycles that characterize planetary processes. One of the main properties

in common between the groundwater (Bekins, 2000) and atmospheric ecosystems is the paucity of vitally important resources to support life. In other ways, the groundwater and atmosphere are very different environments. In the groundwater environment, interstices between the grains of aquifer fill or rocks provide a niche for specific bacteria that, in turn, control hydrogeochemistry, affect geologic processes, and are now recognized to have great impact on human activities (Bekins, 2000). The Earth's groundwater is a major bacterial ecosystem estimated to contain approximately 2.5×10^{30} bacterial cells (Whitman et al., 1998). Further, bacteria in aquifers are isolated and may have very long residence times (Bekins, 2000).

In contrast, bacteria in the atmosphere are present in lower numbers. Air contains about 1×10^4 to 1×10^5 bacterial cells per m^3 (Lighthart, 2000; Griffin et al., 2001). Considering the volume of the troposphere (*circa* $8 \times 10^{18} \text{ m}^3$) this could account for approximately 1×10^{22} to 1×10^{23} bacterial cells in the aerosolized state. Further, bacteria are continually reintroduced or reseeded into the atmosphere by a variety of processes and have on average very short residence times of hours or days (Lighthart, 2000; Griffin et al., 2001).

Microorganisms are reversibly attached to subsurface particles, which causes a retardation of their transport velocity with respect to groundwater flow velocity. The survival time of bacteria and viruses in groundwater is different for the specific species and for the specific groundwater environment (Pekdeger and Matthess, 1983). Unpolluted groundwater systems have scarce trophic resources, and most of the organic matter entering groundwater habitats is mineralized by microorganisms (Bekins, 2000). Anaerobic microbial degradation of recalcitrant organic pollutants is of great

environmental and human significance. In particular, hydrocarbons and halogenated compounds have been found to be transformed and even mineralized in the absence of oxygen (Löffler and Edwards, 2006; van Pee and Unversucht, 2003). The isolation of previously unknown anaerobic hydrocarbon-degrading and reductively dehalogenating bacteria during the last decades provided ultimate proof for the importance of these processes in nature (Holliger et al., 2003; Löffler, 2003).

The groundwater ecosystem portion of this dissertation addressed bacterially-mediated reductive dechlorination of common groundwater pollutants, the chloroethenes. Reductive dechlorination has been studied and applied for *in situ* remediation of chloroethene-contaminated groundwater plumes (Löffler et al., 2003). Through bacterially-mediated reductive dechlorination, tetrachloroethene (PCE) has been shown to be successively reduced to trichloroethene (TCE), the dichloroethene (DCE) isomers (*cis*-DCE, *trans*-1,2-DCE and 1,1-DCE), vinyl chloride (VC) and finally ethene, a nontoxic end product (Freedman and Gossett, 1989). The discovery of bacteria that use chloro-organic compounds as electron acceptors for energy production enabled a better understanding of the major factors controlling the fate of chloroethenes in contaminated groundwater aquifers (Hendrickson et al., 2002; Löffler et al., 2003).

The atmospheric ecosystem portion of this dissertation addressed the hypothesis that air is an active microbial ecosystem by examining the potential for bacteria processes in air and characterizing bacteria in atmospheric water droplets. It has long been thought that air is not a medium of growth for microorganisms, but rather is a passive carrier of particulate matter, dust, and droplets which include microorganisms either alone, or associated with the other particles (Lighthart, 2000). In still air, particles tend to settle

quickly to the Earth's surface leaving the air fairly free of microorganisms. The air after heavy rain is also depleted of microorganisms. Development even of the slightest air currents however, can keep microorganisms suspended in air for protracted periods of time. In general, the air above the warmer regions of Earth harbors a greater number of microorganisms than the air above cooler regions, provided adequate humidity is present (Lighthart, 2000).

The aerosolized microorganisms and their ultimate fate in the atmosphere is governed by a complex set of conditions including sunlight, temperature, humidity, the size of microbe laden particulates, degree of susceptibility or resistance of a particular microbe to the new physical environment, and the ability of the microbe to form resistant spores or cysts (Madigan et al., 2003; Mohr, 2002). Most studies on aerobiology have been focused on identifying microbial species, determining their concentrations and elucidating their transport dynamics in the outdoor and indoor air (Stetzenbach et al., 2004). Recent work has focused on public security and safety concerns such as detection of *Bacillus anthracis* and other human pathogens (Agranovski et al., 2004; Beamer et al., 2004; Higgins et al., 2003). The notion that bacteria in the atmosphere may be active or that they may initiate or mediate specific processes that influence the broader atmospheric environment is a more recent idea that has attracted scientific investigation (Deguillaume et al., 2008).

Microbial biotransformation is chemical transformation made by a microorganism on a chemical compound. Microorganisms utilize chemical compounds as their source of energy, carbon and nutrients for biosynthesis. Sometimes biotransformation occurs fortuitously during microbial activities. Recent methodological breakthroughs in genomic,

metagenomic, proteomic, bioinformatic and other high-throughput analytical methods have enabled detailed insights into biotransformation, transformation pathways and the ability of environmentally relevant microorganisms to adapt to diverse environmental conditions (Kerkhof and Häggblom, 2008).

In the field of environmental microbiology, genome-based global studies open a new era, providing new information to delineate the extent and versatility of biochemical pathways relevant to biotransformation, and providing new insights into the physiological and metabolic adaptation of microorganisms to changing environmental conditions at the molecular level. By increasing our understanding of different pathways and regulatory networks of microorganisms in particular environments and for particular compounds, functional genomic and metagenomic approaches are able to accelerate the development of bioremediation and biotransformation technologies (Kerkhof and Häggblom, 2008). In this dissertation, molecular methodological approaches were utilized to characterize and identify bacterial processes in the subsurface and the atmosphere.

The overall objective of this dissertation was to expand understanding of bacterially-mediated biotransformations in two microbially challenging environments, the subsurface and the atmosphere. This dissertation is divided into two separate studies.

The goal of the first study, described in **Chapter 2**, was to enrich for, isolate and characterize dechlorinating bacteria from a PCE-contaminated aquifer located beneath the Busch Campus of Rutgers University in Piscataway, NJ. The specific objectives were to:

- (1) establish enriched cultures from aquifer material utilizing PCE and VC as the primary electron acceptors;
- (2) identify bacteria present in the original aquifer material and the enrichments established on PCE and VC by using 16S rRNA gene-based community analyses;
- (3) determine whether separate specific *Dehalococcoides* strains are associated with the dechlorination of the separate chloroethenes;
- (4) determine whether reductive dehalogenation is growth-linked and quantify growth yields on the different chloroethenes; and
- (5) characterize the distribution of reductive dehalogenases associated with the enrichments.

The overall goal of the second study, described in **Chapter 3**, was to develop methods to enable investigation of whether bacteria are capable of metabolism and/or growth while airborne and to apply molecular DNA-based methods to characterize bacteria in air and atmospheric water droplets. The specific objectives were to:

- (1) develop methods to determine if airborne microorganisms degrade volatile organic compounds present in the air;
- (2) develop methods to determine if airborne bacteria multiply under some circumstances;
- (3) determine whether airborne bacteria respond to changes in humidity, temperature or presence of chemical substrates; and
- (4) utilize molecular methods to characterize bacterial communities in an atmospheric water sample.

This dissertation is composed of four chapters. **Chapter 1** describes the overview for the dissertation. **Chapter 2** addresses characterization of a highly tetrachloroethene (PCE)-enriched culture developed from a subsurface environment and designated RU11/PCE, which dehalorespires PCE, TCE, *cis*-DCE and VC to ethene. **Chapter 3** describes the development and characterization of compact rotating bioaerosol bioreactor system and the techniques developed to characterize and identify microbial components in atmospheric samples. Finally, **Chapter 4** summarizes the conclusions from this work.

Chapter 2

Characterization of a Novel PCE-Enrichment Culture from PCE-contaminated Groundwater and Sediments

1. Introduction

1.1 Rationale

Halogenated compounds are present naturally in the environment. More than 3,800 living organisms or natural abiotic processes produce halogenated compounds (Asplund and Grimvall, 1991; Gribble, 2003; Keppler et al., 2002). Biologically, halogenated compounds can be produced by seaweed, corals, algae, jellyfish, sponges, fungi, bacteria, insects, and mammals (Asplund and Grimvall, 1991; Gribble, 1996; van Pee and Unversucht, 2003). In nature there also exist fungi and bacteria which can degrade halogenated compounds (Häggbloom et al., 2003). Thus, a biogeochemical cycle exists in which natural halogenated organic compounds are formed, transformed and degraded (Häggbloom et al., 2003). The fate of anthropogenic halogenated pollutants is controlled in part by this cycle.

In the last century, large amounts of halogenated organic compounds have been produced anthropogenically and used for industrial and agricultural applications. Tetrachloroethene (PCE) and trichloroethene (TCE) have been among the most extensively used halogenated compounds. Their major applications are as dry cleaning solvents and metal degreasers. These compounds have been released to the environment by inappropriate disposal and accidental leakage and as a consequence, chlorinated ethenes are among the most widely distributed soil and groundwater contaminants in the US (NRC, 2004). Initially, pump and treat methods were adopted to remediate chlorinated ethene contaminated sites (Abelson, 1990). However, pump and treat is expensive and often ineffective, and may be most useful for controlling contaminant migration (Christ et al., 2005).

Biological reductive dechlorination has been developed and applied for *in situ* remediation of chloroethene-contaminated groundwater plumes (Löffler et al., 2003). Through bacterially-mediated reductive dechlorination, PCE has been shown to be successively reduced to TCE, the dichloroethene (DCE) isomers (*cis*-1,2-DCE, *trans*-1,2-DCE and 1,1-DCE), vinyl chloride (VC) and finally ethene, a nontoxic end product (Freedman and Gossett, 1989). The discovery of bacteria that use chloro-organic compounds as electron acceptors for energy production enabled a better understanding of the major factors controlling the fate of chloroethenes at contaminated sites (Hendrickson et al., 2002; Löffler et al., 2003). Currently, the metabolic reductive dechlorination process is a major focus of bioremediation approaches to contain or remediate chloroethene-contaminated groundwater both in the dissolved and non-aqueous phases (Ellis et al., 2000; Lendvay et al., 2003; Major et al., 2002).

Numerous studies have reported microbial isolates which dehalogenate chlorinated ethenes (Löffler et al., 2003). Some anaerobic dechlorinating bacteria (e.g., *Desulfuromonas*, *Sulfurospirillum multivorans*, and *Dehalobacter*) reductively dechlorinate PCE and TCE to the toxic intermediate *cis*-DCE (Löffler et al., 2003). To date, all scientific studies indicate that reductive dechlorination from *cis*-DCE to ethene is achieved exclusively by the *Dehalococcoides* group of bacteria (He et al., 2003a; He et al., 2003b; Hendrickson et al., 2002; Maymo-Gatell et al., 1997; Sung et al., 2006b). The absence of *Dehalococcoides* spp. at contaminated sites was shown to result in accumulation of the toxic dechlorination daughter products, *cis*-DCE or VC, as a result of incomplete dechlorination (Hendrickson et al., 2002). *Dehalococcoides ethenogenes* strain 195 was the first *Dehalococcoides* isolate described and this organism

dechlorinates PCE to ethene, although the final dechlorination step from VC to ethene is cometabolic (Maymo-Gatell et al., 1997). *Dehalococcoides* sp. strain BAV1 was the first isolate shown to be capable of obtaining energy from reductive dechlorination of VC (He et al., 2003b). Strain BAV1 utilizes all DCE isomers and VC as electron acceptors and it cometabolizes PCE and TCE in the presence of DCE isomers or VC (He et al., 2003b). *Dehalococcoides* sp. strain GT can reduce TCE, *cis*-DCE, and VC as electron acceptors and it dechlorinates PCE cometabolically (Sung et al., 2006b). No single isolate has been found that grows on all four chloroethene compounds.

Despite the observed variety of metabolic functions, *Dehalococcoides* strains share highly similar 16S rRNA gene sequences. The 16S rRNA gene sequences of strain BAV1, strain FL2 and *Dehalococcoides* sp. strain CBDB1, an isolate that uses chlorinated benzenes as growth-linked electron acceptors (Bunge et al., 2003), are greater than 99.9% identical over 1400 bp. Further, the 16S rRNA gene sequences of strain FL2, strain GT, and strain CBDB1 are identical to that of KB-1/VC, a *Dehalococcoides* strain found in a highly enriched TCE-to-ethene-dechlorinating culture (Duhamel et al., 2004). Because of the highly conserved nature of the 16S rRNA gene, phylogeny alone is an inadequate predictor of the metabolic activities of *Dehalococcoides*.

The inadequacy of the 16S rRNA gene for characterizing dehalogenating potential has resulted in development of alternative methods for characterizing the *Dehalococcoides* species present at a site. Interest has focused instead on characterization of strains by assessing the presence of functional genes that encode for reductive dehalogenases, the enzymes that catalyze the removal of halogens from halogenated electron acceptors. To date, the primary functional genes that have been investigated are

tceA, *pceA*, *bvcA*, and *vcrA*, the genes encoding for dehalogenases that dechlorinate TCE, PCE, VC, and VC, respectively. Recently, quantitative polymerase chain reaction (qPCR) analysis protocols have been developed for detection and enumeration of the reductive dehalogenase genes, and this has provided important information about different dehalogenating cultures (He et al., 2005; Magnuson et al., 2000b; Ritalahti et al., 2006b).

There is still a great deal that is not known about the dechlorinating bacteria, in particular the *Dehalococcoides*. More information about specific strains found in contaminated environments and the reductive dehalogenases associated with these strains is needed to fully understand their presence and activity in the environment.

1.2. Objectives and experimental strategy

The overall goal of this study was to enrich for, isolate and characterize dechlorinating bacteria from a PCE-contaminated aquifer located beneath the Busch Campus of Rutgers University in Piscataway, NJ. The specific objectives were to:

- (1) establish enriched cultures from aquifer material utilizing PCE and VC as the primary electron acceptors;
- (2) identify bacteria present in the original aquifer material and the enrichments established on PCE and VC using 16S rRNA gene based community analyses;
- (3) determine whether separate specific *Dehalococcoides* strains are associated with the dechlorination of the separate chloroethenes;
- (4) determine whether reductive dehalogenation is growth-linked and quantify growth yields on the different chloroethenes; and

(5) characterize the distribution of reductive dehalogenases associated with the enrichments.

Initial enrichment cultures were developed from groundwater and aquifer sediment using PCE and VC as the primary electron acceptors. A highly enriched PCE-dechlorinating culture, designated RU11/PCE, that exhibited growth-linked reductive dechlorination on PCE, TCE, *cis*-DCE, and VC was ultimately developed. During reductive dechlorination of PCE and TCE to ethene by RU11/PCE, little accumulation of the toxic intermediates *cis*-DCE and VC was observed. Cloning and sequencing of 16S rRNA genes, denaturing gradient gel electrophoresis analyses of PCR amplified 16S rRNA genes (PCR-DGGE), and sequencing of 16S rRNA genes directly from genomic DNA extracted from the enriched culture, were used to verify purity of the *Dehalococcoides*-containing RU11/PCE enrichment culture. Quantitative PCR was used to estimate the growth yields for subcultures amended with each of the chlorinated ethenes and to investigate dehalogenating abilities of *Dehalococcoides* in the enrichment.

2. Literature Review

2.1. Chlorinated ethenes

PCE and TCE have been used extensively for dry cleaning and mechanical degreasing since the 1930s (NRC, 2004) because of their desirable physico-chemical characteristics. However, frequent use, improper disposal practices and accidental releases of these chlorinated solvents have resulted in the widespread contamination of subsurface environments (Lee et al., 1998; Löffler and Edwards, 2006; Stroo et al., 2003). In the environment, biotic and abiotic transformation of PCE and TCE often resulted in accumulation of more toxic products such as *cis*-DCE, *trans*-1,2-dichloroethene (*trans*-DCE) and VC (Bradley, 2003). As a result, chlorinated ethenes, PCE, TCE, DCE isomers, and VC, have been among the most common contaminants found in groundwater and aquifer sediments (Lee et al., 1998; NRC, 2004).

Because of the toxicity and suspected carcinogenicity of chlorinated ethenes, the U.S. Environmental Protection Agency (EPA) established regulatory standards for these compounds in the late 1980s (ATSDR, 1997a; ATSDR, 1997b). The current drinking water maximum contaminant levels (MCLs) for PCE, TCE, *cis*-DCE, and *trans*-DCE are 5 ppb, 5 ppb, 70 ppb, and 100 ppb, respectively (US EPA; <http://www.epa.gov/safewater/contaminants.2009>). PCE and TCE have been found in 771 and 852 sites of the 1,430 National Priorities List sites identified by the EPA, respectively (ATSDR, 1997a; ATSDR, 1997b).

Freedman and Gossett first reported that under anaerobic conditions, PCE and TCE can be reductively dechlorinated in a stepwise manner to ethene as shown in **Figure 2.1** (Freedman and Gossett, 1989).

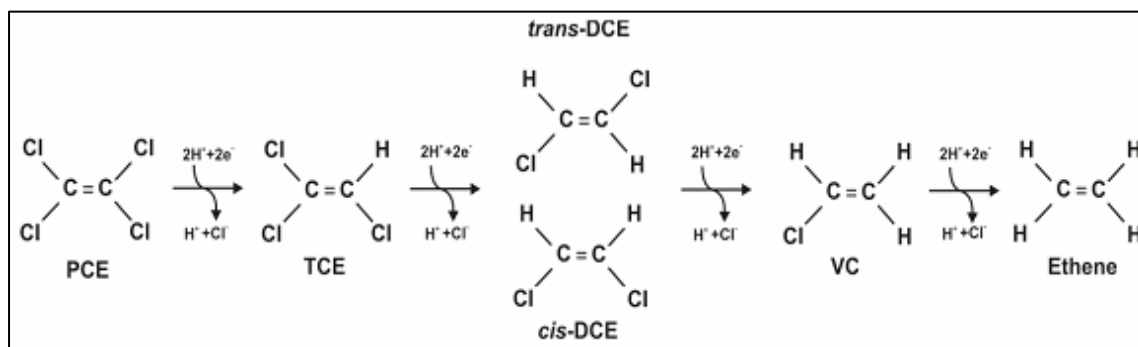


Figure 2.1. Anaerobic reductive dechlorination pathway of chlorinated ethenes showing the stepwise removal of chlorine and the requirement for an electron source (Freedman and Gossett, 1989).

Each of the successive dechlorinating steps requires two electrons (for one chlorine which is replaced by one hydrogen atom), and therefore constitutes a reductive reaction. Intermediates in the pathway include TCE, DCE isomers, and VC (Fetzner, 1998; Freedman and Gossett, 1989; Lee et al., 1998). The *cis*-DCE is toxic and a suspected carcinogen (ATSDR, 1996) and VC is a proven human carcinogen (ATSDR, 2006). Hence, incomplete dechlorination may not achieve detoxification of chloroethene-contaminated sites, unless the *cis*-DCE and VC are subsequently degraded or removed by other processes. Because of its status as a known human carcinogen, VC is the highest ranked organic compound on the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) priority list (ATSDR, 2007).

PCE and TCE are dense non aqueous phase liquids (DNAPLs). Remediation of PCE and TCE is challenging because of their volatility, low aqueous solubility, hydrophobicity, and the maintenance of a separate DNAPL phase in the subsurface

(Christ et al., 2005; Mercer and Cohen, 1990). Traditionally, pump and treat methods were adopted to remediate contaminated sites (Abelson, 1990). Pump and treat remediation consists of pumping the contaminated water out of the aquifer and treating it *ex situ* via air stripping, activated carbon sorption, or other means (Durant et al., 2004; NRC, 2004). These methods are expensive and often ineffective, especially where contaminant concentrations are low. Furthermore, because pump and treat technology is a method that remediates only the plume of dissolved contaminant in the aqueous phase, it can not treat the DNAPL phase or the reservoir of contaminant sorbed to the aquifer material (Christ et al., 2005). In most cases in contaminated groundwater, pure DNAPL serves as a continuous contaminant source (EPA, 2003), along with contaminants which have sorbed to clays in the aquifer and later diffuse back into the dissolved phase (EPA, 2003; Parker et al., 2008). Additional physicochemical remediation methods are numerous and include surfactant flushing, soil vapor extraction, chemical oxidation and reduction by metallic iron (EPA, 2003; NRC, 2004).

2.2. Biological transformation of chlorinated ethenes

Chlorinated ethenes may be aerobically degraded and anaerobically dechlorinated by microorganisms. For example, VC is mineralized to carbon dioxide by aerobic bacteria such as *Mycobacterium* sp. (Hartmans and De Bont, 1992), *Rhodococcus* sp. (Malachowsky et al., 1994), *Actinomycetales* sp. (Phelps et al., 1991), *Nitrosomonas* (Vannelli et al., 1990) and *Pseudomonas* sp. (Verge and Freedman, 2000; Verge et al., 2000). *Polaromonas* sp. strain JS666 utilizes *cis*-DCE as a sole carbon and energy source under aerobic conditions (Coleman et al., 2002). Methanotrophic microorganisms also

utilize monooxygenases to cometabolically oxidize TCE through the formation of epoxide at expense of methane (Verge and Freedman, 2000; Vogel et al., 2002). The disadvantage of aerobic co-metabolic degradation is the need for a co-substrate. Further, since DCE or VC are usually produced in anaerobic zones, contaminant migration to areas of higher redox potential is needed before these aerobic processes could occur.

PCE and TCE can be reductively dechlorinated under anaerobic conditions to sequentially form less chlorinated daughter products. During metabolic microbial reductive dechlorination, chlorinated organic compounds serve as terminal electron acceptors in a respiratory process carried out by specialized bacteria (for reviews see (Löffler and Edwards, 2006; Löffler et al., 2003)). Reductive dechlorination couples the reduction of chlorinated compounds to the oxidation of suitable substrates (i.e. electron donors). Complete dechlorination of PCE to ethene results in contaminant detoxification, since ethene is considered an environmentally acceptable and safe product. Biological dechlorination utilizing microbial populations has been studied and applied for *in situ* remediation of chloroethene-contaminated groundwater plumes extensively in the last decade (Löffler and Edwards, 2006).

Respiratory reductive dechlorination is a process in which microorganisms are able to use chlorinated compounds as electron acceptors in their energy metabolism (Holliger et al., 2003). Over the last two decades, considerable effort has been devoted to identifying and isolating microorganisms capable of metabolic reductive dechlorination. Numerous bacterial isolates capable of dechlorinating chlorinated compounds have been reported (Holliger et al., 2003).

Anaerobic microorganisms, such as methanogens (Cabirol et al., 1998; Fathepure et al., 1987), sulfate reducers and homoacetogens (Egli et al., 1988; Terzenbach and Blaut, 1994), can also reductively dechlorinate PCE and TCE in a cometabolic process. Metal containing enzymes such as vitamin B12, coenzyme F430 and hematin in methanogens and sulfate reducers can additionally catalyze reductive dechlorination of chlorinated ethenes (Gantzer and Wackett, 2002).

Isolated chlororespiring bacteria which gain energy from the process (see **Table 2.1**) are members of phylogenetically diverse bacterial groups including the Low G+C Gram positives, δ -proteobacteria, ϵ -proteobacteria, and green non-sulfur bacteria or chloroflexi. Most of the anaerobic dechlorinating bacteria that have been isolated (e.g., *Desulfuromonas*, *Sulfurospirillum multivorans*, and *Dehalobacter*) reductively dechlorinate PCE and TCE to the toxic intermediate *cis*-DCE. *Desulfitobacterium* sp. strain PCE1 is a Gram-positive bacterium that can dechlorinate PCE to TCE and small amount of DCE (Gerritse et al., 1996). This strain has a wide substrate range and can utilize hydrogen, lactate, pyruvate, butyrate, formate, ethanol and serine as electron donors; and PCE, 2-chlorophenol, 2,4,6-trichlorophenol, 3-chloro-4-hydroxyphenylacetate, sulfite, thiosulfate, fumarate, cysteate, and isothionate as electron acceptors (Gerritse et al., 1996). *Desulfitobacterium* sp. strain Viet1 has a similar substrate range as strain PCE1 and is not affected by 2-bromoethanesulfonic acid (BES) (Löffler et al., 1997). Strain PCE-S utilizes PCE, TCE, sulfite, and fumarate as electron acceptors using formate and pyruvate as electron donors. *Desulfitobacterium frappieri* strain TCE1 also has a similar substrate range as strain PCE1 (Gerritse et al., 1999).

Dehalobacter restrictus strain PERK23 is a strictly hydrogenotrophic bacterial species, which transforms PCE or TCE to *cis*-DCE (Holliger et al., 1993). Acetate is required as a carbon source and the addition of thiamine, cyanocobalamin, arginine, histidine and threonine were necessary for growth (Holliger et al., 1998). *Dehalobacter* sp. strain TEA uses H₂ as an electron donor and acetate and carbon dioxide as carbon sources. This strain was isolated from an anaerobic attached-attached film reactor with charcoal attachment media and inoculated with contaminated groundwater. Its 16S rRNA gene sequence is 99.7% identical to the sequence of strain PERK23 (Wild et al., 1996).

Clostridium bifermentans strain DPH-1, a Gram-positive anaerobic bacterium, dechlorinates PCE and TCE to *cis*-DCE and uses H₂, glucose, and yeast extract as electron donors (Chang et al., 2000).

Desulfuromonas chloroethenica strain TT4B (Krumholz, 1997) and *Desulfuromonas michiganensis* (Sung et al., 2003) dechlorinate PCE and TCE to *cis*-DCE using acetate as the electron donor. *Desulfuromonas michiganensis* strain BB1 and strain BRS1 were isolated from pristine river sediment and chloroethene-contaminated aquifer material, respectively (Sung et al., 2003). Besides PCE and TCE, strains BB1 and BRK1 can reduce ferric iron, sulfur, fumarate and malate as electron acceptors. They use acetate, lactate, pyruvate, succinate, malate and fumarate as electron donors (Sung et al., 2003).

Sulfurospirillum multivorans (formerly *Dehalospirillum multivorans*), a Gram-negative spirillum, dechlorinates PCE and TCE to *cis*-DCE using H₂ or formate as electron donors and acetate as a carbon source. *Sulfurospirillum multivorans* can use

fumarate, arsenate, selenate and nitrate as electron acceptors (Neumann et al., 1994; Scholz-Muramatsu et al., 1995).

Current information suggests that reductive dechlorination of *cis*-DCE to VC and ethene is mediated exclusively by the *Dehalococcoides* spp. (see **Table 2.2**). *Dehalococcoides* spp. are strict hydrogenotrophic bacteria which can dechlorinate all chlorinated ethenes as well as other halogenated compounds as electron acceptors. *Dehalococcoides ethenogenes* strain 195 was the first *Dehalococcoides* isolate described, and to date is the only one to dechlorinate PCE to ethene, although the final dechlorination step from VC to ethene is cometabolic (Maymo-Gatell et al., 1997). This strain exhibits a wide substrate range, including chlorobenzenes, chlorophenols, polychlorinated dibenzo-*p*-dioxins and furans, polychlorinated biphenyls and polybrominated diphenyl ethers (Fennell et al., 2004; Fung et al., 2007; He et al., 2007; He et al., 2006; Liu and Fennell, 2008). Indeed, in general, *Dehalococcoides* populations show a remarkable range of dechlorinating capabilities. *Dehalococcoides* sp. strain CBDB1 can dechlorinate chlorinated benzenes (Adrian et al., 2000) and polychlorinated dibenzo-*p*-dioxins (Bunge et al., 2003). *Dehalococcoides* sp. strain BAV1 was the first isolate capable of obtaining energy from VC reductive dechlorination (He et al., 2005). Strain BAV1 utilized all DCE isomers and VC as electron acceptors and cometabolized PCE and TCE in the presence of DCE isomers or VC (He et al., 2003b). *Dehalococcoides* sp. strain VS also grew on VC (Cupples et al., 2003). Isolate FL2 can dechlorinate PCE to ethene, though the PCE-to-TCE and VC-to-ethene steps are cometabolic and require the presence of one of the growth-supporting electron acceptors TCE, *cis*-DCE, or *trans*-

1,2-dichloroethene (*trans*-DCE) (He et al., 2003b). To date, however, no single *Dehalococcoides* isolate has been found to grow on all four chloroethene compounds.

Because of the differing physiological capabilities of the dehalogenating bacteria, it is likely that in the environment a variety of strains co-exist to completely dechlorinate the chloroethenes (Löffler and Edwards, 2006). Indeed *Dehalococcoides* spp. with different substrate ranges and other strains such as *Dehalobacter* sp. may compete for both electron donors and acceptors while bringing about dechlorination of environmental contaminants (Becker, 2006).

Table 2.1. Growth-linked PCE-dechlorinating isolates other than *Dehalococcoides* spp. (adapted and updated from (Löffler, 2003))

Lineage	Organism	Dechlorination activity	Electron donors	Ref.
Low G+C Gram-positive bacteria	<i>Desulfotobacterium hafniense</i> strain Y51	PCE to <i>cis</i> -DCE	H ₂	(Suyama et al., 2001)
	<i>Desulfotobacterium</i> sp. strain Viet1	PCE to TCE	H ₂	(Löffler et al., 1997)
	<i>Dehalobacter restrictus</i> strain PER-K23	PCE to <i>cis</i> -DCE	H ₂	(Holliger et al., 1998)
	<i>Clostridium bifermentans</i> strain DPH1	PCE to <i>cis</i> -DCE	H ₂	(Chang et al., 2000)
Proteobacteria, δ -subdivision	<i>Desulfuromonas chloroethenica</i> strain TT4B	PCE to <i>cis</i> -DCE	acetate	(Krumholz, 1997)
	<i>Desulfuromonas michiganensis</i> strain BB1	PCE to <i>cis</i> -DCE	acetate	(Sung et al., 2003)
	<i>Desulfuromonas michiganensis</i> strain BRS1	PCE to <i>cis</i> -DCE	acetate	(Sung et al., 2003)
	<i>Geobacter lovleyi</i> strain SZ	PCE to <i>cis</i> -DCE	acetate, H ₂	(Sung et al., 2006a)
Proteobacteria, ϵ -subdivision	<i>Sulfurospirillum multivorans</i>	PCE to <i>cis</i> -DCE	H ₂	(Neumann et al., 1994; Scholz-Muramatsu and Neumann, 1995)
	<i>Sulfurospirillum halorespirans</i>	PCE to <i>cis</i> -DCE	H ₂	(Luijten et al., 2003)
Proteobacteria, γ -subdivision	<i>Enterobacter agglomerans</i> strain MS-1 ^a	PCE to <i>cis</i> -DCE	Acetate ^b	(Sharma and McCarty, 1996)

^a Growth linked to reductive dechlorination has not been unequivocally demonstrated.

^b H₂ was not tested.

Table 2.2. Properties of *Dehalococcoides* strains dechlorinating chlorinated ethenes (adapted from (Löffler and Edwards, 2006))^a.

<i>Dehalococcoides</i> strain	Metabolic chlorinated ethene electron acceptors	Cometabolized chlorinated ethenes	Major end products	Ref.
strain 195	PCE, TCE, <i>cis</i> -DCE, 1,1-DCE	<i>trans</i> -DCE, VC	VC, ethene	(Maymo-Gatell et al., 1997)
strain FL2	TCE, <i>cis</i> -DCE, <i>trans</i> -DCE	PCE, VC	VC, ethene	(He et al., 2003a)
strain BAV1	<i>cis</i> -DCE, <i>trans</i> -DCE, 1,1-DCE, VC	PCE, TCE	Ethene	(He et al., 2003a; He et al., 2003b)
strain VS	TCE, <i>cis</i> -DCE, 1,1-DCE, VC	ND	Ethene	(Cupples et al., 2003)
strain GT	TCE, <i>cis</i> -DCE, 1,1-DCE, VC	None	Ethene	(Sung et al., 2006b)
strain KB-1/VC	TCE, <i>cis</i> -DCE, VC	ND	ethene	(Duhamel et al., 2004)

^a ND, not determined

2.3. Reductive dehalogenases

Transformation of chlorinated ethenes during microbial reductive dechlorination is catalyzed by a novel class of redox enzymes, the reductive dehalogenases (RDases) (Löffler et al., 2003). A variety of RDases with a wide range of substrate specificities have been purified and characterized from a number of halorespiring microorganisms. PCE-to-*cis*-DCE reductive dechlorinating enzyme systems and/or the encoding genes have been characterized from *Sulfurospirillum multivorans* (Neumann et al., 1996; Neumann et al., 1998), *Dehalobacter restrictus* (Maillard et al., 2003; Schumacher et al., 1997), *Desulfitobacterium* sp. strain PCE-S (Miller et al., 1997), *Clostridium bifermentans* (Okeke et al., 2001), and *Dehalococcoides ethenogenes* strain 195 (Magnuson et al., 2000; Magnuson et al., 1998; Nijenhuis and Zinder, 2005). All characterized reductive dehalogenases share common features in that they have iron sulfur binding motifs and a corrinoid cofactor, and a twin arginine transport (TAT) signal peptide sequence that is targeted for excretion to the periplasm (Berks et al., 2000). Physiological studies of whole cells and cell extracts have suggested that the expression of different dechlorinating enzymes depends on the type and concentration of the initial dechlorinating substrate (Gerritse et al., 1999; Nijenhuis and Zinder, 2005).

In addition, several RDase genes involved in chlorinated ethene dechlorination have been identified in *Dehalococcoides* spp. A TCE-to-VC RDase (TceA) was identified and characterized from *Dehalococcoides ethenogenes* strain 195 (Magnuson et al., 2000). This RDase reductively dechlorinates TCE, *cis*-DCE, and 1,1-DCE at rates of 5 to 12 $\mu\text{mol/min}$, dechlorinates VC and *trans*-DCE at much lower rates and cannot dechlorinate PCE (Magnuson et al., 2000). The gene encoding the TCE RDase, *tceA*, was identified in *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp. strain

FL2 (He et al., 2005). A *cis*-DCE-to-ethene RDase (VcrA) was partially purified from strain VS (Muller et al., 2004), and the gene encoding for this RDase (*vcrA*) was also identified in strain GT (Sung et al., 2006b). The *bvcA* gene, implicated in VC-to-ethene reductive dechlorination was detected in strain BAV1 and several VC-respiring mixed cultures (Krajmalnik-Brown et al., 2004). Low growth yields of dechlorinating microorganisms, and the hydrophobic nature and oxygen sensitivity of RDases have made purification of chlorinated ethene RDases laborious and impractical. Large amounts of information on the dechlorinating activities can be obtained using molecular investigations of the RDase genes associated with chlorinated ethene dechlorination processes. Identification of RDase genes has allowed for the development of RDase gene-targeted molecular tools specific to critical dechlorination steps and more accurate characterization of the metabolic activity of the *Dehalococcoides* population (Holmes et al., 2006; Ritalahti et al., 2006). In addition to the RDase genes whose functions are known, multiple nonidentical putative RDase genes have also been described (Holscher et al., 2004; Kube et al., 2005; Seshadri et al., 2005; Waller et al., 2005). The genome sequences of strain 195 and strain CBDB1 revealed 17 (Seshadri et al., 2005) and 32 putative RDase genes (Kube et al., 2005) respectively. For strains BAV1 and FL2, 11 and 14 putative RDase genes, respectively, have been found (Holscher et al., 2004; Krajmalnik-Brown et al., 2004).

The molecular characteristics of isolated dehalogenases, including the presence of cofactors, are summarized in **Table 2.3**.

Table 2.3. Characteristics of reductive dehalogenases and reductive dehalogenase genes. Adapted and updated from (Holliger et al., 2003)

Enzyme		Organisms	Cofactors	Ref.
PCE RDase	PceA	<i>Dehalococcoides ethenogenes</i> 195	Corrinoid ^a Fe/S	(Magnuson et al., 1998)
PCE/TCE RDase	PceA-Sm	<i>Sulfurospirillum multivorans</i>	1 Corrinoid ^{a,b} 8Fe/8Sb	(Neumann et al., 1996)
	PceA-Dr	<i>Dehalobacter restrictus</i>	1 Corrinoid ^{a,b,c} 4Fe/4S ^c	(Maillard et al., 2003)
		<i>Desulfitobacterium</i> sp. PCE-S	1 Corrinoid ^{a,b} 8Fe/8S ^d Corrinoid ^a	(Miller et al., 1998) (Okeke et al., 2001)
		<i>Clostridium bifermentans</i>		
TCE RDase	TceA (<i>tceA</i>)	<i>Dehalococcoides ethenogenes</i> 195	Corrinoid ^a Fe/S	(Magnuson et al., 2000b; Magnuson et al., 1998)
3-chlorobenzene RDase		<i>Desulfomonile tiedjei</i>	Heme ^e	(Ni et al., 1995)
<i>o</i> -chlorophenol RDase	CprA	<i>Desulfitobacterium dehalogenans</i>	1 Corrinoid ^{c,b} 1 4Fe/4S ^c 1 3Fe/3S ^c 1 Corrinoid ^a	(van de Pas et al., 1999)
		<i>Desulfitobacterium hafniense</i>	12Fe/13S ^d	(Christiansen et al., 1998)
VC RDase	BvcA (<i>bvcA</i>)	<i>Dehalococcoides</i> sp. BAV1		(Krajmalnik-Brown et al., 2004)
VC RDase	VcrA (<i>vcrA</i>)	<i>Dehalococcoides</i> sp. VS		(Muller et al., 2004)

^a Indicated by photo-reversible inhibition of the reduced enzyme by iodo-alkanes.

^b Quantified by analysis of cobalt content and by extraction of corrinoid from purified enzyme followed by spectroscopic analysis.

^c Determined by EPR spectroscopy.

^d Quantified by atomic absorption spectroscopy and by standard procedure for acid-labile sulfide.

^e Indicated by optical spectroscopic analysis.

2.4. Bioremediation of chlorinated ethene contaminated sites

Bioremediation is a remediation technique that utilizes living organisms such as bacteria, fungi or plants as the catalysts for detoxification reactions that attempt to return the natural environment altered by contaminants to a satisfactory state. Bioremediation can be divided into three categories: natural attenuation, biostimulation, and bioaugmentation. As a result of recent successes, bioremediation is becoming increasingly accepted as a viable remediation strategy (William and Ronald, 2006). Dechlorinating microorganisms are native to many sites contaminated with chlorinated ethenes, but the dechlorinating bacteria are often patchily distributed throughout the aquifer and/or are present in very low numbers (Fennell et al., 2001; Lendvay et al., 2003; Major et al., 2002).

At sites where native dechlorinators occur, lack of electron donors, and/or unfavorable geochemical conditions such as non-neutral pH or oxidative redox potential may limit reductive dechlorination and remediation of the sites. Biostimulation has been implemented successfully at the field scale by amending organic or inorganic substrates to promote complete dechlorination of PCE or TCE to ethene (Lendvay et al., 2003). Many organic substrates such as alcohols, organic acids, vegetable oil and complex organic materials, have been proposed and employed as biostimulatory agents (William and Ronald, 2006). Degradation of these compounds induces anaerobiosis and their fermentation supplies H_2 and acetate which may be used as electron donors by dechlorinating bacteria. However, this approach may be insufficient to sustain desirable dechlorination rates and only works at sites where native dechlorinators, especially *Dehalococcoides* spp., which are known to carry out complete dechlorination to ethene, are present.

As an alternative approach, robust PCE-to-ethene dechlorinating consortia may be grown *ex situ*, transported to the contaminated site, and injected into the subsurface in a process called bioaugmentation (He et al., 2003a). This approach, which is usually accompanied by biostimulation, has been implemented at numerous sites (Lendvay et al., 2003) and bioaugmentation inocula are commercially available (Bio-Dechlor INOCULUM from Regenesis, <http://www.regenesis.com/products/bioAug/biode>; KB-1® from SiREM, <http://www.siremlab.com/kb1guarantee>, 2009). Application of bioaugmentation requires techniques that minimize exposure of the bioaugmentation inoculum to air (Durant et al., 2004). Another challenge for successful bioaugmentation is the effective distribution of the dechlorinating bacteria throughout the targeted remedial zone. Adhesion, attachment, and colonization of bacteria in subsurface environments should be understood for successful application. Successful bioaugmentation with a *Dehalococcoides*-containing culture (KB-1) was reported in a pilot scale field test (Major et al., 2002). In that study, it was demonstrated that the augmentation of the culture to the contaminated site, where only incomplete dechlorination of chlorinated ethenes had been detected, resulted in the stimulation of dechlorination beyond *cis*-DCE to ethene.

Inoculation of dechlorinating cultures (Adamson et al., 2004), and a combination of physicochemical remediation and bioaugmentation using *Dehalococcoides*-containing cultures (Amos et al., 2007) were shown to be effective in DNAPL source zone remediation. Approaches to analyzing biodegradation potential of the contaminated sites have also been developed. Bioremediation techniques such as natural attenuation, biostimulation and bioaugmentation, require assessing and monitoring of the microbial

populations of interest to inform site management to achieve successful bioremediation. The presence, growth, and distribution of microorganisms in the sites can be monitored through cultivation-based methods and molecular analysis techniques. Cultivation methods are useful to study the physiology and biochemistry of microorganisms, however, these methods are limiting because these require more time and labor and can be biased by the type of cultivation media and methods used (Amann et al., 1995). Cultivation methods usually underestimate the native microbial populations because less than 10% of microorganisms in the natural environment are thought to be culturable under laboratory conditions (Amann et al., 1995).

2.5. PCR analysis of dechlorinating microbial populations

The application of cultivation-independent molecular biological tools has been adopted and has improved the monitoring of microbial contaminant detoxification processes during bioremediation. Nucleic acid-based molecular biological tools allow for specific and sometimes quantitative detection of key bacteria involved in detoxification processes (Löffler and Edwards, 2006). The most common target for nucleic acid-based analyses is the bacterial rRNA gene (SERDP and ESTCP, 2005). Phylogenetic information obtained from 16S rRNA gene analysis, however, is not useful for prediction of metabolic function in *Dehalococcoides* spp. because the strains share highly similar 16S rRNA gene sequences. For example, the 16S rRNA sequences of strain BAV1, strain FL2 and strain CBDB1 are greater than 99.9% identical (Adrian et al., 2000; He et al., 2003b; He et al., 2005). Further, the 16S rRNA gene sequences of FL2 and CBDB1 are identical to that of KB-1/VC, a highly enriched TCE-to-ethene-dechlorinating strain

(Adrian et al., 2000; Duhamel et al., 2004; He et al., 2005). When 16S rRNA gene-targeted molecular biological tools fail to provide phenotypic information, alternative targets for identification are needed. Use of reductive dehalogenases genes such as *tceA*, *bvcA*, and *vcrA* associated with chlorinated ethene transformation has allowed for design of molecular biomarkers specific to critical dechlorination steps (Holmes et al., 2006; Ritalahti et al., 2006). Both 16S rRNA gene-based and functional gene-based analyses have been used to understand microbial processes involved in contaminant detoxification and have further enhanced the ability of site managers to effectively implement reliable, cost-effective bioremediation approaches (Fennell et al., 2001; Himmelheber et al., 2007; Rahm et al., 2006).

Although specific genes can be used as molecular biomarkers indicating metabolic potential, detection of genes does not always indicate that the desired metabolic activity is occurring or that the target populations are active. Unlike DNA detection, detection of RNA is considered to be a more direct measure of microbial activity (Löffler and Edwards, 2006). In RNA biomarker analysis, functional gene transcription is monitored by detecting or enumerating messenger RNA (mRNA) transcripts. Typically, mRNA is extracted from sample material and then converted to cDNA by reverse transcription (RT) (Johnson et al., 2005; Lee et al., 2006) and the characterization of cDNA via a variety of analyses then proceeds as with DNA. The lack of established protocols for RNA biomarker analysis either from field or laboratory samples and concerns related to mRNA stability make interpreting and comparing results from RNA biomarker analysis difficult. Detailed studies are needed to address the current limitations of RNA biomarker analysis and to increase the resolution and applicability of

this approach. Despite the current limitations, RNA-targeted molecular biomarkers have already proven useful in laboratory experiments and current studies are evaluating the applicability of RNA-targeted molecular biomarkers for field assessment and determining *in situ* microbial activity (Amos et al., 2008).

PCR protocols have been designed that specifically amplify the 16S rRNA gene of many dechlorinating organisms, including *Dehalococcoides* spp. (Fennell et al., 2001; He et al., 2003b; Hendrickson et al., 2002; Löffler et al., 2000), *Desulfuromonas* spp. (Löffler et al., 2000), *Sulfurospirillum* spp. (Ebersole and Hendrickson, 2005), *Dehalobacter* spp. (Löffler et al., 2000), and *Desulfitobacterium* spp. (el Fantroussi et al., 1997). Additional protocols have been designed to specifically target RDase genes including *pceA* of *Sulfurospirillum multivorans* (Regeard et al., 2004), *pceA* of *Dehalobacter restrictus* (Regeard et al., 2004) and *tceA* of *Dehalococcoides ethenogenes* strain 195 (Krajmalnik-Brown et al., 2007; Magnuson et al., 2000; Regeard et al., 2004). Application of direct PCR (Fennell et al., 2001) or nested PCR (Löffler et al., 2000) with DNA extracted from bacterial cultures, groundwater samples, or sediments provides specific information regarding the presence or apparent absence of a particular organism or biomarker gene, and thus gives an indication of the likelihood of specific dechlorinating activity.

While conventional PCR can provide only qualitative information, quantitative PCR (qPCR) analysis provides an additional level of precision by enumerating target genes. qPCR analysis is similar to PCR, but the reaction components also include a fluorescent reporter. During qPCR, the fluorescence increases at each amplification cycle, and the amount of fluorescence can be correlated to the number of initial target genes

present in the PCR reaction. Many qPCR protocols have been developed for specific detection and quantification of dechlorinating organisms, including *Dehalococcoides* spp. (He et al., 2003b; Holmes et al., 2006; Ritalahti et al., 2006; Smits et al., 2004), *Dehalobacter* spp. (Smits et al., 2004), *Desulfitobacterium* spp. (Smits et al., 2004) and *Geobacter* spp. (Duhamel and Edwards, 2006). Protocols have also been designed to quantify RDase genes such as *tceA*, *bvcA*, and *vcrA* (Holmes et al., 2006; Ritalahti et al., 2006). qPCR analysis has a dynamic range spanning six to eight orders-of-magnitude and is generally as sensitive as direct PCR and sometimes nested PCR. Recently qPCR analysis has extended to quantify RNA biomarkers via reverse transcription (RT)-qPCR analysis (Johnson et al., 2005; Lee et al., 2006; Rahm et al., 2006).

Dehalococcoides species are difficult to both obtain and grow in pure culture, and so are often studied as members of consortia using molecular techniques. In particular, a significant number of qPCR assays targeting *Dehalococcoides* spp. have been reported (He et al., 2003b; Holmes et al., 2006; Ritalahti et al., 2006; Smits et al., 2004). Initial qPCR methods targeted the 16S rRNA gene, however, because strains with the same 16S rRNA gene sequence can have different dehalogenating abilities, reductive dehalogenase genes are now used as the most appropriate qPCR target (Lee et al., 2008; Sung et al., 2006b). Quantitative PCR has been critical to our current understanding of *Dehalococcoides* populations; it has provided information on their growth characteristics, dehalogenating abilities and effective use in bioremediation efforts. Future qPCR research directions will likely involve method standardization, as well as continued research on the functional genes associated with *Dehalococcoides* populations.

3. Materials and Methods

3.1. Chemicals

PCE (>98% purity), TCE (>98% purity), butyric acid (>99% purity), and sodium lactate (>99% purity) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The *cis*-DCE was obtained from Supelco Co. (Bellefonte, PA, USA). Gaseous VC ($\geq 99.5\%$ purity) was obtained from Fluka Chemical Corp. (Ronkonkoma, NY, USA), and methane (99% purity) and ethene (99% purity) were purchased from Matheson (Matheson Tri-Gas, Inc., Montgomeryville, PA, USA). Prepurified N₂ and a 70% N₂/30% CO₂ gas mixture purchased from Air Gas (Piscataway, NJ, USA) were used as anaerobic purge gases.

3.2. Analytical procedures

Chlorinated ethenes, ethene and methane were determined using a Agilent Technology 6890N (Agilent Technologies, Inc. Santa Clara, CA, USA) gas chromatograph (GC) with a flame ionization detector (FID). 100 μ L headspace samples were injected into the GC-FID equipped with a GS-GasPro capillary column (30 m x 0.32 mm I.D.; Agilent Technologies, Inc. Santa Clara, CA, USA) with helium as the carrier gas at constant pressure (19 PSI). The oven temperature was programmed to hold at 50°C for 2 min, increase to 180°C at 15°C/min, and then hold at 180°C for 2 min. Retention times of individual standard compounds were 0.9, 1.6, 6.0, 10.4, 11.3 and 13.5 min for methane, ethene, *trans*-DCE, *cis*-DCE, 1,1-DCE, TCE and PCE, respectively. The major DCE isomer detected throughout this study was *cis*-DCE; only trace amounts of *trans*-DCE or 1,1-DCE were detected in initial microcosms, and these were not

quantified. Calibration for *cis*-DCE, TCE, and PCE was performed using aqueous external standards prepared by adding a known mass of a methanol stock solution containing PCE, TCE and *cis*-DCE, in known gravimetric concentrations, to 100 mL of autoclaved groundwater and sediment mixture. The gaseous VC, ethene, and methane were added to these external standards via a gastight syringe at atmospheric pressure, and the concentrations added were determined by the ideal gas law (Freedman and Gossett, 1989).

Organic acids (lactate, butyrate, and acetate) were quantified using a Beckman Coulter[®] System Gold[™] high performance liquid chromatography (HPLC) (Beckman-Coulter, Inc., Fullerton, CA). Aqueous samples (1 mL) were centrifuged and the supernatant was filtered (0.2 μ m) into HPLC vials. Samples were analyzed with an HPX-87H column (Bio-Rad, Hercules, CA, USA) using a flow rate of 1 mL/min, 0.005 mM sulfuric acid as the eluent, and a UV detector at a wavelength of 210 nm. Calibration curves for the organic acids were prepared using a dilution series over a concentration range from 0.1 to 10 mM for each compound.

3.3. Enrichment set-up and growth conditions

The chloroethene-dechlorinating microbial enrichments were derived from groundwater and sediment retrieved from an aquifer. Groundwater and sediments were obtained from a PCE-contaminated fractured rock aquifer on the Rutgers University Busch Campus in Piscataway, NJ. This site is part of the Brunswick (Passaic) formation that makes up much of northern New Jersey [<http://www.state.nj.us/dep/njgs/>]. Sterile polyethylene tubing was extended into a groundwater, monitoring well screened at 15 ft

below ground surface. During groundwater pumping, the tubing was extended to the bottom of the well to obtain fine sediments that accumulated there. Groundwater and sediment were collected in autoclaved amber glass jars that were filled to overflowing, sealed with a positive meniscus to exclude air, and refrigerated at 4°C until use. Based on visual observation, the samples consisted of less than approximately 1% volume to volume (vol/vol) sediment, with the remainder groundwater.

For preparation of enrichment cultures, approximately 100 mL of the groundwater with 1% sediment (vol/vol) was transferred to pre-autoclaved 160 mL serum bottles in a glove bag (Cole-Parmer, Vernon Hills, IL, USA) purged with N₂ (prepurified grade). Serum bottles were sealed with Teflon®-lined butyl rubber stoppers (Supelco Inc., Bellefonte, PA, USA) and aluminum caps (Supelco Inc.) in the glove bag. A total of 15 bottles were set up and divided into five different treatments (**Table 2.4**). Lactate and butyric acid (2.5 mM final concentration each) were added as electron and hydrogen donors. Sterile controls were prepared by autoclaving microcosms for 40 min at 120 °C over three successive days. Chlorinated ethenes, ethene and methane were present in the microcosms upon setup because of the contamination and dechlorination products originally present in the groundwater aquifer. Concentrations of chlorinated ethenes in microcosms were measured weekly by analyzing headspace samples using a GC-FID as described in **section 3.2**. After complete dechlorination of all the initially present chlorinated ethenes, electron acceptor (2 µL of neat PCE or 5 mL of VC) and electron donors were amended to the microcosms, according to the protocol (**Table 2.4**).

After complete dechlorination of amended PCE or VC and depletion of electron donors, PCE or VC-dechlorinating microcosms were transferred (10%, vol/vol) to sterile

anaerobic defined mineral medium that had been purged with N₂-CO₂ (70:30 vol/vol). The mineral salts medium contained the following (per liter): 1 g of NaCl, 0.5 g of MgCl₂·6H₂O, 0.5 g of KH₂PO₄, 0.2 g of NH₄Cl, 0.3 g of KCl, 0.015 g of CaCl₂·2H₂O, and 1 mg of resazurin (Löffler et al., 1996). After the medium was boiled and cooled to room temperature under N₂ purging, NaHCO₃ (30 mM final concentration) was added to the medium, and the pH was checked to ensure it was 7.2 to 7.3. Next, 90-mL portions were dispensed into 160-mL bottles purged with N₂-CO₂ (70:30, vol/vol) and the bottles were sealed with Teflon-coated butyl stoppers, autoclaved for 45 min, and cooled to room temperature (Fennell et al., 2001). Routinely, L-cysteine (0.5 mM final concentration) and Na₂S 9H₂O (0.4 mM final concentration) were added as reductants. Each transfer culture received 1 mg/mL of ampicillin and 0.25 mM 2-bromoethanesulfonic acid (BES) to inhibit the growth of Gram-positive and certain Gram-negative bacteria, and methanogens, respectively (Maymo-Gatell et al., 1997). As an electron acceptor, 4 µL of neat PCE or 5 to 10 mL of gaseous VC resulting in 40 µmol PCE or 80 to 160 µmol VC per bottle, respectively, was added. Finally, inoculum (10%, vol/vol) was added to each bottle. Transferred cultures were incubated in the dark at 25°C without shaking. Sequential transfers (10%, vol/vol) from VC-to-ethene-dechlorinating microcosms to a defined medium, as described, over approximately 4 years (3 transfers) continued to dechlorinate VC to ethene. Sequential transfers (10%, vol/vol) from PCE-to-ethene-dechlorinating microcosms to a defined medium, as described, over approximately 4 years (11 transfers) yielded a sediment-free, non-methanogenic, ethene-producing enrichment culture, referred to as RU11/PCE.

Table 2.4. Protocol for initial microcosms prepared in triplicate.

Bottle set	Electron donors ^a (Lactate and Butyrate 1.5 mM, each)	PCE ^a (0.2-0.3 mM)	VC ^a (1.5 mM)	Autoclaved
Autoclaved control	No	No	No	Yes
Live control	No	No	No	No
Electron donors only	Yes	No	No	No
Electron donors + PCE	Yes	Yes	No	No
Electron donors + VC	Yes	No	Yes	No

^a electron donors and PCE/VC were added only after the initially present chloroethenes were dechlorinated

3.4. DNA isolation

Genomic DNA for molecular analyses was collected from duplicate 1 to 5 mL culture samples that were removed from the enrichment cultures using a sterile syringe equipped with a 25 G sterile needle and pre-flushed in sterile, anoxic N₂-CO₂ (70:30, vol/vol). For preparation of genomic DNA from the original groundwater, 400 mL of groundwater was processed to recover cells. Cells were harvested using either filtration onto a 0.2 µm membrane filter (SUPOR 200, Pall Gelman, East Hills, NY, USA) or by centrifugation at 16,000 x g for 20 minutes at 4 °C. The filtered cells were washed twice with 10 mL of sterile distilled water. The harvested cells were frozen at -20 °C until DNA extraction. Microbial genomic DNA was extracted from frozen cells using a MoBio Ultraclean DNA extraction kit (Mo-Bio Inc., Solana Beach, CA, USA), as recommended by the manufacturer with the following modifications. The cells collected through centrifugation were resuspended in 1 mL of a bead-beating solution included in the kit and the cell suspension was transferred to bead-beating tubes. At the end of the extraction

process, DNA was eluted from the spin filters with 30 μ L of sterile water instead of the elution buffer provided with the kit. The reproducibility of this DNA extraction method was evaluated by extracting replicate samples. The eluted DNA was stored at -20°C until use.

3.5. PCR methods for 16S rRNA, housekeeping genes and reductive dehalogenase genes

Genomic DNA from the initial microcosms was used as the template with PCR primer pair Bac27f/Bac1527f (**Table 2.5**), targeting the universal bacterial 16S rRNA gene, using PCR conditions as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C (60 s), 55°C (45 s), and 72°C (90 s), with a final extension at 72°C for 7 min (Löffler et al., 2000). Nested PCR with the PCR primers Dhc 730f/Dhc 1350f targeting the *Dehalococcoides* 16S rRNA gene (Fennell et al., 2001), was performed using as template the amplicons from the PCR of the genomic DNA from initial microcosm under the following conditions: initial 94°C denaturation step (3 min), followed by 30 cycles of 94°C (45 s), 58°C (30 s), and 72°C (90 s), with a final extension at 72°C for 7 min (**Table 2.5**). Each 25 μ L PCR reaction mixture contained the following: 1x PCR buffer (USB Corporation, Cleveland, OH, USA), 2.5 mM of MgCl_2 (USB), 20 nmol of deoxynucleoside triphosphate (USB), 10 pmol of each primer, 1.5 U of Taq polymerase (USB), and 1 to 4 μ L of template DNA. All Primers were obtained from Integrated DNA Technology (Coralville, IA, USA).

A PCR approach was utilized to investigate the purity of 11th generation PCE-amended cultures (RU11/PCE) using bacterial housekeeping genes. PCR primer pairs

were utilized to amplify the RNA polymerase gene, *rpoB*; the ATP synthase delta subunit gene, *atpD*; the superoxide dismutase gene, *sod*; the DNA repair protein gene, *recA*; and the DNA gyrase subunit B gene, *gyrB* (**Table 2.6**). PCR primer pairs were designed using PrimerQuest (Integrated DNA Technology, Coralville, IA, USA) The thermal cycler program was as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C (45 s), 60 °C (45 s) for *rpoB*, *atpD*, and *recA* genes and 58 °C for the *sod* gene, and 72 °C (60 s), with a final extension at 72 °C for 3 min. The thermal cycle for the *gyrB* gene was as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C (60 s), 60 °C (60 s), and 72 °C (120 s), with a final extension at 72 °C for 7 min. Each 25 µL PCR reaction mixture contained the following: 1x PCR buffer (USB Corporation, Cleveland, OH, USA), 2.5 mM of MgCl₂ (USB), 20 nmol of deoxynucleoside triphosphate (USB), 10 pmol of each primer, 1.5 U of Taq polymerase (USB), and 1 of template DNA. The PCR amplicons were purified and sequenced to determine whether more than one gene, and thus more than one strain, was detected.

PCR for detecting dehalogenase genes (*tceA*, *vcrA*, *bvcA* and specific RDase genes found in the KB-1 culture) was also performed using genomic DNA from our enrichment cultures after five, eight and 11 generations, as template. The PCR primer information for RDase gene detection is summarized in **Table 2.7**. The thermal cycler program was as follows: initial 94 °C denaturation step (3 min), followed by 30 cycles of 94 °C (30 s), 60 °C (45 s), and 72 °C (120 s), with a final extension at 72°C for 5 min (Waller et al., 2005). Each 25 µL PCR reaction mixture contained the following: 1x PCR buffer (USB Corp.), 2.5 mM of MgCl₂ (USB), 20 nmol of deoxynucleoside triphosphate

(USB Corp.), 10 pmol of each primer, 1.5 U of Taq polymerase (USB), and 1 to 2 μ L of template DNA.

An aliquot of each PCR product was electrophorized on a 1.2% agarose gel, stained in 0.1% ethidium bromide solution and visualized using UV on a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA, USA). Gels were analyzed against a size standard to determine if the correct fragment size had been amplified under each condition. Some of the RDase PCR amplicons showing positive bands on the gels were purified and sequenced for identification.

Table 2.5. Primer sequences used for amplification of 16S rRNA genes.

Primer	Sequence (5' → 3')	Target gene (expected amplicon size)	Ref.
Bac27f Univ1527r	AGAGTTTGATCMTGGCTCAG AAGGAAGGTGWTCARCC	Bacterial 16S rRNA gene (1500 bp)	(Lane, 1991)
Dhc730f Dhc1350r	AAGGCGGTTTTCTAGGTT GGTTGGCACATCGACTTC	<i>Dehalococcoides</i> 16S rRNA gene (630 bp)	(Fenne ll et al., 2001)

Table 2.6. Primer sequences used for analysis of bacterial housekeeping genes.

Primer	Sequence (5' → 3')	Target gene (expected amplicon size)	Ref.
Dehalo rpoBf Dehalo rpoBr	TRGCCTTTATGAGCTGGCAGGGTT TTTGTCACCCACTGAAACCTTGCG	Bacterial RNA polymerase gene (500 bp)	This study
Dehalo recAf Dehalo recAr	CAACAYATTATTGCCCAGGCCCAA AATTCGGCTCCTGAAAGGRGGA	Bacterial DNA repair protein gene (500 bp)	This study
Dehalo atpDf Dehalo atpDr	TAACGTGYTGGGTGAASCTCTGGA AACAGGAGCACGTCCTGTCTTTCT	Bacterial ATP synthase gene (500 bp)	This study
Dehalo sodf Dehalo sodr	TACCAGCTCTGGGAARCNGACTTT CGTTTNAGYCCGTAATCTGTCA	Bacterial superoxide dismutase gene (200 bp)	This study
UP-1 UP2r	GAAGTCATCATGACCGTTCTGCAY- GCNGGNGGNAARTTYGA AGCAGGGTACGGATGTGCGAGCCR- TCNACRTCNGCRTCNGTCAT	Bacterial DNA gyrase subunit B gene (1100 bp)	(Yama moto and Haraya ma, 1995)

Table 2.7. Primer sequences used for detection of reductive dehalogenase genes

Primer	Sequence (5' → 3')	Target gene (expected amplicon size)	Ref.
tceAf tceAr	GCTTTGGCGGTGATGATAAG TCCACCACCCATCGAGTTAT	<i>tceA</i> (212 bp)	(Ahn et al., 2008)
tceA_797r tceA_2490r	ACGCCAAAGTGCGAAAAGC TAATCTATTCCATCCTTTCTC	<i>tceA</i> (1732 bp)	(Magnuson et al., 2000)
bvcAf bvcAr	TGCCTCAAGTACAGGTGGT ATTGTGGAGGACCTACCT	<i>bvcA</i> (839 bp)	(Krajmalnik-Brown et al., 2004)
vcrABf vcrABr	CTATGAAGGCCCTCCAGATGC GTAACAGCCCCAATATGCAAGTA	<i>vcrAB</i> (1482 bp)	(Muller et al., 2004)
rdhA1_246f rdhA1_336r	ATCGGAGCTGCACAAGTAGG TCTTGTGAGCGGTGTCTTTG	<i>rdhA1</i> (90 bp)	(Waller et al., 2005)
rdhA2_720f rdhA2_985r	CAAAGGAGATGTTCCGGTGT CAGGTGGAAAAGACCGGTTA	<i>rdhA2</i> (265 bp)	
rdhA3_1149f rdhA3_1379r	CATTCTCCGGGAAGAAAACA CCAGGCTTCCTTGTCTTCAG	<i>rdhA3</i> (230 bp)	
rdhA4_754f rdhA4_925r	TTGTTATGCCGCCAATATGA TCTATCCATTTCGCCCAGAC	<i>rdhA4</i> (170 bp)	
rdhA5_1017f rdhA5_1137r	GATGCAGGCATTTACCGTTT GTCTCTTTGCCTTCGGTCAG	<i>rdhA5</i> (120 bp)	
rdhA6_318f rdhA6_555r	ATTTAGCGTGGGCAAAACAG CCTTCCCACCTTGGGTATTT	<i>rdhA6</i> (215 bp)	
rdhA7_1391f rdhA7_1539r	GCTAAAGAGCCGTCATCCTG GCAGTAACAACAGCCCCAAT	<i>rdhA7</i> (150 bp)	
rdhA8_845f rdhA8_1016r	CCCAAGGTAGGTGTGCAGAT CCCGGTTAGTTACCCCGTAT	<i>rdhA8</i> (160 bp)	
rdhA9_251f rdhA9_425r	CTGACCTTGAAACCCCTGAA TTGCCACCCATTTCATATT	<i>rdhA9</i> (170 bp)	
rdhA10_710f rdhA10_860r	GCTGAAACACCCACCAAAC CGACAAAGGGGAATCTTTGA	<i>rdhA10</i> (150 bp)	
rdhA11_429f rdhA11_609r	TAATGGCAACCGGAGGTAAG TCTACCGGTATGGCCTGAAC	<i>rdhA11</i> (180 bp)	
rdhA12_864f rdhA12_994r	AGGAGTTCCTGTGGGGACTT TTTGGGGGTCATAACTGCTC	<i>rdhA12</i> (130 bp)	
rdhA13_1356f rdhA13_1493r	CAGGGTACCTGTCCCTTCAA AGGGTTCTTCCGTCCGTACT	<i>rdhA13</i> (140 bp)	
rdhA14_642f rdhA14_846r	GAAAGCTCAGCCGATGACTC TGGTTGAGGTAGGGTGAAGG	<i>rdhA14</i> (200 bp)	

3.6. PCR-denaturing gradient gel electrophoresis (PCR-DGGE)

PCR-DGGE was performed for bacterial community analysis of the original groundwater and for enrichments after 5, 8 and 11 transfers. PCR-DGGE was also performed for the 11th generation subcultures tested for growth on all the chloroethenes (see **section 3.8**). Universal bacterial 16S rRNA genes and *Dehalococcoides*-specific 16S rRNA genes were amplified using the Bac338f-GC and Univ519r primer sets and the Dhc1f-GC and Dhc259r primer sets, respectively (**Table 2.5**). Each 50 μ L PCR contained the following (all reagents from USB Corp., Cleveland, OH, USA): 1x PCR buffer, 2.5 mM of MgCl₂, 20 nmol of deoxynucleoside triphosphate, 10 pmol of each primer, 1.5 U of Taq polymerase, and 2 to 4 μ L of template DNA. The thermal cycling program was as follows: initial denaturation for 5 min at 94 °C; 25 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec; and a 7 min final extension at 72 °C. The expected PCR amplicon size was verified with agarose gel electrophoresis on a 1.2% gel prior to DGGE. DGGE of PCR-amplified fragments was performed on an 8% polyacrylamide gel with a 30 to 60% urea-formamide gradient for 4 h at 150 V and 60°C with the DCode mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA).

The gel was stained in 0.1% ethidium bromide solution and visualized using UV on a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories). Each prominent band detected in the DGGE gel was excised using a sterile razor. Using the excised gel fragments as DNA template, a second PCR-DGGE was performed under the same conditions described above and the purity of each band was confirmed by noting a single DNA band after repeating the DGGE using the same conditions.

Table 2.8. Primer sequences used for PCR-DGGE analysis.

Primer	Sequence (5' → 3')	Target gene (expected amplicon size)	Ref.
Bac338f	CTCCTACGGGAGGCAGCAG	200 bp	(Nakat su et al., 2000a)
Bac338f-GC	CGCCCGCCGCGCCCCGCGCCCGTCCCGC- CGCCCCCGCCCTCCTACGGGAGGCAGCAG		
Univ519r	ATTACGCGGCTGCTGG		
Dhc1f	GATGAACGCTAGCGGCG	200 bp	(Duha mel et al., 2004)
Dhc1f-GC	CGCCCGCCGCGCGCGGGCGGGCGGGGCG- GGGGCACGGGGGGGATGAACGCTAGCG- GCG		
Dhc259r	CAGACCAGCTACCGATCGAA		

3.7. Quantitative PCR (qPCR)

Segments (V3 region) of bacterial 16S rRNA genes and *Dehalococcoides*-specific 16S rRNA genes were amplified by using primer sequences (Bac338f/Univ519r and Dhc1f/Dhc259r) shown in **Table 2.8**. Primers were obtained from Integrated DNA Technology (Coralville, IA, USA). Approximately 200 to 250-bp fragments of the 16S rRNA gene from 338 to 519 and 1 to 259 (according to *Escherichia coli* position) respectively, were amplified using the universal primers and *Dehalococcoides* spp. specific primers. The PCRs were performed in a total volume of 24 µL reaction mixture containing 12.5 mL of 2 × iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) [containing: the hot-start enzyme, iTaq™ DNA polymerase; SYBR PCR buffer; dNTP mix; SYBR I; 20 nM fluorescein for dynamic well factor collection; and 6 mM MgCl₂], 0.2 µM forward primer and 0.2 µM reverse primer. One µL of the DNA sample was added to each PCR mixture. The standards and the samples were included in duplicate for each run. The amplification reaction was performed with an iCycler iQ™

thermal cycler (Bio-Rad Laboratories) using the following program: 10 min at 94 °C; 40 cycles of (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s). Data analysis was performed using iCycler iQ™ Real-Time Detection system software (Bio-Rad Laboratories). All reactions were analyzed by melting curve measurement by a 0.5 °C per cycle decrease from 94 °C to 72 °C, following the amplification steps, to confirm that only one PCR product was synthesized. Standard curves for universal bacterial qPCR and *Dehalococcoides*-specific qPCE were prepared with a dilution series of quantified genomic DNA from *Pseudomonas fluorescens* and *Dehalococcoides ethenogenes* strain 195, respectively. The linear range for quantification was 10^2 to 10^9 gene copies μL^{-1} DNA. Genome and genomic analyses demonstrated previously that the 16S rRNA gene exists as a single copy on *Dehalococcoides* spp. genomes (Kube et al., 2005; Ritalahti et al., 2006; Seshadri et al., 2005).

3.8. Dechlorination yield determination

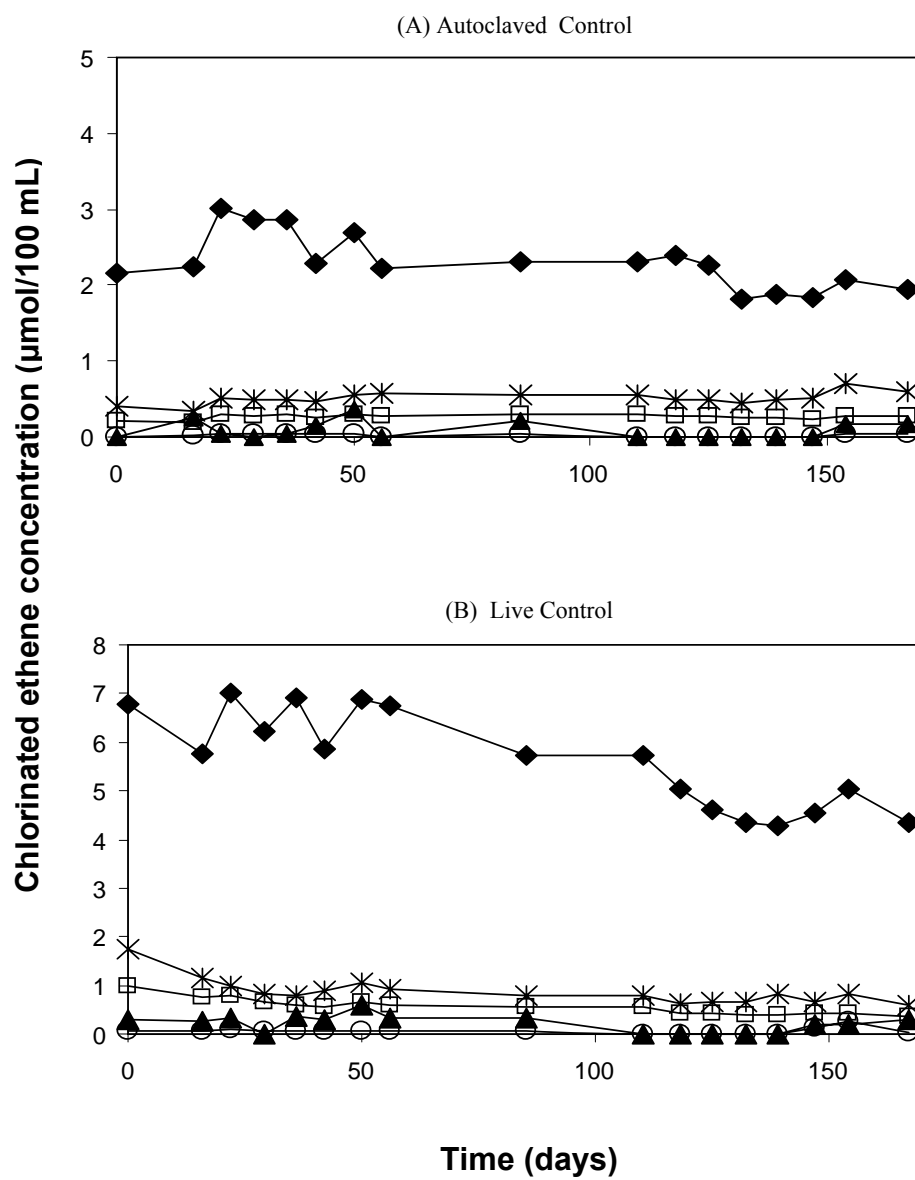
Triplicate RU11/PCE subcultures were set up for each treatment amended with either PCE, TCE, *cis*-DCE or VC as the only halogenated substrate in 60 mL serum bottles with 34 mL of 0.5 mM acetate-amended medium containing 0.4 to 6 mM of each chlorinated ethene and 400 μmol of H_2 . After two to three hours of equilibration time, 10% (vol/vol) inoculum was added and initial gas chromatography measurements were performed. Duplicate 1 mL culture samples were removed for DNA extraction. Headspace samples were monitored by gas chromatography every 3 to 4 days. After the chlorinated ethenes were consumed, duplicate 1 mL of culture samples were again removed for DNA extraction.

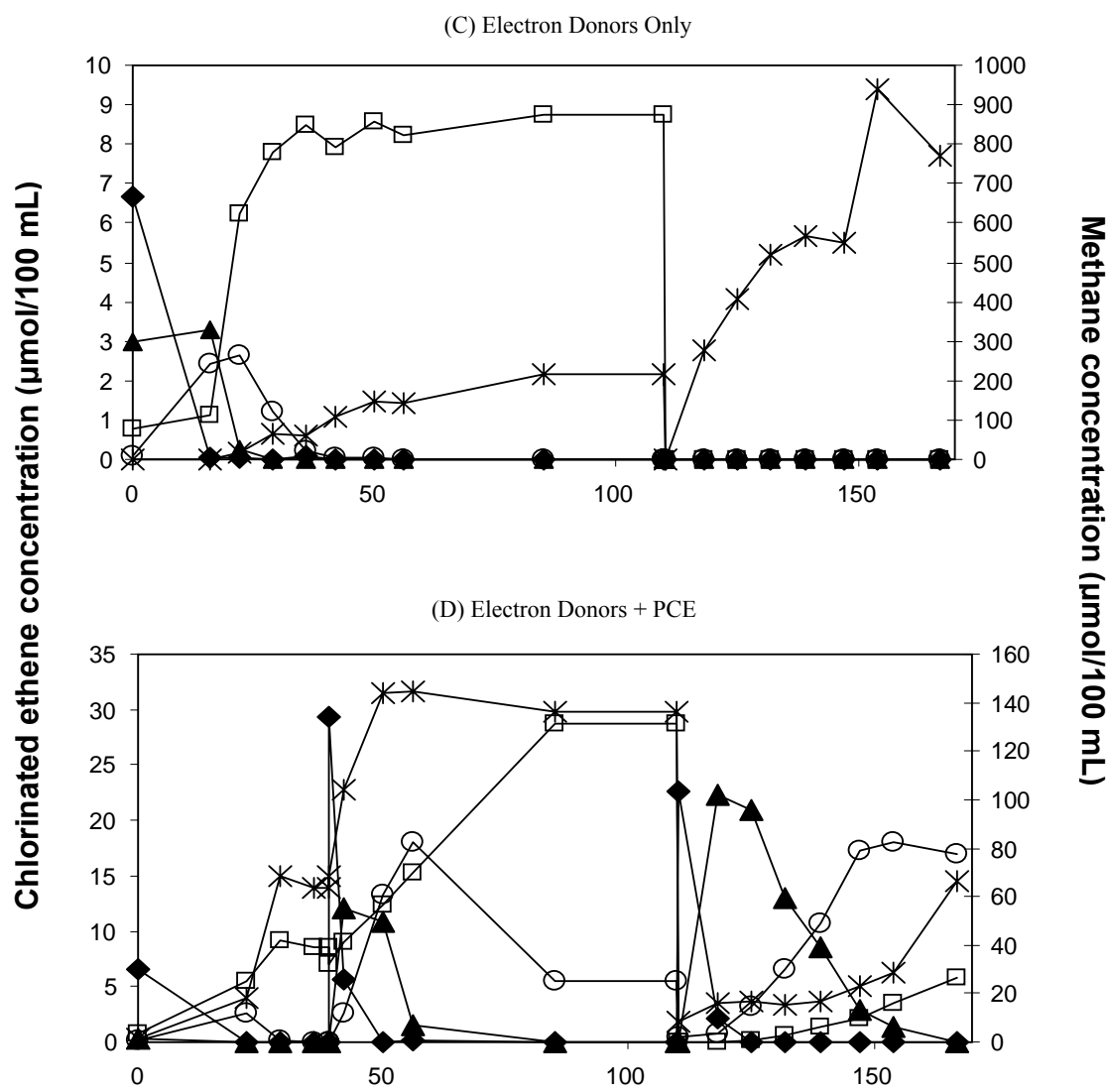
4. Results

4.1. Dechlorination in Rutgers University, Busch Campus groundwater microcosms

Figure 2.2 shows the dechlorination of chlorinated ethenes observed in an initial replicate microcosm from each of the five sets of microcosm bottles (triplicates for each treatment) established as shown in **Table 2.4**. On Day 0, microcosms contained PCE and *cis*-DCE that were present in the original groundwater. In the microcosms to which lactate and butyric acid had been added as electron donors, the original PCE was dechlorinated to ethene by the first observation point (Day 14). Note that TCE was detected in only trace amounts during dechlorination of the PCE and is not displayed in **Figure 2.2**. The only DCE isomer detected in the microcosms both in the original groundwater and as a product of dechlorination of added PCE was *cis*-DCE. Little dechlorination was observed in live controls not receiving electron donors or in autoclaved controls in which all live microorganisms had been killed. The microcosms to which no electron donor was added remained pink, indicative of oxidized conditions. This suggests that the original groundwater as collected from the well may have contained inadequate electron donor to drive reductive processes in the microcosms. Methane production was observed in the electron donor-amended microcosms. PCE and VC added to the PCE- and VC-amended microcosms on Days 40 and 110 were subsequently dechlorinated to ethene. The rate of PCE and VC dechlorination after re-amendment on Days 40 and 110 were comparable, which suggested that VC dechlorination to ethene might not be rate-limiting for this microbial population. These

results show that a robust dechlorinating population already existed at the site which was highly contaminated with PCE.





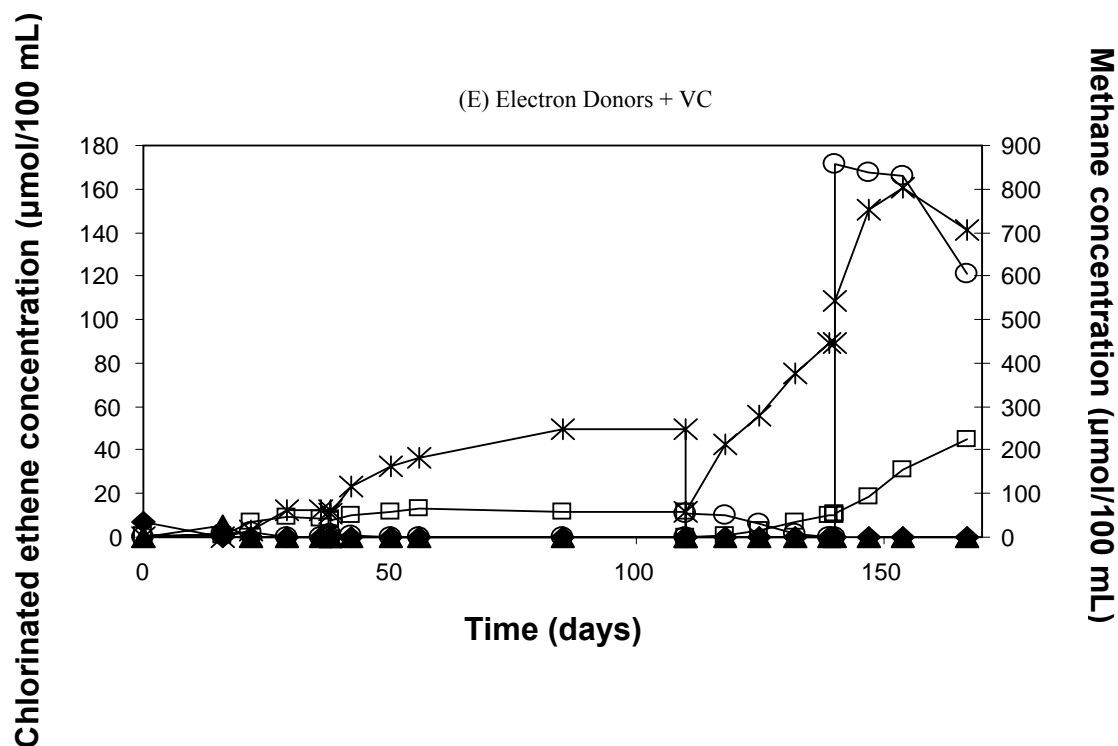


Figure 2.2. Dechlorination profiles from a replicate groundwater microcosm of each treatment established using sediment-groundwater mixtures from a contaminated aquifer at Rutgers University, Piscataway, NJ. (A) Autoclaved controls; (B) live control; (C) electron donors only control; (D) electron donors plus PCE; and (E) electron donors plus VC. Electron donors were lactic and butyric acids. PCE \blacklozenge ; TCE (not shown); *cis*-DCE \blacktriangle ; VC \circ ; ethene \square ; methane $*$.

4.2. Development of PCE- and VC-enrichments

Enrichment cultures were continually re-amended with either PCE or VC and transferred to fresh medium periodically as described in **section 3.3**, and maintained for over 4 years in defined mineral medium. A total of 3 transfers were accomplished for the VC-amended enrichments, which dechlorinated VC to ethene stoichiometrically, but these were not characterized in further detail beyond the second transfer. A total of 11 serial transfers, resulting in a dilution factor of 10^{11} were performed for the PCE-amended enrichments. The 11th generation culture, designated RU11/PCE stoichiometrically dechlorinated all chlorinated ethenes, from PCE to VC, completely to ethene in the presence of hydrogen as an electron donor and with acetate as a carbon source.

To control microbial activities other than dechlorinating bacteria, 2-bromoethanesulfonic acid (BES) and ampicillin were added from the time of the third transfer onward. BES is a structural analog to coenzyme M, the cofactor required for the terminal step in methanogenesis, and thereby inhibits this process (Gunsalus et al., 1978). Ampicillin is a bacterial inhibitor of peptidoglycan cross-linking (Madigan et al., 2003), but perhaps because of the *Dehalococcoides* spp.' unique cell wall structure (Maymo-Gatell et al., 1997), ampicillin had no impact on dechlorination rates. Similar to cultures containing *Dehalococcoides* spp. reported previously, neither 2-bromoethanesulfonic acid (BES) nor ampicillin affected dechlorination activities, at the concentrations used in this study. Methanogenesis activity present in the initial microcosms was inhibited, and eventually excluded by amendment with BES at each transfer. PCE dechlorination proceeded in the absence of methanogenesis and at increasing rates in the 10% (vol/vol)

transfer cultures. Numerous attempts were made to isolate PCE- and VC-dechlorinators using dilution to extinction methods, but these methods were not successful and have not been described.

4.2. Community analysis of original groundwater and the dechlorinating cultures

To characterize the microbial community composition of the original groundwater and the enrichment cultures, PCR-DGGE of bacterial 16S rRNA gene fragments was used. In DGGE, each band in a lane theoretically represents a different organism in the culture. However, in general, one organism may have multiple 16S rRNA gene copies with slightly different sequences, which may cause multiple bands for one organism. Further heteroduplexes, which occur during re-annealing of two very similar DNA strands amplified during PCR, could also result in multiple DGGE bands for the same strain (Ferris and Ward, 1997).

Bands of DNA detectable by eye were excised, reamplified using the same primers (**Table 2-5**), and sequenced. The sequences were compared to known sequenced strains or phylotypes using BLASTN [<http://blast.ncbi.nlm.nih.gov.ezproxy.lib.utexas.edu/Blast.cgi>]. **Figure 2.3** shows the results of the DGGE profile and the DNA sequences associated with each DGGE band from the original groundwater. The results indicate relatively low complexity in the bacterial community present in the original groundwater and sediments. *Dehalococcoides*-like bacteria were detected by PCR using *Dehalococcoides*-specific PCR primers in the original microcosms and in first-generation transfers enriched on PCE and VC (**Figure 2.4**).

The bacterial communities as assessed by DGGE in second generation PCE- and VC-amended enrichment cultures are shown in **Figure 2.5**. The results revealed that these early enrichments (second generation) contained only a few species of bacteria, since few bands were detected. Bacteria closely related to *D. ethenogenes* strain 195, *Dehalococcoides* strain BAV1 and *Dehalococcoides* strain CBDB1, based on 16S rRNA gene sequences, were detected in these second generation enrichments. An interesting observation was that two distinct bands corresponding to two separate *Dehalococcoides* spp. were detected in the second generation enrichment cultures maintained on PCE or VC (**Figure 2.5**).

After 11 transfers, DGGE analysis of the RU11/PCE enrichment using universal PCR primers for bacteria, 338f and 519r, yielded a single DNA band. Further, DGGE analysis with the *Dehalococcoides*-specific primers 1f-GC and 259r yielded a single band. The results shown in **Figure 2.6** suggest that the RU11/PCE culture was composed of one genus of bacteria, *Dehalococcoides*, and only one *Dehalococcoides* 16S rRNA gene was present in the culture.

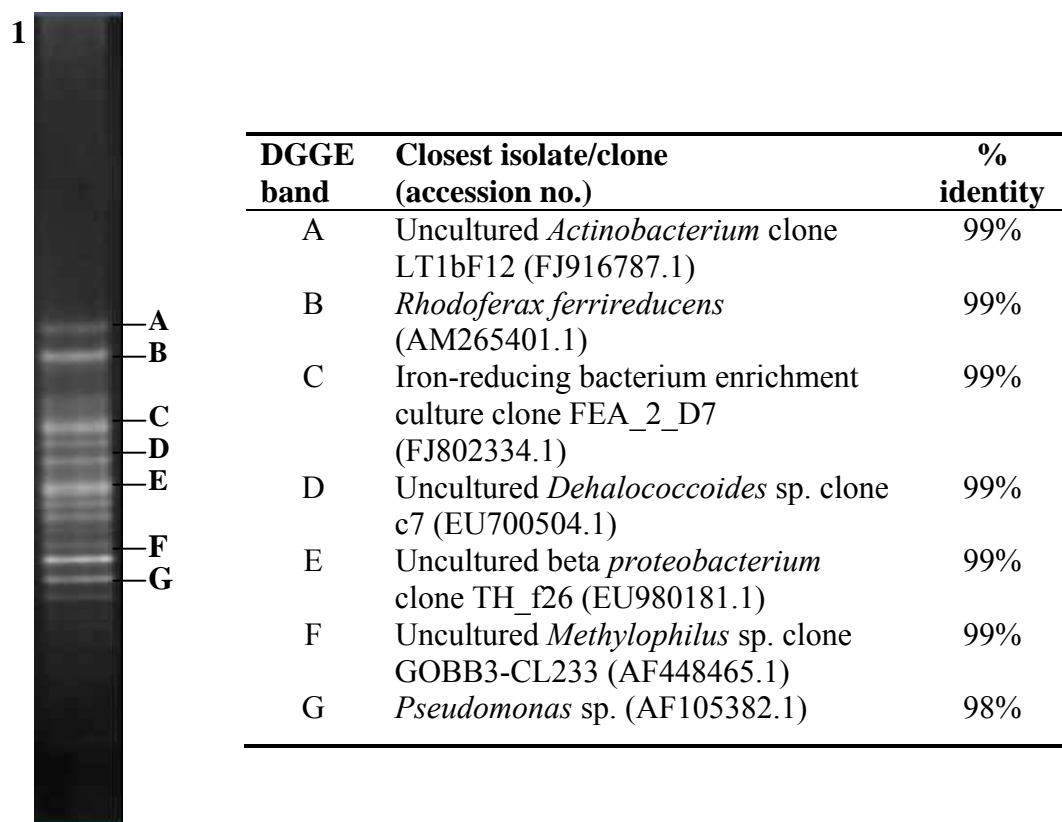


Figure 2.3. PCR-DGGE profile and DGGE band sequencing results of original contaminated groundwater recovered from an aquifer underlying Rutgers University, Piscataway, NJ. Seven bands were excised and their sequences obtained (approximately 150 bp).

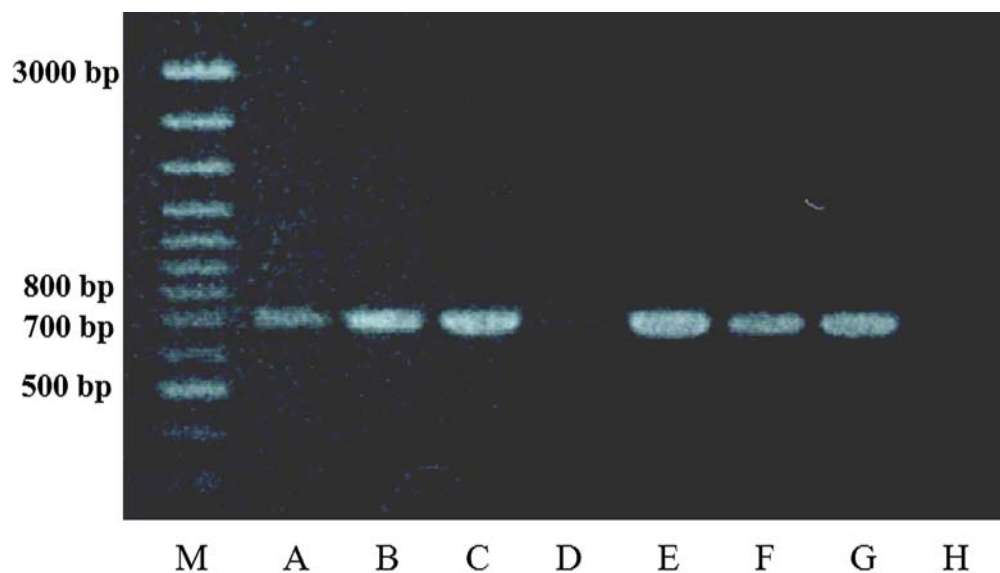
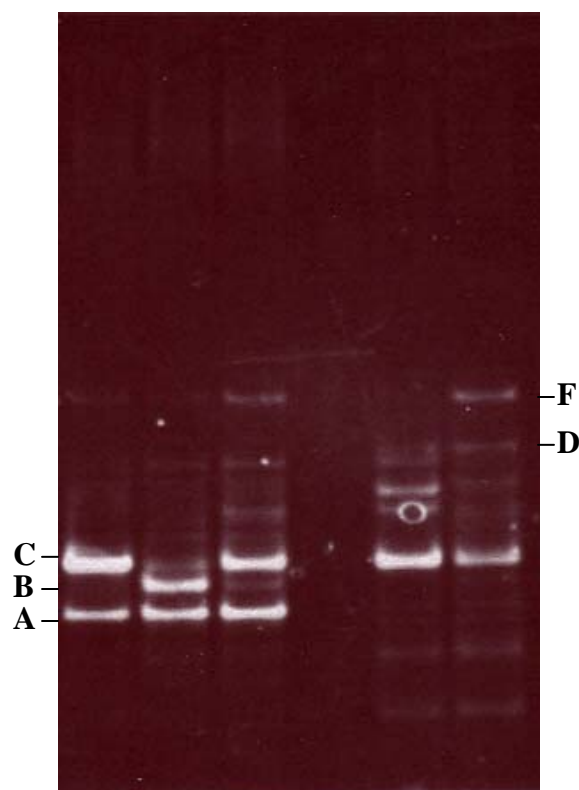


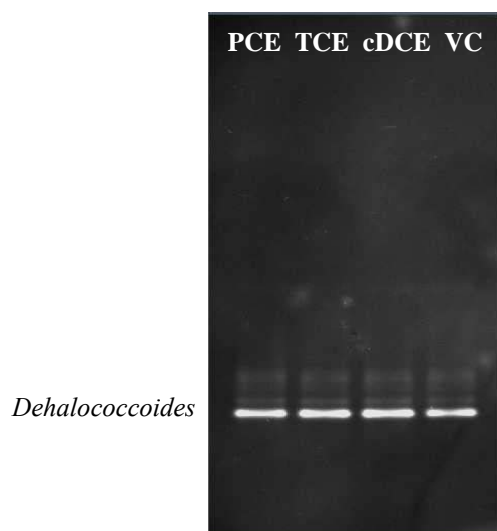
Figure 2.4. PCR amplification of genomic DNA from initial microcosms (A, B, C, and D) and 1st generation transfers (E and F) of groundwater from a contaminated aquifer at Rutgers University, Piscataway, NJ using primers targeting *Dehalococcoides* spp. M, 100 bp DNA molecular marker; A, electron donor; B, electron donor + PCE; C, electron donor + VC; D, live control microcosms; E, PCE-amended 1st generation transfer; F, VC-amended 1st generation transfer; G, positive control for PCR; and H, negative control.



DGGE band	Closest isolate/clone (accession no.)	% identity
A	Uncultured <i>Geobacter</i> sp. clone (EU266833.1)	97%
B	<i>Dehalococcoides ethenogenes</i> 195 (CP000027.1)	98%
C	<i>Dehalococcoides</i> sp. BAV1 (CP000688.1)	98%
D	Uncultured <i>Firmicutes</i> bacterium (CU920003.1)	96%
E	Uncultured <i>planctomycete</i> clone JY1a (DQ393189.1)	97%

Figure 2.5. PCR-DGGE profile and DGGE band sequencing results of second generation enrichments of groundwater and sediment recovered from an aquifer underlying Rutgers University, Piscataway, NJ. Twelve bands were excised and their sequences obtained (approximately 150 bp).

(A)



(B)

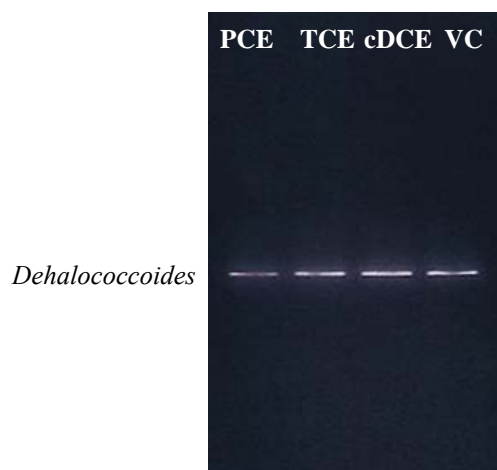


Figure 2.6. PCR-DGGE of genomic DNA from RU11/PCE cultures using primers targeting general bacteria using a universal bacterial primer pair (A) and *Dehalococcoides* spp. using a *Dehalococcoides*-specific primer pair (B). PCR amplicons were separated on 8 % acrylamide gels with a 20-60% denaturing gradient.

4.3. Detection of reductive dehalogenase genes

Primers for fourteen distinct reductive dehalogenase genes from the KB-1 culture (Waller et al., 2005) were used for screening RDase genes in the fifth, eighth and 11th (RU11/PCE) generation enrichment cultures. As reported in (Waller et al., 2005), each of these RDase genes were found to be highly similar to previously identified RDase genes in one or more *Dehalococcoides* strains. Sequences with over 99% identity on a nucleotide basis differ by only one or two amino acids in the enzyme. The KB-1 culture shares nine highly similar RDase genes with strain CBDB1, eight with strain FL2, three with strain 195, and two with strain BAV1 (Waller et al., 2005).

The RU11/PCE culture contained ten RDase genes highly similar to those found in the KB-1 culture, five highly similar to those found in strain FL2, four highly similar to those found in strain CBDB1, three highly similar to those found in strain 195 and one highly similar to those found in strain BAV1. With genomic DNA from the 8th generation PCE enrichment culture, two more RDase genes (*rdh3* and *rdh8* as denoted for the KB-1 culture) were detected; however these apparently disappeared through transfer and were not detected in RU11/PCE. These results suggest that multiple RDases existed simultaneously in the original groundwater and initial microcosms.

Among the three *Dehalococcoides* reductive dehalogenase genes most well characterized, only the *vcrA* gene, involved in VC dechlorination in strain VS, was detected in the RU11/PCE culture. Amplification of genomic DNA from subcultures of RU11/PCE that were established for determining growth yields (PCE-, TCE-, *cis*-DCE- and VC-amended as described in **sections 3.8 and 4.4**) with *vcrA* specific primers

yielded products of the expected size (1458 bp). Sequence analysis confirmed high similarity (99%) to the *vcrA* gene of strain VS (Muller et al., 2004).

In contrast, no PCR product was detected using genomic DNA from RU11/PCE as the template for the *bvcA* primer pair (*bvcAF/bvcAR*) (Krajmalnik-Brown et al., 2004) or the *tceA* primer pair (*tceAF/tceAR* and *tceA_797F/tceA_2490R*) (Ahn et al., 2008; Magnuson et al., 2000). The KB-1 *rdhA6* gene (*bvcA*-like gene) was also not detected in RU11/PCE. However, in fifth generation PCE-enrichments *bvcA* was detected using the *bvcA* primer pair (*bvcAF/bvcAR*). These data suggest that there were at least two distinct *Dehalococcoides* strains present in the initial microcosms and that one strain containing the *bvcA*-like gene was diluted out and excluded over the four years of enrichment and transfer.

4.4. Use of bacterial housekeeping genes to assess purity of RU11/PCE

Based on the inconclusive nature of the 16S rRNA gene analysis and the dehalogenase gene analyses we also assessed culture purity using the bacterial housekeeping genes: RNA polymerase gene, *rpoB*; ATP synthase delta subunit gene, *atpD*; superoxide dismutase gene, *sod*; the DNA repair protein gene, *recA*; and the DNA gyrase subunit B gene, *gyrB*. PCR analyses using primers targeting *rpoB*, *atpD* and *recA* were unsuccessful using genomic DNA from the RU11/PCE culture. The PCR targeting the *sod* gene and the *gyrB* gene generated a single DNA band. The PCR amplicons were purified and sequenced. The DNA fragments were 98 to 99% identical to the *sod* genes and the *gyrB* gene from strain BAV1 and strain CBDB1.

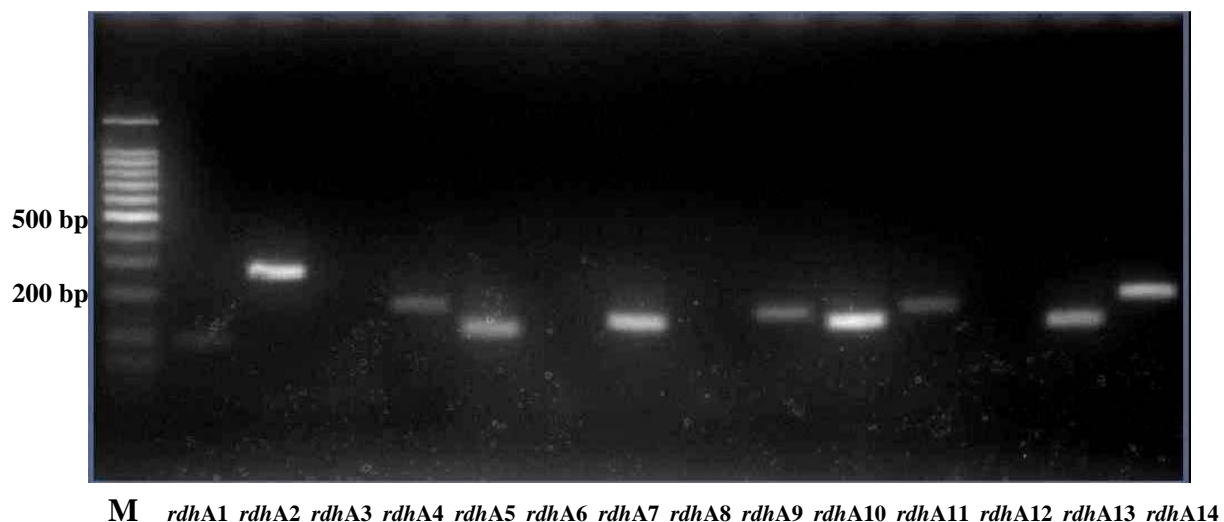
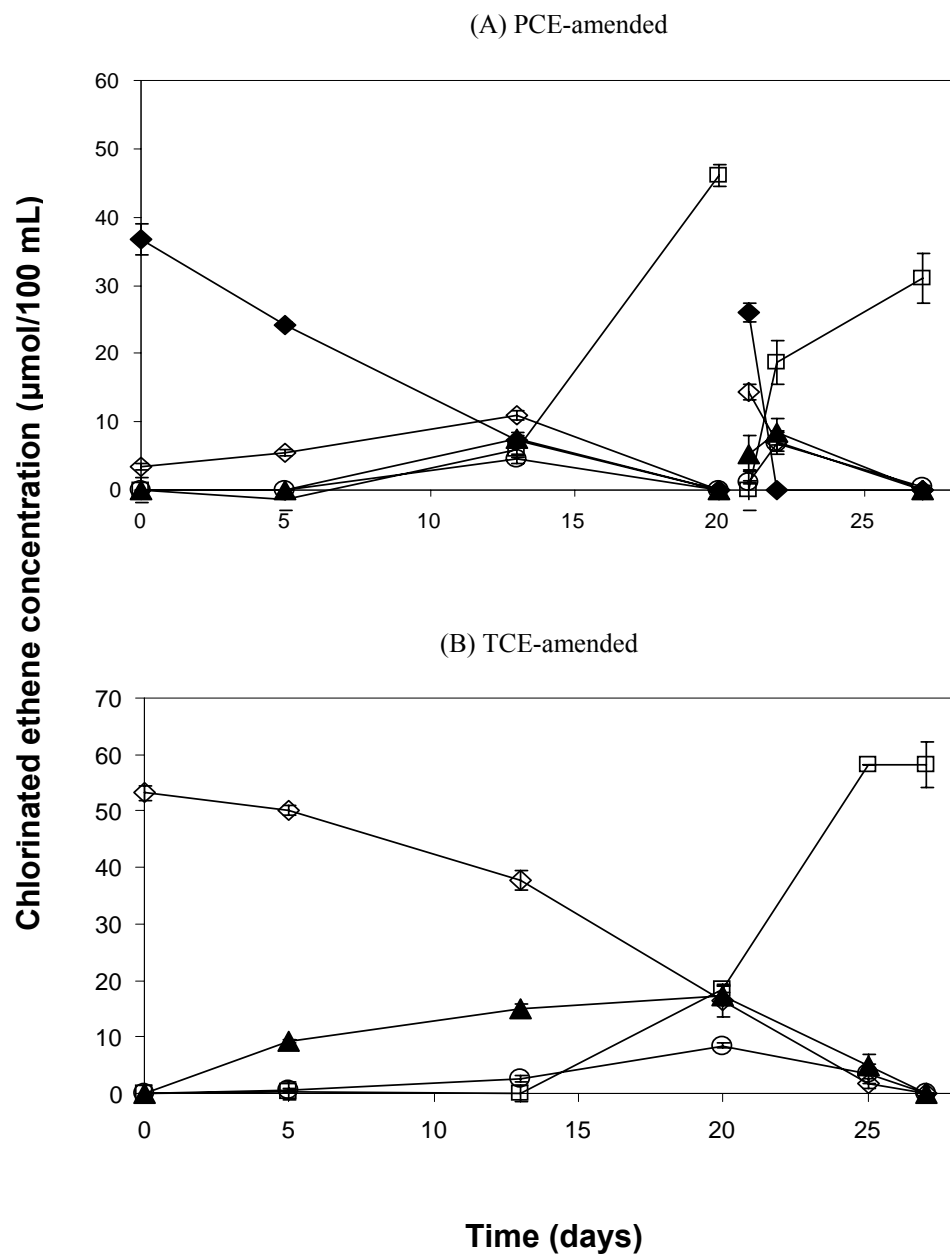


Figure 2.7. PCR amplification of genomic DNA from the 11th generation PCE-amended enrichment (RU11/PCE) using primers targeting reductive dehalogenase genes in *Dehalococcoides* strain KB-1. M is the 100 bp DNA molecular marker.



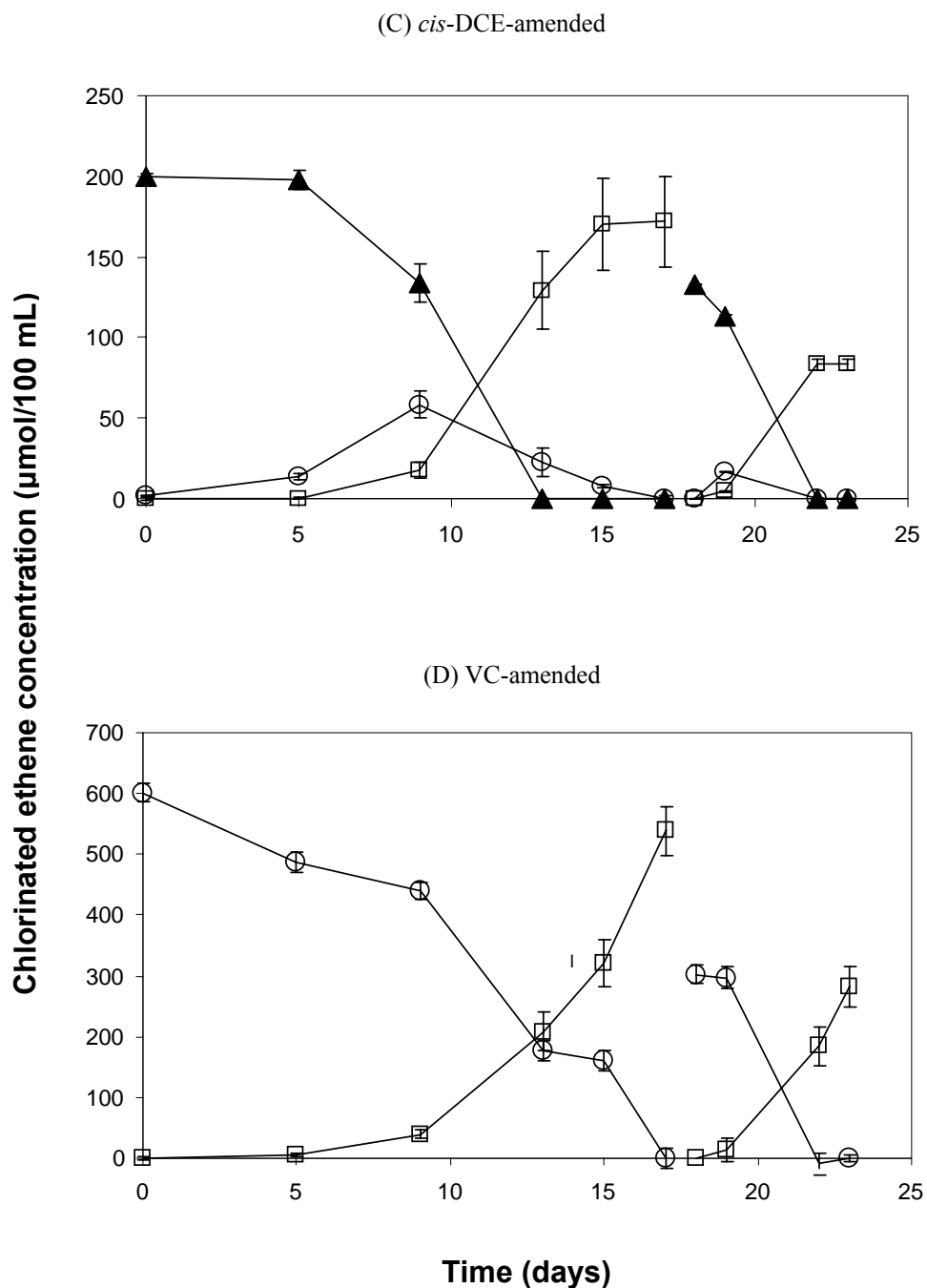


Figure 2.8. Dechlorination profiles from RU11/PCE subcultures amended with different chloroethene substrates. (A) PCE-amended; (B) TCE-amended; (C) *cis*-DCE-amended; and (D) VC-amended subculture. PCE \blacklozenge ; TCE \diamond ; *cis*-1,2-DCE \blacktriangle ; VC \circ ; ethene \square . Symbols are averages of triplicates and error bars are one standard deviation.

4.4. Growth yield

The growth yields on chlorinated ethenes for the *Dehalococcoides* sp./spp. present in the RU11/PCE culture were determined using qPCR. As shown in **Figure 2.8**, subcultures dechlorinated their respective substrates (PCE, TCE, *cis*-DCE and VC) to ethene. A lag of approximately six days occurred prior to substantial dechlorination of *cis*-DCE.

Using qPCR the average cell yields (*Dehalococcoides* 16S rRNA gene copies/ $\mu\text{mol Cl}^-$ produced) were determined to be $(1.06 \pm 0.25) \times 10^8$ for PCE, $(0.76 \pm 0.16) \times 10^8$ for TCE, $(0.75 \pm 0.28) \times 10^8$ for *cis*-DCE, and $(6.16 \pm 1.26) \times 10^7$ for VC, when using *Dehalococcoides*-specific primers. Growth yields measured using universal bacterial primers were similar to those from *Dehalococcoides*-specific qPCR. On an electron equivalent or Cl^- basis, the yields on all the chlorinated ethenes were similar, indicating that the *Dehalococcoides* strain(s) in this enrichment culture gained energy for growth from all dechlorination steps from PCE to ethene (**Table 2.9**).

Table 2.9. Comparison of yields of RU11/PCE on chloroethene substrates.

Subst -rate	Strain/ Culture	Bacterial 16S rRNA gene copies/ μ mol chlorinated ethene	<i>Dehalococcoides</i> 16S rRNA gene copies/ μ mol chlorinated ethene	<i>Dehalococcoides</i> 16S rRNA gene copies/ μ mol Cl ⁻ ^a	Ref.
PCE	RU11/ PCE	$(3.05 \pm 0.45) \times 10^8$	$(4.25 \pm 0.98) \times 10^8$	$(1.06 \pm 0.25) \times 10^8$	This study
TCE	RU11/ PCE	$(1.81 \pm 0.48) \times 10^8$	$(2.33 \pm 0.47) \times 10^8$	$(0.76 \pm 0.16) \times 10^8$	This study
DCE	RU11/ PCE	$(1.31 \pm 0.60) \times 10^8$	$(1.49 \pm 0.56) \times 10^8$	$(0.75 \pm 0.28) \times 10^8$	This study
VC	RU11/ PCE	$(9.70 \pm 6.37) \times 10^7$	$(6.16 \pm 1.26) \times 10^7$	$(6.16 \pm 1.26) \times 10^7$	This study
VC	VS	-	$(5.2 \pm 1.5) \times 10^8$	$(5.2 \pm 1.5) \times 10^8$	(Cupples et al., 2003)
VC	BAV1	-	$(6.3 \pm 0.3) \times 10^7$	$(6.3 \pm 0.3) \times 10^7$	(He et al., 2003a)
VC	KB- 1/VC	-	$(5.6 \pm 1.4) \times 10^8$	$(5.6 \pm 1.4) \times 10^8$	(Duhamel et al., 2004)
VC	GT	-	$(2.5 \pm 0.13) \times 10^8$	$(2.5 \pm 0.13) \times 10^8$	(Sung et al., 2006b)

^a for VC dechlorination, moles of ethene produced are equal to the moles of Cl⁻ produced.

5. Discussion

A new *Dehalococcoides*-containing culture, designated as RU11/PCE, was established from chloroethene-contaminated groundwater and sediments from a contaminated aquifer underlying the Busch Campus of Rutgers University in New Brunswick, NJ. Similar to other *Dehalococcoides* isolates, this highly purified culture required hydrogen as an electron donor and used either acetate or lactate as carbon source. Importantly, the RU11/PCE culture is able to carry out the complete PCE-to-ethene reductive dechlorination pathway and gain energy from dehalorespiration of all of four chlorinated ethenes.

Upon initial set up of the original groundwater microcosms from which RU11/PCE was developed, complete dechlorination of PCE to ethene was observed. Further, *Dehalococcoides* sp. was detected in the original microcosms using PCR, and community analysis by PCR-DGGE revealed the presence of multiple *Dehalococcoides* spp. in second generation enrichments. The groundwater community also contained phylotypes closely related to *Pseudomonas* sp. (AF105382), *Rodoferrax ferrireducens* (AM265401), and *Dehalococcoides* sp. (EU700504), among other bacterial species. The original microcosms were methanogenic, but this activity was selectively lost over several generations by the addition of BES. Thus, the original groundwater community was indicative of reduced, favorable conditions for dechlorinating bacteria.

Over 11 generations, PCE-amended cultures were enriched and community analysis by PCR-DGGE and direct DNA extraction and sequencing, revealed one 16S rRNA gene in the culture while complete dechlorination of PCE to ethene was maintained. Measurements of the growth yield of RU11/PCE on the chlorinated ethenes

confirmed that this culture can grow on all four chlorinated ethenes (**Table 2.6**). The growth yields on PCE, TCE, *cis*-DCE and VC were comparable to those reported previously for other *Dehalococcoides* strains and ranged from approximately 0.6×10^8 to 1×10^8 16S rRNA gene copies/ $\mu\text{mol Cl}^-$, for VC and PCE, respectively. Growth yields of *Dehalococcoides* organisms on chlorinated ethenes were determined using qPCR approaches because their fastidious growth, small cell size, and disk-shaped morphology impair traditional procedures (e.g., microscopic counts, protein measurements, determining dry weight, etc.) to estimate biomass.

We measured both *Dehalococcoides* specific 16S rRNA gene copy numbers and universal bacterial 16S rRNA gene copy numbers in RU11/PCE during the subculture experiments to determine growth yield (**Table 2.6**). The qPCR results indicate that the number of total bacterial 16S rRNA gene copies were similar to that of total *Dehalococcoides* 16S rRNA gene copies, confirming the purity of the culture with respect to the presence of bacteria other than *Dehalococcoides* and confirming the cellular yield on each of the chloroethenes. This analysis also suggested that the *Dehalococcoides* sp. or spp. in RU11/PCE contain a single 16S rRNA gene, as has been reported for other strains.

The *Dehalococcoides* bacteria have been divided into three subgroups, Victoria, Pinellas and Cornell by Hendrickson et al. (Hendrickson et al., 2002) based on 16S rRNA gene sequence differences. However, with the currently limited physiological information available for each of the strains, it is not obvious that this grouping reflects the similarity of physiological properties of the *Dehalococcoides* members. For example the 16S rRNA gene sequence of strain CBDB1, of the Pinellas subgroup, which was isolated on

chlorobenzenes and dehalorespires polychlorinated dibenzo-*p*-dioxins is 100% identical to that of strain FL2, a TCE and DCE dehalorespirer (Adrian et al., 2000; Sung et al., 2003). Further information is needed regarding the functionality of each strain as represented by the presence and expression of their suite of multiple dehalogenase genes, in order to better classify these organisms (Holscher et al., 2004).

The difficulty in culturing and isolating *Dehalococcoides* populations has hindered a comprehensive understanding of their distinctive characteristics. In this study, the purity of the RU11/PCE enrichment culture was examined through DNA-based approaches. We performed 16S rRNA gene based analyses using genomic DNA from the culture including direct sequencing, qPCR and PCR-DGGE. Like others, we also found that 16S rRNA gene-based analyses were not effective for unequivocally proving the purity of a *Dehalococcoides* culture. In our efforts to isolate a new strain of *Dehalococcoides*, we derived a culture that contained a single 16S rRNA gene sequence which is identical to that of strain CBDB1, strain GT and KB-1/VC over 1500 bp. Obviously, all 16S rRNA gene-based assays would detect a single sequence and suggest culture purity. Unfortunately, as mentioned above strains of *Dehalococcoides* with different functional abilities can have identical 16S rRNA gene sequences. We further assesses culture purity using bacterial housekeeping genes (*rpoB*, *atpD*, *sod*, *recA* and *gyrB*). PCR using the primers targeting *ropB*, *atpD*, and *recA* was unsuccessful using the genomic DNA from RU11/PCE as template. PCR targeting the *sod* gene and *gyrB* gene yielded amplicons. The *sod* gene and *gyrB* gene DNA fragments from RU11/PCE were 98 to 99% identical to the *sod* gene and *gyrB* gene from strain BAV1 and strain CBDB1. According to the GenBank database, two physiologically different *Dehalococcoides*

strains, CBDB1 and BAV1, share 99% identical sequences in *rpoB*, *recA*, *atpD*, *gyrB* and *sod* genes. Because strain BAV1 and strain CBDB1 share such highly similar sequences over the amplified region using the *sod* and *gyrB* primers, information on purity with respect to whether one or more *Dehalococcoides* strains were present in RU11/PCE was unsuccessful using this approach.

RU11/PCE contains RDase genes that are highly similar to those in strain CBDB1, FL2, BAV1, 195 and mixed cultures KB1 and VS. In combination with previously reported RDase gene sequences, these results suggest that a pool of *Dehalococcoides* RDase genes exists and that each strain shares some of these genes.

Interestingly, we observed the apparent lack of *tceA* in RU11/PCE, similar to strain GT, implying that this strain has a different PCE or TCE RDase (Sung et al., 2006b). In a study of the transcriptional expression of the RDase-encoding *tceA* and *vcrA* genes (Lee et al., 2006), a significant fraction of the TCE dechlorination activity appeared to come from non-*tceA* reductases. Besides RDases from non-*Dehalococcoides* spp., it is possible that multiple *Dehalococcoides* reductases other than *tceA* and *vcrA* are participating in chlorinated ethene reduction. Annotation of the strain 195 genome revealed 17 intact putative RDases (Seshadri et al., 2005), however the actual role and importance of each of these reductases is not yet fully understood. A transcriptional analysis of the *Dehalococcoides*-containing enrichment culture KB-1 provided evidence that multiple RDase homolog genes were transcribed in the presence of chlorinated organics (Waller et al., 2005). Therefore multiple RDases may be involved in chlorinated ethene reduction and to fully understand the reactions a more global transcription analysis

is required. Further, a recent study found that less than 50% of *Dehalococcoides* in field samples could be accounted for by the three known RDases (Ritalahti et al., 2006).

Summarizing the results from 16S rRNA analyses, functional gene screening, and growth yield measurements, it is most likely that RU11/PCE culture is composed of a single strain of *Dehalococcoides* which shows unique functionality to dechlorinate and grow on all chloroethenes. Even if this culture is not a pure culture, its dehalogenase gene profile indicates that RU11/PCE contains a novel *Dehalococcoides* strain or strains which is not overlapping with previously reported *Dehalococcoides* isolates/ cultures (Waller et al., 2005).

Future investigation of RU11/PCE is warranted. Its ability to dechlorinate PCE completely to ethene would be useful for bioaugmentation purposes. Currently, strains containing a VC RDase are highly desirable for bioaugmentation purposes, since VC is a known human carcinogen and may drive management of contaminated sites. Areas to be investigated should include expanding what is known about its substrate specificity for RU11/PCE and identifying the specific dehalogenases involved in dehalorespiration of different compounds. Identification of genes specific for each substrate is of great need as a site management and monitoring tool (Waller et al., 2005).

Chapter 3

Detection of Bacteria in the Atmosphere and Development of Methods for Measurement of Their Activity

Portions of this chapter concerning the design and characterization of the gas-phase bioreactors appeared in Krumins, V., E.-K. Son, G. Mainelis, and D. E. Fennell. 2008. Retention of Inactivated Bioaerosols and Ethene in a Rotating Bioreactor Constructed for Bioaerosol Activity Studies. *CLEAN - Soil, Air, Water*, 36, 593-600.

3-1. Development of Methods to Measure Bacterial Activity in Air

1. Introduction

1.1. Rationale

The study of airborne microorganisms and their effect on human health and the environment is known as aerobiology. In recent years, aerobiology research has increased primarily because of growing awareness of the variety of health problems potentially caused by airborne microorganisms (Bitton, 2002). Microorganisms of terrestrial or aquatic origin can be aerosolized by a variety of mechanisms (Mohr, 2002). Location and environmental conditions such as humidity, density and temperature have a great influence on the number and types of microorganisms in the air (Jones and Harrison, 2004). Aerial bacteria may exist as single cells; however they are more often associated with bioaerosol particles consisting of aggregates of bacteria, cellular remains, fungal or plant components, mineral dust and water (Stetzenbach et al., 2004). Liquid droplets produced by water bodies may contain one or more bacteria depending on the droplet size. Bioaerosols may range from 0.3 to 100 μm in diameter (Stetzenbach et al., 2004).

The atmosphere is a harsh environment for microorganisms and death may occur through desiccation, chemical reactions involving radicals, and UV damage. However, association with other airborne particulate matter offers microbes some protection from UV radiation (Lighthart, 2000). Because of the hostile nature of the atmospheric environment, the traditional paradigm for air holds that it is an unsuitable growth medium for bacteria and serves only as a transitory conveyor (Madigan et al., 2003; Mohr, 2002). Most aerobiology research focuses on presence, transmission, and die-off of pathogenic or allergenic microorganisms that affect humans, animals or plants (Stetzenbach et al.,

2004). Recent work has focused on public security and safety concerns such as detection of *Bacillus anthracis* and other pathogens (Agranovski et al., 2004; Beamer et al., 2004; Higgins et al., 2003).

This study describes development of methods to determine whether airborne bacteria could be metabolically active. There are several reasons that potential activity of airborne bacteria has relevance. The atmosphere contains many compounds that are known to support microbial growth. The estimated global emissions of non-methane volatile organic compounds (VOCs) are estimated to be 142 Tg yr⁻¹ (Pandis and Seinfeld, 1998). Air concentrations of VOCs are low, but significant enough to be of concern for human health—100 µg L⁻¹ for butane (Pandis and Seinfeld, 1998); 30 µg m⁻³ each for benzene, toluene and xylenes (Schneider et al., 2001); and 21 µg L⁻¹ for acetic acid. Aerial VOC concentrations are comparable to aqueous concentrations found in aquatic oligotrophic environments (Hama and Handa, 1980) known to support diverse bacterial communities (Page et al., 2004; Pearce, 2003; Stockner et al., 2005). Bacterial growth was supported by VOC substrates that partitioned into liquid growth medium from ambient air (Geller, 1983). Similarly, organic compounds may be concentrated on aerosolized particles via sorption or condensation (Saxena and Hildemann, 1996), and this mechanism could increase the availability of aerial substrates to airborne microorganisms. Indeed, the fine particulate matter found in air, PM₁₀ and PM_{2.5} (diameters less than 10 µm and 2.5 µm, respectively), consist of 20 to 50 % hydrophobic and hydrophilic organic compounds, many of which are known bacterial substrates (Saxena and Hildemann, 1996).

Are the ubiquitous and varied organic compounds in air present at concentrations which can support bacterial life and growth? **Equation (3-**

$S_{min} = K \frac{b}{\mu_{max} - b}$ <p>Where:</p> <p style="margin-left: 40px;"> $K =$ half-velocity coefficient ($\text{mg}\cdot\text{L}^{-1}$); $b =$ decay coefficient (d^{-1}); and $\mu_{max} =$ maximum specific growth rate (d^{-1}). </p>	Equation (3-1.1)
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1.1) is a simplification of the Monod kinetic model that may be used to describe the minimum substrate concentration (S_{min}) that will support bacterial growth (Rittmann and McCarty, 2001). Using parameters ($K = 2.8$ to $37 \mu\text{g L}^{-1}$; $b = 0.002 \text{ hr}^{-1}$; and $\mu_{max} = 0.4 \text{ hr}^{-1}$) for aerobic toluene degradation under substrate limited conditions (Button et al., 1981; Duetz et al., 1994), results in an S_{min} of approximately 0.02 to $0.2 \mu\text{g L}^{-1}$. These concentrations are similar in order of magnitude to that of many VOCs found in bulk air, as mentioned above. Degradation of combinations of substrates may proceed at levels below individual S_{min} values, indicating that bacteria have the ability to conserve energy from combinations of substrates that individually have concentrations less than S_{min} Harms, 1996; Kovarova-Kovar and Egli, 1998; Namkung and Rittmann, 1987).

Very little is known about the growth and activity of microorganisms in the air. Key questions include whether a) airborne bacteria are active and b) airborne bacteria degrade contaminants or incorporate carbon or nutrients. A series of experiments in the 1970s that were intended to determine the risk of biocontamination of the gaseous Jovian atmosphere by space probes sent from Earth examined reproduction of airborne bacteria. Glucose transformation (Dimmick et al., 1975), incorporation of thymidine (Straat et al., 1977) and cell doubling (Dimmick et al., 1979a; Dimmick et al., 1979b) were observed in aerosolized *Serratia marcescens* bacteria suspended in rotating drum aerosol chambers. To our knowledge, these are the only studies to have focused on bacterial growth and

activity *in situ* in the bulk aerial environment, albeit in a model system. Investigation of microorganisms collected from the atmosphere in cloud droplets is more prevalent. For example, Sattler et al. (Sattler et al., 2001) have reported that bacteria in cloud droplets could grow at temperatures of 0°C and estimated that carbon processing by cloud-borne bacteria could range from 1 to 10 Tg yr⁻¹.

To the best of our knowledge, there has been no exploration of microbial *activity* and *growth* in natural bulk air. This activity could be especially important in atmospheric environments rich in organic pollutants. Air contains about 1×10^4 to 1×10^5 bacterial cells m⁻³ (Bauer et al., 2002; Griffin et al., 2001; Lighthart, 2000; Sahu et al., 2005). Considering the volume of the troposphere (circa 8×10^{18} m³) this could account for approximately

1×10^{22} to 1×10^{23} total bacterial cells in the aerosolized state. These populations are

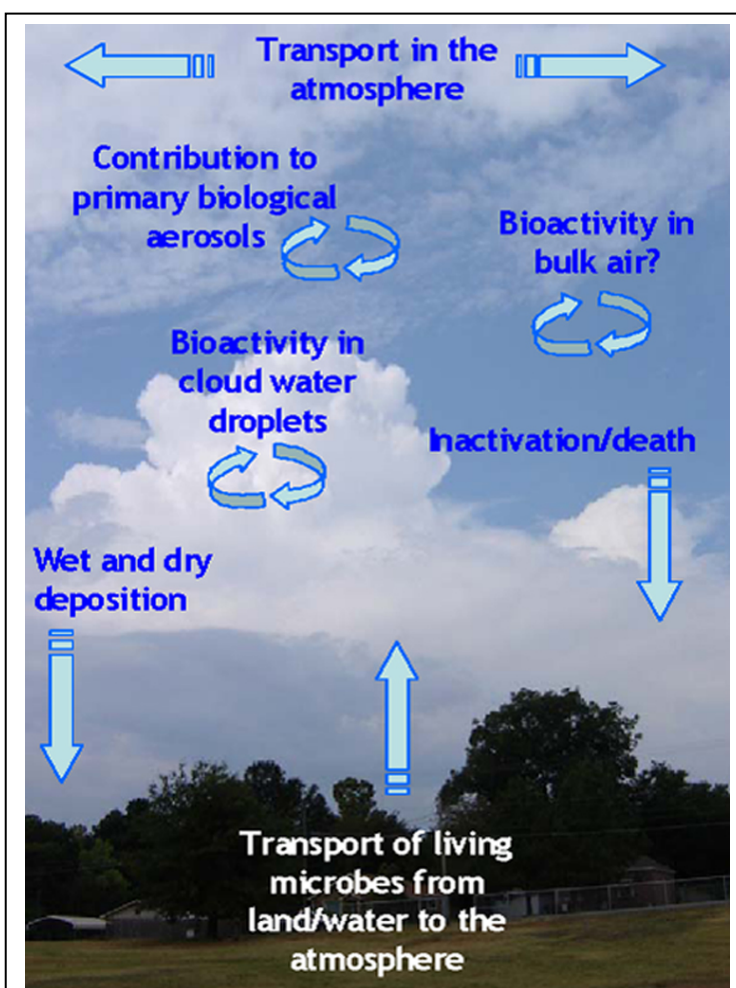


Figure 3-1.1. Proposed fates and roles of microorganisms in the atmosphere.

comparable to bacterial quantities present in the gut of certain animal species such as termites or swine (Whitman et al., 1998). The latter ecosystems have a significant impact on carbon cycling in the global environment and thus it is anticipated that bacteria in the airborne state may also have a similar impact (**Figure 3-1.1**). Emerging atmospheric and climate research suggests that airborne microorganisms could influence atmospheric chemistry, physics and climate during long-range vertical and horizontal transport (Deguillaume et al., 2008).

1.2. Objectives and experimental strategy

In most, if not all of the ecological niches on Earth, bacteria are the primary mediators of biological processes and have great impact on the Earth's environment (Whitman et al., 1998). The activity of bacteria in groundwater and sediments, for example in degradation processes, is known to have tremendous impact on the human environment. The enrichment of bacteria from soils, sediment or groundwater is performed using microcosms or enrichment cultures containing inoculum from the affected environmental medium. These studies have resulted in discovery of novel bacteria occupying specific niches and novel biological activities that have great impact on environmental quality. A variety of microbiological and molecular techniques may be used to identify and assign activity to specific community members.

This study addressed the hypothesis that air contains an active microbial ecosystem. The presence of bacteria and presence of suitable substrates at concentrations conducive to their growth create a possibility of an active living ecosystem, and not just a collection of dormant cells that are passively transported. Could an enrichment approach

also be applied to air to study the bacterial community? The overall goal of the study was to develop methods to enable investigation of whether bacteria are capable of metabolism and/or growth while airborne. The specific objectives were to:

- (1) develop methods to determine if airborne microorganisms degrade volatile organic compounds present in the air;
- (2) develop methods to determine if airborne bacteria multiply under some circumstances; and
- (3) determine whether airborne bacteria respond to changes in humidity, temperature or presence of chemical substrates.

To achieve the objectives of the study, compact rotating bioaerosol bioreactors were developed to keep bacteria suspended for days while measuring their activity. The reactor design is a rotating drum system similar to that described by Goldberg et al. (Goldberg et al., 1958); however, the bioreactor was rolled in a rack, rather than being mounted on a rotating shaft. This adaptation allowed for use of off-the-shelf drums with minimum modification: the only requirement was that the drum has a circular cross-section. As a result, the bioreactors in this study were relatively inexpensive (approximately US\$2000 per reactor).

The reactor system was also equipped with a virtual impactor positioned at the reactor inlet to have the capability to increase the particle concentration when filling the reactor. Concentrating the bioaerosol particles from ambient air would increase the concentration of bacteria inside the reactor and would thus increase the sensitivity of activity tests with ambient airborne bacteria. The effectiveness of the concentrator in filling the reactors was tested with ambient air.

We evaluated the capability of the reactors to retain ethene (a model VOC) and a bioaerosol of inactivated (killed) *Pseudomonas fluorescens* bacteria (ATCC 13525). Ethene was chosen as a model compound because of its relevance as an atmospheric VOC (Altuzar et al., 2005; Baker et al., 2008; Smidt et al., 2005), its biodegradability by bacteria, including *Pseudomonas* species (Ginkel and Bont, 1986; Koziollek et al., 1999; Shennan, 2006; Verce et al., 2001), its small size (and thus suitability for testing the ability of the reactor to maintain integrity), its expected low reactivity with reactor components, and the relatively simple and in-house analytics needed for its detection. Ethene is produced biogenically by plants, has anthropogenic origins as a combustion by-product, and undergoes destructive reactions with NO_x, ozone, atomic oxygen and hydroxyl radicals in the atmosphere (Altuzar et al., 2005; Baker et al., 2008; Pandis and Seinfeld, 1998; Smidt et al., 2005). In terrestrial and aquatic environments ethene is used as a sole carbon and energy source under aerobic conditions by certain species of bacteria (Ginkel and Bont, 1986; Koziollek et al., 1999; Shennan, 2006; Verce et al., 2001). *P. fluorescens* (ATCC 13525) (Stanier et al., 1966) is a widely-distributed Gram-negative bacterium and was chosen as a model bacterial species because of its availability, ease of growth and our past experience working with this strain as a bioaerosol (An et al., 2006; An et al., 2004; Yao and Mainelis, 2006; Yao and Mainelis, 2007).

To test airborne bacterial growth and activity in the bioreactor system, two microorganisms were chosen as model microbes: Gram-negative, ethene-degrading *Xanthobacter autotrophicus* (ATCC BAA-1158) and Gram-positive *Bacillus subtilis* (obtained from US Army Edgewood Laboratories). *X. autotrophicus* was isolated from soil and is known to utilize ethene and propene (Van Ginkel et al., 1987). *B. subtilis* is a

common soil bacteria, which has been widely studied for a variety of purposes and which is often used as a model microorganism in aerobiological studies (An et al., 2004; Wang et al., 2001; Yao et al., 2005). *X. autotrophicus* was aerosolized in the presence or absence of gaseous ethene as a carbon source and *B. subtilis* was aerosolized in the presence or absence of aqueous diluted tryptic soy broth (TSB) as a nutrient and carbon source.

Finally, a quantitative polymerase chain reaction (qPCR) method was developed and used along with an ATP assay, acridine orange staining and microscopy, and traditional plate counts to enumerate bacteria in the airborne state within the reactors under different conditions.

2. Literature Review

2.1. Bioaerosols

Biological aerosols (bioaerosols) are collections of biological material in the air. Bioaerosols may be single microorganisms or aggregates of microorganisms attached to solid or liquid particles suspended in the air (Stetzenbach et al., 2004). Bioaerosols can consist of bacteria, yeasts, fungi, spores of bacteria and fungi, microbial fragments, toxins, metabolites, viruses, parasites and pollen (Matthias-Maser and Jaenicke, 1995; Matthias-Maser et al., 2000). Microorganisms in bioaerosols may attach to dust particles or may survive as free floating particles surrounded by a coating of dried organic or inorganic material. Bioaerosols are typically 0.3 to 100 μm in diameter; however, particles of size fraction 0.5 to 5.0 μm generally remain in the air longer, whereas larger particles are deposited on surfaces more rapidly (Mohr, 2002). Biological material makes up 10 to 28 % by volume of the total airborne particulate matter (Matthias-Maser et al., 2000). In the atmospheric environment, particles may be characterized either as primary or secondary aerosols, also in addition to being characterized according to size fraction. Primary aerosols are those that are emitted from a source to the atmosphere, while secondary aerosols are those formed from condensation of chemical species brought about by chemical reactions in the gaseous phase. A recent measurement at Lake Baikal, Russia reported that about 15 to 35 % of atmospheric aerosol is composed of primary biological aerosols (Jaenicke, 2005). Further, the emission rate of primary biological aerosols is estimated to be approximately 1000 Tg yr^{-1} compared with 3300 Tg yr^{-1} for sea salt and 2000 Tg yr^{-1} for mineral dust (Jaenicke, 2005).

Several studies have reported that a major fraction of biological aerosols consists of fungal spores. The mass concentration and characteristic cell concentration of fungal spores is roughly 10^4 cells m^{-3} or $1 \mu\text{g m}^{-3}$ (Bauer et al., 2008; Elbert et al., 2007). The concentration of airborne bacteria is estimated to be 10^4 to 10^5 cells m^{-3} (Lighthart, 2000), far lower concentrations than those found in the ocean or freshwater bodies. Bauer et al. (Bauer et al., 2002) showed that the average mass concentrations of bacteria amounted to just 0.01 % of the organic carbon in cloud, snow, rain and aerosol samples. Bacteria range from 0.25 to 5 μm in diameter and are commonly spheres (cocci), rods (bacilli) or spirals (Lighthart and Mohr, 1994). The physical behavior of airborne bacteria in the atmosphere is determined by their aerodynamic diameter defined as the diameter of the equivalent particle, which behaves in the same way as the particle in question but is spherical and has a density of one g m^{-3} (Jones and Harrison, 2004).

A survey of the culturable airborne bacteria at four different locations in Oregon, USA, identified the majority of bacteria at inland sites as being associated with particles with greater than 3 μm aerodynamic diameter (Shaffer and Lighthart, 1997). Association of living cells with other particles may influence the length of time that the cell remains viable, in addition to controlling the time that a particle remains suspended in the atmosphere. The interaction of a particle with its environment depends on its surface area, while its ability to sustain damage depends on its volume, resulting in smaller aerosols being more susceptible to environmental damage such as oxidation, irradiation, desiccation and dehydration due to increase in the surface area (Jones and Harrison, 2004).

2.2. Atmosphere as a substrate sink for airborne bacteria

Global emissions of non-methane volatile organic compounds (VOCs) are estimated to be 142 Tg yr^{-1} (Pandis and Seinfeld, 1998). Concentrations of various VOCs have been widely measured in outdoor air and vary substantially on a geographical, temporal and seasonal basis. In a study of VOC measurement in 28 US cities, ethane varied from 560 to 8740 pptv; combined benzene, toluene, ethylbenzene, and xylene was 1 to 4 ppbv; and isoprene was up to 2590 pptv (9). Benzene was reportedly present at up to 3 ppbv and toluene at up to 7 ppbv in some urban environments (Schneider et al., 2001). Ethene was reported to be from less than 1 ppbv to greater than 60 ppbv (Altuzar et al., 2005; Baker et al., 2008; Smidt et al., 2005). Concentrations of carboxylic acids may be higher, for example 8500 ppmv acetic acid was reported (Saxena and Hildemann, 1996). The fine particulate matter (PM) found in air, PM₁₀ and PM_{2.5} (diameters less than 10 and 2.5 μm , respectively), consist of 20 to 50 % hydrophobic and hydrophilic organic compounds, many of which are bacterial substrates (Saxena and Hildemann, 1996).

In many cases, the concentrations of VOCs in the atmosphere noted above are low relative to concentrations that may be encountered in terrestrial aquatic environments, especially near sources of contamination. However, VOCs likely sorb onto particles or dissolve into water droplets, therefore increasing their concentrations in airborne particles relative to that in the bulk air (Saxena and Hildemann, 1996). Even at the lower concentrations observed in the aerial environment there is precedent from literature regarding terrestrial aquatic environments that suggest that these lower concentrations could support bacterial activity. A study on the dissolution of atmospheric organic carbon

into sterile mineral medium demonstrated that the dissolution rate of airborne organic carbon and the concentration of dissolved organic carbon (DOC) achieved were enough to support the growth of oligotrophic bacteria (Geller et al., 1983). It is possible, therefore, that atmospheric background levels of VOCs either directly adsorbed to bacterial cells or to hydrometeors containing cells may support metabolism of airborne microorganisms. Indeed there have been several studies of microbial activity in cloud or fog droplets. Fuzzi et al. (Fuzzi et al., 1997) identified three bacterial genera (*Pseudomonas*, *Bacillus* and *Acinetobacter*) by using a culture-based method for samples of fog droplets and reported that the concentration of airborne bacteria and yeasts was up to two orders of magnitude greater in foggy conditions than in clear air conditions. Bacterial growth in cloud droplets was estimated to account for carbon assimilation of 1 to 10 Tg yr⁻¹ (Sattler et al., 2001). Bauer et al. (Bauer et al., 2002) estimated the average number of bacteria in cloud water at Mount Rax in Austria during spring 1999 and 2000 as 5.9×10^3 cells m⁻³. Amato et al. (Amato, 2007) identified diverse bacterial populations present in cloud droplets. These authors speculated that cloud isolates could transform relevant compounds at temperatures and environmental conditions relevant to the cloud environment (Amato et al., 2007c). In a study on nitrogen processing in clouds, nitrifying bacteria were detected in cloud droplets, which suggested that nitrogen biotransformation could even occur in clouds (Hill et al., 2007).

2.3. Evidence for growth and activity of airborne bacteria

Most aerobiology research has focused on presence, transmission, and decay of airborne pathogenic microorganisms or allergens which are involved in human health

effects. Because of ultraviolet radiation, desiccating conditions, and the presence of free radicals, the atmosphere is a harsh environment for microorganisms airborne and microorganisms are mostly assumed to be inactive or resting (Gregory, 1973). Thus, the possibility that bacteria suspended in natural ambient air can metabolize substrates, grow, or divide has not been considered until very recently. However, most airborne bacteria are present as aggregates in bioaerosols and the association with particles provides bacteria with protection from UV damage and desiccation stress (Griffin et al., 2001; Stetzenbach et al., 2004). Furthermore, aerial environments transiently contain moisture, nutrients and many organic compounds (as mentioned in 2.2) that are known to support microbial growth.

In the 1970s studies were carried out at the Naval Biomedical Research Facility to evaluate the potential for bacterial survival in the Jovian atmosphere in an effort to determine the risk that space exploration might infect Jupiter. It was shown that *Serratia marcescens* was capable of glucose transformation (Dimmick et al., 1975), incorporation of thymidine into DNA (Straat et al., 1977), and cell doubling (Dimmick et al., 1979a; Dimmick et al., 1979b) while airborne. In these experiments, bacterial cultures and solutions of nonvolatile substrates (glucose and thymidine) were aerosolized separately, and the aerosols were mixed. Therefore the bacteria were only exposed to the substrates if they collided with a substrate droplet at the time of aerosol mixing. To carry out such experiments, the bacteria were kept airborne for several hours by using a slowly rotating reactor design which allowed maintenance of particles airborne much longer than upflow air reactors (Goldberg et al., 1958). In the original design, Goldberg et al. (Goldberg et al., 1958) used 0.61 m long by 1.83 m diameter (1.6 m³) rotating reactors to maintain

relatively constant airborne pathogen concentrations for animal exposure tests. A facility containing eight 1.0 m³ rotating bioreactors was constructed at the Naval Biomedical Research Facility for studies of pathogen survival and transmission (Goldberg, 1971). In a variety of studies “Goldberg drum” designs have been used to study airborne microorganisms (Cox, 1976; Griffiths et al., 2001), environmental effects on microbial survival (Lighthart, 1973; Sattar et al., 1984; Songer, 1967), or to maintain relatively constant bioaerosol concentrations for animal exposure studies (Goldberg, 1971).

Clouds, fog and rain can be a biologically favorable medium by protecting airborne bacteria from desiccation. Recent studies demonstrated that viable microorganisms are present in clouds, fog and rain and they have potential to influence atmospheric physics and chemistry (Cote et al., 2008). Amato et al. (Amato, 2007) identified 60 microbial strains from cloud water and showed their ability to degrade some of main atmospheric organic acids to more volatile compounds. Furthermore, the concentrations of adenosine triphosphate (ATP) in cloud water suggested that major fraction of bacteria in clouds are metabolically active (Amato et al., 2007c). It has been reported that aerosolized bacteria play an role in ice nucleation for precipitation by using protein or proteinaceous compounds (Christner et al., 2008a).

The scientific information summarized above raises the possibility that the presence of bacteria and sufficient concentrations of suitable substrates make the ambient air an active ecosystem. Atmospheric bacteria may be metabolically active and air may not be just a medium through which they are passively transported. If microbial metabolism and/or growth do in fact occur, it would represent an unrecognized sink for

environmental contaminants, could be a factor in the global carbon cycle and have great implications for our understanding of the atmosphere as a part of the biosphere.

2.4. Microbiological analysis of bioaerosols

To elucidate the possibility of microbial growth and/or metabolism and the potential role of airborne bacteria in atmospheric transformation, accurate and reproducible identification and quantification methods of microorganisms in the air are required. Various sampling and analytical methods have been utilized for air sampling and analysis to provide information regarding concentration and composition of microorganisms in bioaerosols.

Sample collection methods affect what type of sample analysis methods may be subsequently utilized. Impaction, impingement, and filtration are the most common air sampling methods (Bitton, 2002). Overall, impaction is the most frequently used method for bioaerosol collection (Bitton, 2002; Buttner, 2002). Impaction is a method whereby air is collected from the atmosphere and the flow is directed perpendicular to a collection surface coated with agar, gelatin or other media (Bitton, 2002). The particles in the air are propelled via momentum related to their mass to impact and be retained on the surface collector. The impaction method allows culture-based analysis and usually has a very low upper detection limit. During impingement sampling, air is forcibly bubbled through a liquid and the aerosols are transferred into the liquid (Buttner and Stetzenbach, 1991; Buttner and Stetzenbach, 1993). Because aerosols are collected in a liquid medium, samples can be diluted and easily used for further analytical methods, including culture, microscopy, immunoassay, flow cytometry and molecular methods (Buttner and Stetzenbach, 1991; Buttner and Stetzenbach, 1993; Cox and Wathes, 1995). The filtration

method collects airborne microorganisms from the air directly onto a filter or membrane with a specific pore size. Filtration sampling is simple and the costs are low, but, this method results in desiccation of vegetative bacterial cells which may be damaging and preclude later culturing (Cox and Wathes, 1995).

Conventional methods for identifying and enumerating airborne bacteria and other microorganisms rely on microscopic or cultural techniques (Buttner, 2002). These conventional methods are time-consuming, laborious and sometimes inaccurate. Microscopy is not species-specific and may be unreliable for detection of small and nondescript microorganisms. Culture-based techniques are unsuitable for detection of microorganisms that are slow growing or nonculturable *in vitro* and the choice of medium may influence which species can grow (Cox, 1989). In many complex natural environments, for example, less than one percent of the viable microbes present can be cultured under standard conditions (Pace, 1997).

Recently, culture-independent molecular methods for microbial identification and quantification have been developed and applied in the context of aerobiology (Alvarez et al., 1994). As the potential of these techniques for detection of airborne microbes has been recognized, there have been many reports on progress in this area (Stetzenbach et al., 2004). DNA-based detection methods offer greater potential for sensitive and specific detection, and some progress has been made in the detection of airborne bacteria using these techniques (Mukoda et al., 1994; Schafer et al., 1998). Polymerase chain reaction (PCR) amplification provides a qualitative and semi-quantitative assessment (Saiki et al., 1985). Recently, real-time quantitative PCR using fluorogenic probes has been developed and used for detection for airborne bacteria and fungi (Claudio Orlando et al., 1998;

Cruz-Perez, 2001; Orlando et al., 1998)). Several molecular techniques use ribosomal RNA (rRNA) gene sequences as tools for species identification by means of phylogenetic sequence analysis or quantification of total bacterial/fungal population numbers (Stetzenbach et al., 2004). Ribosomal RNA genes can also be quantified by real-time, quantitative PCR methods directly from mixed-community DNA preparations (Stetzenbach et al., 2004). Recently, Brodie et al. (Brodie et al., 2007) utilized gene chips, clonal libraries and culture methods to enumerate diversity and fluctuation in air from an urban environment.

3. Materials and Methods

3.1. Test microorganisms

We used *Pseudomonas fluorescens* (ATCC 13525, American Type Culture Collection, Manassas, VA), *Xanthobacter autotrophicus* (ATCC BAA-1158, American Type Culture Collection, Manassas, VA) and *Bacillus subtilis* var. *niger* from the US Army Edgewood Laboratories (Edgewood Research, Development and Engineering Center, Aberdeen Proving Ground, MD, USA) for the various experiments described in this dissertation. The *P. fluorescens* and the *B. subtilis* var. *niger* used in our experiments were cultured in Nutrient Broth (NB) and Trypticase Soy Broth (TSB) (Becton Dickinson Microbiological System, Sparks, MD, USA), respectively at 37 °C. *Xanthobacter autotrophicus* was maintained on NB. For live aerosol growth tests, *X. autotrophicus* was transferred and grown in 160 mL serum bottles in minimal salts medium containing (per L in deionized water): 1.55 g of K₂PO₄, 0.85 g of NaH₂PO₄, 2.0 g of NH₄Cl, 0,075 g of MgCl₂.6H₂O, 0.1 g of (NH₄)₂SO₄, 0.1 g of yeast extract, and 0.2 mL of a trace element solution (Wiegant and De Bont, 1980). Gaseous ethene was added to the bottle headspace to give a final concentration of approximately 43,000 ppmv (50,000 mg/m³) in the gas phase and the cultures were incubated at 30 °C at 120 rpm.

3.2. Rotating bioreactors

3.2.1. Theory (Krumins et al., 2008)

One of the main challenges involved in studying potential metabolic activity of airborne bacteria is the need to keep them airborne for a prolonged period of time, i.e., several days. Particles suspended in quiescent air will settle under the influence of gravity

at a rate determined by the Stokes equation. In a rotating volume of air (as inside a rotating drum), the direction in which the particles settle is constantly changing, so there is no net downward motion of the particles. Rather, particles trace a circular orbit with respect to the drum, the radius of which depends on the rotation speed of the drum and the Stokes velocity of the particle as described by Goldberg (Goldberg, 1971):

$$r_0 = \frac{d_p^2 \cdot g \cdot \rho \cdot C_c}{18 \cdot \mu \cdot \omega} \quad \text{Equation (3-1.2)}$$

Where r_0 is the particle orbit radius (m);

d_p is particle diameter (m);

g is the gravitational constant (9.81 m/s²);

ρ is the particle density (kg/m³);

C_c is the slip correction factor (Gruel et al., 1987) (unitless, not included in Goldberg's (Goldberg, 1971) formulation);

μ is dynamic viscosity of the air (Pa·s; approximately 1.83x10⁻⁵ Pa·s at 22°C);

and

ω is the drum rotation rate (rad/s).

Particles located within one orbit radius of the drum wall at the start of rotation will hit the wall and be lost, but the other particles remain suspended. Over time, these particles will move outward under centrifugal force generated by the rotation of the drum (Goldberg, 1971). The outward particle velocity caused by centrifugal acceleration is:

$$V(r) = \frac{m \cdot \omega^2 \cdot r \cdot C_c}{3 \cdot \pi \cdot \mu \cdot d_p} \quad \text{Equation (3-1.3)}$$

Where V is the outward net radial particle velocity (m/s);

m is the particle mass (kg); and

r is the radial distance of the particle from the drum center line (m).

Goldberg (Goldberg, 1971) reported that particles remain uniformly distributed throughout the rotating drum volume. That simplification led to a first order decay model for particle suspension that depends on the particle properties and rotation rate of the drum:

$$C(t) = C(0) \exp^{-2 \cdot \tau \cdot \omega^2 \cdot t} \quad \text{Equation (3-1.4)}$$

Where $C(t)$ is the concentration of particles (number/vol) of a given size at time t ;

$$\tau \text{ is relaxation time, defined as } \tau = \frac{\rho \cdot d_p^2 \cdot C_c}{18 \cdot \mu} \text{ (s); and} \quad \text{Equation (3-1.5)}$$

t is time (s).

Gruel et al. (Gruel et al., 1987) and Asgharian and Moss (Asgharian and Moss, 1992) pointed out that once a particle migrates to within r_0 of the reactor wall, it will hit the wall within the next revolution. In effect, the useful volume of the reactor is decreased by a factor of $1 - (R - r_0)^2 / R^2$, which depends on the reactor diameter R , particle properties, and the drum rotation rate. By including this factor, both groups calculated the “optimal” rotation rate for maintaining particles in suspension. Asgharian and Moss (Asgharian and Moss, 1992) determined that the optimal rotation rate for maintaining 1 μm particles in a 1 m diameter drum for 96 hours is 0.30 rpm. However they also indicated that retention of this particle size is fairly insensitive to rotation speed over a wide range of ω . It is worth noting that once the particles are lost to the wall, forces required to detach them are substantial. According to Hinds (Hinds, 1999), adhesion force for 1.0 μm particles is 10^{-7} N, while, for comparison, gravitational force is 5×10^{-15} N.

3.2.2 Development of rotating bioreactors

A rotating bioaerosol bioreactor system was developed and characterized as part of this study. This system, described in (Krumins et al., 2008) consisted of two stainless steel drums (reactors) cradled between and rotated by two axles (**Figure 3-1.2**). The two reactors (reactor 1 and reactor 2) were maintained at a constant temperature in a controlled-environment chamber (model GC-96-CW, Environmental Growth Chambers, Chagrin Falls, OH, USA). Humidity inside the reactors was adjusted during filling; leakage of water vapor (humidity) in or out of the reactors was minimized by setting the humidity control of the controlled-environment chamber to the desired humidity level in the reactors.

Each reactor vessel consisted of a 0.32 m³ stainless steel open-head drum, 1.02 m long by 0.66 m diameter. This was the largest size that could be purchased off-the-shelf in stainless steel. An electrically conductive material was chosen in order to minimize potential image forces due to the net negative charge carried by aerosolized bacteria (Mainelis et al., 2001). A neoprene gasket was used to minimize leakage around the interface of the lid and the rolled lip of the drum. Three holes were cut into the drum and lid to allow for filling, sampling, and instrumentation. Two 21 mm diameter (1/2" NPT) bulkhead fittings were installed, one in the drum bottom, and one located approximately half-way radially from the center of the lid. A third 19 mm diameter hole was installed in the lid for a duct-mounted temperature and relative humidity probe. A 21 mm cross fitting was installed on the bulkhead in the drum lid. One port of the cross was fitted with a Teflon®-lined septum, through which a needle attached to a syringe could be inserted

for sampling or for injecting a VOC into the reactor. A second port was used for filling the reactor. The final port of the cross was connected to the pressure tap of the pressure transducer, located inside the electrical enclosure.

A 200 x 150 x 100 mm NEMA 1 electrical enclosure was mounted to the drum lid and housed a battery-powered datalogger (model HOBO U12-013, Onset Inc., Pocasset, MA, USA), a differential pressure transducer with a range of ± 3.7 kPa (± 15 in. H₂O) (model PX275-30DI, Omega Engineering Inc., Stamford, CT, USA), and a temperature/humidity transmitter (model HX94CW, Omega Engineering Inc., Stamford, CT, USA). The temperature/humidity probe was mounted through the lid into the drum. The pressure and temperature/humidity transmitters were powered by two 6V batteries.

A major design feature distinguished this reactor system from that of Goldberg et al. (Dimmick et al., 1979a; Goldberg et al., 1958). Rather than rotating about a shaft, the reactors were cradled between two axles in a roller frame. Power from a 37 W (1/20 hp) gear motor, with 90 rpm output, was transmitted through a V-belt drive to the drive shaft (**Figure 3-1.2**). Urethane drive rollers (64 mm diameter) transferred rotational energy to the reactors. Idler rollers spun freely on the other shafts. This adaptation could allow a variety of reactors with round cross section to be rotated without modification (i.e., without installing and aligning a new shaft), although the rotation rate is related to the reactor diameter.

In our experiments, the drum was set to rotate at 1.3 rpm, which would yield 1.0 percent loss of 1 μ m particles per day. Taking into account that an additional 0.3 percent of the particles were removed per day during sampling, the resulting theoretical half-life for 1 μ m particles was 54 days.

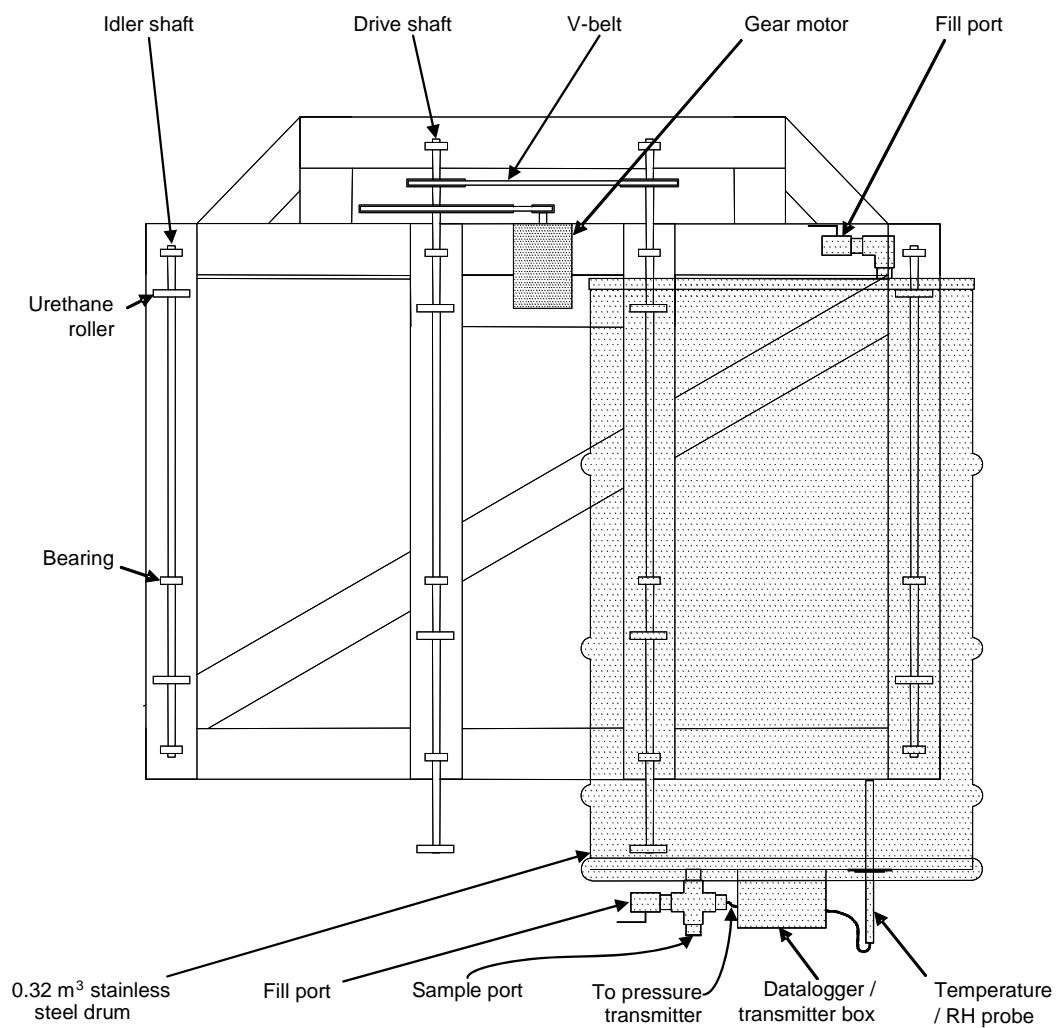


Figure 3-1.2. Schematic of the rotating bioaerosol bioreactor system (drawing courtesy of Dr. Valdis Krumins (Krumins et al., 2008)).

Before running the tests, the drums were opened and the interiors wiped clean with 70 % isopropanol, then the lids were replaced and the drums were flushed with three volumes of ambient air filtered through a 0.45 μm filter.

3.2.3. Bioreactor environmental conditions

Experiments were performed in a controlled-environment chamber (model GC-96-CW, Environmental Growth Chambers, Chagrin Falls, OH, USA) with a 12 hr light:dark cycle. The temperature was 22 °C for the ethene retention test and inactivated bioaerosol particle suspension test, and 30 °C for the airborne bacteria growth test. The relative humidity (RH) in the chamber was 70 % during all the tests with *P. fluorescens* and *X. autotrophicus*, 30 % for the particle concentration tests and 85 to 90 % during the tests with *B. subtilis*.

3.2.4. Incorporation of airborne particle concentrator in the bioreactor system

The use of airborne bacteria at their average natural ambient abundance levels of 10^4 to 10^5 bacteria per m^3 , (Lighthart, 2000) in the 0.32 m^3 reactors could limit the extent of detectable bacterial concentrations, bacterial community changes or VOC consumption that could be observed. To increase the concentration of bacteria contained in natural air and thus to increase sensitivity of detecting their activity we incorporated a particle concentrator for filling the reactors with natural air. The concentrator is a virtual impactor designed and constructed by Dr. Constantinos Sioutas of the Southern California Particle Center, University of Southern California, Los Angeles, CA, USA (Kim et al., 2000). For filling the reactor using the concentrator, a major flow of 95 L min^{-1} was pulled through

the virtual impactor using a pump while the product flow of 5 L min^{-1} enriched with aerosol particles, was drawn through the reactor by a second vacuum pump.

3.2.5. Introduction to and recovery from reactors of aerosolized bacteria

For aerosolization, *P. fluorescens* and *B. subtilis* were grown overnight (not exceeding 16 hours) in fresh medium for each experiment. *X. autotrophicus* was transferred to minimal salts medium containing ethene, incubated for 2 to 3 days and then the ethene degrading ability of the culture was checked by gas chromatography. The cells were harvested by centrifugation at 7000 g for 5 min at 4°C (BR4; Jouan, Winchester, VA), and the pellet was resuspended in distilled water. After a second centrifugation, the final pellet was resuspended with 50 mL of distilled water or a diluted growth medium.

The cell suspensions were aerosolized using a Collison nebulizer with 5 L min^{-1} of $0.45 \mu\text{m}$ filtered pre-purified nitrogen (Airgas East, Piscataway, NJ, USA). The flow from the aerosolizer was mixed with 45 L min^{-1} of $0.45 \mu\text{m}$ filtered ambient air to dry the airborne particles of associated water, which would affect particle sizes and agglomeration. In the live aerosol test with *B. subtilis*, purified air (Airgas East, Piscataway, NJ, USA) was used as a drying gas instead of ambient air. The bioaerosol was delivered to the reactor through sterile 9.5 mm inside diameter Tygon® tubing. To ensure even filling of the reactors, one reactor was filled for 3 minutes, followed by the other reactor. Because the reactors were rolling during filling, the fill tubing was uncoiled as it was switched from one reactor to the other every 3 min. Each reactor was filled for a total of 30 minutes. Particle counts were measured immediately after filling, one hour later, and every 24 hours thereafter. Air samples for particle counts were collected from

each reactor by attaching a 9.5 mm sterile ID tube to the reactor outlet and collecting a 1 L sample at a 2.8 L min^{-1} flow rate by the particle counter. During particle measurement, the reactor rotation was not stopped.

The bioaerosols were sampled using a liquid impingement microbial sampler, the BioSampler (SKC Inc., Eighty Four, PA), equipped with 5 mL sampling cup and operated at a flow rate of 12.5 L min^{-1} . The BioSampler has established performance characteristics and features of higher collection efficiency for bacterial cells (Rule et al., 2007). During each test, airborne bacteria were collected by the BioSampler into 5 mL of sterile phosphate buffered saline (PBS) solution for 2 min. In our experiments the bacterial concentration of the original liquid culture before aerosolization was determined by spectrophotometry to be approximately 10^8 cells/mL.

After each air sample was collected from the bioreactor, the cell suspension in 5 mL PBS solution was processed as follows:

(1) 1 mL of sample suspension was transferred to sterilized microcentrifuge tubes and a 0.1 mL aliquot was used to obtain 10^{-1} to 10^{-3} PBS-based serial dilutions. Next, 0.1 mL of each of these dilutions was plated to obtain colony forming units (CFU) counts.

(2) 1 mL of sample suspension was transferred to sterilized microcentrifuge tubes and used to prepare 10-fold water-based serial dilutions. Aliquots of these dilutions were used to determine the concentration of collected bacteria using epifluorescence microscopy following Acridine Orange staining.

(3) 1 mL of sample suspension was transferred to sterilized microcentrifuge tubes and processed immediately for quantitative polymerase chain reaction (qPCR).

(4) 1 mL of sample suspension was transferred to sterilized microcentrifuge tubes and stored for ATP determination.

The portions of the collected suspension utilized for genomic DNA extraction or cell lysis before the qPCR reaction and for ATP measurement were immediately centrifuged at 16000 x g for 20 min at 4 °C.

3.3 Experiments performed in rotating bioreactors

A series of experiments were performed in rotating biological reactors to characterize the reactors and to examine the activity of bacteria in the airborne state. An experimental protocol describing an overview of these tests is shown in **Table 3-1.1**.

Table 3-1.1. Experimental protocol of studies performed in rotating bioreactors.

Question/Task	Bacteria	Substrate	Assay
(1) Retention of volatile organic substrates by rotating bioreactors	None	ethene	VOC loss
(2) Retention of aerosolized bacteria by rotating bioreactors	Inactivated <i>Pseudomonas fluorescens</i>	none	Particle loss
(3) Culturability of bacteria aerosolized with and without substrate	Live <i>Bacillus subtilis</i>	± tryptic soy broth	Bacterial concentration VOC loss
(4) Activity and growth of bacteria in the presence of volatile growth substrates	Live <i>Xanthobacter autotrophicus</i>	± ethene	Bacterial concentration Bacterial activity
(5) Activity and growth of bacteria in the presence of aqueous-phase dissolved substrates	Live <i>Bacillus subtilis</i>	± tryptic soy broth	Bacterial concentration

3.3.1. Retention of volatile organic substrates by rotating bioreactors

Ethene was used to test the ability of the bioreactors to retain volatile organic compounds. Ethene was selected as a model VOC because of its volatility and relatively simple structure. One hundred eighty milliliters (180 mL) ethene (99%, Matheson Tri-Gas, Inc., Montgomeryville, PA, USA) was injected into each reactor to produce a gaseous concentration of 560 ppmv (650 mg m^{-3}). The rolling speed of the reactors was 1.3 rpm. The ethene concentration was monitored daily for 15 days. Triplicate 1 mL gas samples for ethene analysis were removed via the sampling port without stopping the drum rotation using a 1 mL series A-2 gastight syringe (VICI Precision Sampling, Baton Rouge, LA).

The ethene content of gas samples removed from the reactors was determined using an Agilent 6890 gas chromatograph equipped with a GS-GasPro (Agilent Technologies, Inc. Santa Clara, CA) column (30 m x 0.32 μm I.D.) and a flame ionization detector. The column was held at 50°C for 2 min. The integrated chromatographic peak areas of the samples were compared to a five point linear calibration curve. The ethene calibration curve was obtained by measuring the integrated chromatographic peak area of ethene from 0.1 mL head space of 160 mL serum bottles containing 500, 1000, 2500, 5000, and 7500 mg m^{-3} ethene at 22°C.

3.3.2. Retention of aerosolized bacteria by rotating bioreactors

Pseudomonas fluorescens cultures were used for particle suspension tests. The bacteria were killed by addition of 3.7 % (final concentration, volume/volume) formalin. The cells were then harvested by centrifugation, and the pellet was resuspended in sterile

distilled water. This suspension was aerosolized using the system described in section 3.2.5. Particle counts were measured immediately after filling, one hour later, and every 24 hours thereafter, as described in section 3.4.1.

3.4. Analytical methods

3.4.1. Aerial particle counts

Particle counts were measured using a hand-held particle counter (ART Instruments HHPC-6, Grants Pass, OR). Air samples were collected by attaching a 9.5 mm ID tube to the drum outlet and collecting a 1 L sample at a 2.8 L min⁻¹ flow rate. During collection of air for particle counts from rotating reactors, the rotation was not interrupted.

3.4.2. DNA sample preparation

The microcentrifuge tubes containing 1 mL airborne bacteria samples collected by liquid impingement were subjected to centrifugation at 16000 x g for 20 min at 4 °C. Supernatant was removed from the microcentrifuge tubes. A Mo-Bio Power Soil kit (Mo-Bio Inc., Solana Beach, CA, USA) was employed to extract total DNA from the cell pellet. The procedure was slightly modified from that provided by the manufacturer in the following ways. The bacterial cell pellet was resuspended with a bead-beating solution and transferred to a bead-beating tube. Cell lysis was initiated by addition of lysozyme solution at a final concentration of 5 mg mL⁻¹. The bead-beating tubes containing bacterial cells and lysozyme were incubated for 30 min at 37 °C. Proteinase K (final concentration, 2 mg mL⁻¹) and the C1 solution (SDS solution) from the Mo-Bio Power

Soil kit were added to the tubes. The tubes were incubated for 30 min at 50 °C. For further cell lysis, samples were subjected to three cycles of freezing at -80 °C, and thawing at 65 °C. After the final cycle, the bead-beating tubes containing samples were subjected to horizontal vortexing for 10 min. Nucleic acids were cleaned and concentrated in accordance with the Mo-Bio DNA kit protocols. Final DNA was eluted in 30 µL of nuclease-free water.

3.4.3. Whole cell lysate preparation

To develop a more rapid bacterial DNA detection protocol, the results of DNA quantification using DNA extract obtained using the Mo-Bio kit as described in section 3.4.2 or a whole cell lysate were compared. The whole cell lysate was prepared by centrifuging the collected airborne bacterial cells (*X. autotrophicus*) and then resuspending the cell pellet in 50 µL of nuclease-free water. Next, the cell suspensions underwent three cycles of freezing at -80 °C, and thawing at 65 °C. Final cell lysis was performed by heating the cells for 10 min at 94 °C.

3.4.4. Quantitative polymerase chain reaction analysis

A Bio-Rad IQ-5 cyclor system was used for qPCR analysis. A segment (V3 region) of the bacterial 16S rRNA gene was amplified by using the primer sequences shown in **Table 3-1.2**. Primers were obtained from Integrated DNA Technology (Coralville, IA, USA). A 200-bp fragment of the bacterial 16S rRNA gene bp 338 to 519 (according to *Escherichia coli* position) was amplified with the universal primers. The PCR reaction was performed in a total volume of 24 µL reaction mixture using 12.5 mL

of $2 \times$ iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) [containing: the hot-start enzyme, iTaqTM DNA polymerase; SYBR PCR buffer; dNTP mix; SYBR I; 20 nM fluorescein for dynamic well factor collection; and 6 mM MgCl₂], 0.2 μ M forward primer (Bac338f), and 0.2 μ M reverse primer (Univ519r). One μ L of the DNA extract or whole cell lysate was added to the each PCR mixture. The standards and the samples were included in duplicate for each run. The amplification reaction was performed with a iCycler iQTM thermal cycler (Bio-Rad Laboratories, Hercules, CA) using the following program: 10 min at 94 °C; then 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Data analysis was performed using iCycler iQTM Real-Time detection system software. All reactions were subjected to melting curve analysis using a 0.5 °C decrease from 94 °C to 72 °C following the amplification steps to confirm that only one PCR product was synthesized.

As a standard for the qPCR assay, a fragment of the V3 region of the 16S rRNA gene of *P. fluorescens* was amplified and pCR®2.1 vectors (Invitrogen, Carlsbad, CA) containing the PCR amplicons were transformed into chemically competent *E. coli*, TOP10 cells (Invitrogen, Carlsbad, CA), using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Transformed cells were grown on Luria-Bertani agar plates at 37 °C overnight and plasmid vectors were recovered from the transformed cells using QIAGEN plasmid purification kits (QIAGEN, Valencia, CA). The concentration of plasmid DNA in the final eluate was determined by imaging and analyzing electrophoretic gels using Quantity One 1-D gel analysis software (Bio-Rad, Hercules, CA). These DNA solutions were used as standards of 10^2 to 10^8 gene copies per μ L for qPCR.

Table 3-1.2. PCR primers used in quantitative analyses for airborne bacteria.

Primer	Sequence (5' → 3')	Target gene (expected amplicon size)	Ref.
Bac338f Univ519r	CTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	200 bp	(Nakatsu et al., 2000b)
M13 -20f M13r	GTAAAACGACGGCCAGT TTCACACAGGAAACAG	Dependent upon insert size	TOPO® TA Cloning (Invitrogen, Carlsbad, CA)

3.4.5. Culturable bacterial colony counts

Aerosol samples were serially diluted from 10^{-1} up to 10^{-5} with sterile PBS solution. A 0.1 ml volume of each dilution was plated in triplicate onto Trypticase Soy Agar (TSA) or Nutrient Agar (NA) plates. The plates were incubated at 37°C for *B. subtilis* or 30 °C for *X. autotrophicus* for 3 to 5 days and the number of colony forming units (CFUs) was counted manually.

3.4.6. Total bacterial count by epifluorescence microscopy

Total numbers of bacteria in liquid suspensions were determined by epifluorescence microscopy using an Axioskop 20 microscope (Carl Zeiss Inc., Thornwood, NY). For epifluorescence microscopy, two replicate slides were prepared by filtering 1 mL aliquot of a selected dilution through a black polycarbonate filter (Fisher Scientific, Suwannee, GA) and then staining with 1 mL of Acridine Orange solution (Becton Dickinson Microbiology Systems, Sparks, MD) for 10 min. At least 20 microscopic fields were counted using an oil-immersion objective at X 1000 magnification.

3.4.7. ATP assay

A model 20/20ⁿ luminometer (Turner Biosystems Inc., Sunnyvale, CA) and Promega BacTiter-Glo ATP Assay bioluminescence detection kit (Promega Corp., Madison, WI) was used as a sensitive and rapid method for quantifying ATP as described in Seshadri et al. (Seshadri, 2008). The luminometer is capable of measuring as little as 3 amol of ATP. A 100 µL sample containing bacterial suspension of bioaerosols collected in the BioSampler was treated with BacTiter-Glo™ to release the ATP from the cells which in turn reacted with luciferin/luciferase present in the reagent to produce luminescence. The luminometer response (RLU) was compared to a bacterial standard curve established using acridine orange counts and the luminometer output (RLU) as described in Seshadri et al. (Seshadri et al., 2008).

3.5. Data Analysis

The culturability of the aerosolized cells in each sample was evaluated by comparing the culturable bacterial number, CFUs, to the total bacterial numbers determined from the qPCR assay and from epifluorescence microscopy according to **Equation (3-1.6)**.

$$\% \text{Culturability} = \frac{\text{CFU}}{\text{total bacterial number}} \times 100 \quad \text{Equation (3-1.6)}$$

A t-test was performed using Microsoft Excel® to compare the culturability of cells recovered from the gas-phase bioreactors from *B. subtilis* suspensions aerosolized with or without TSB growth medium. A significance level of 0.05 was used.

4. Results

4.1. Operation of Rotating Bioaerosol Bioreactors

4.1.1. Reactor environmental control (Krumins et al., 2008)

Data collected from the onboard dataloggers on reactor 1 for a control run in the presence of inactivated bioaerosol are presented in **Figure 3-1.3**. Data were automatically collected and stored at two minute intervals, but for clarity only every 60th reading for pressure and every 120th reading for temperature and humidity are presented. The relative humidity was 58.5 ± 1.4 % (average \pm standard deviation) inside the reactor, while the temperature was 21.94 ± 0.04 °C. On average, there was a slight positive pressure ($+0.039 \pm 0.018$ kPa) inside the reactor relative to the chamber during the test. Data collected during other control runs exhibited similar results (data not shown).

4.1.2. Ethene retention tests (Krumins et al., 2008)

The ethene concentration in reactor 1 during the control test with killed bioaerosol is shown in **Figure 3-1.4**. Data are presented as averages of triplicate measurements \pm one standard deviation for each time point. The data were fit with a first order loss model ($r^2 = 0.59$), with a loss constant, k , of 0.040 d^{-1} . The loss constants from two control tests in reactor 2 were 0.055 d^{-1} ($r^2 = 0.45$) and 0.059 d^{-1} ($r^2 = 0.6$). The average loss rate constant estimated by fitting data from all three runs was $0.051 \pm 0.010 \text{ d}^{-1}$ (average \pm standard deviation). Thus, abiotic loss resulted in an ethene half-life of 13.6 days, or approximately 5.0 % of the ethene lost per day

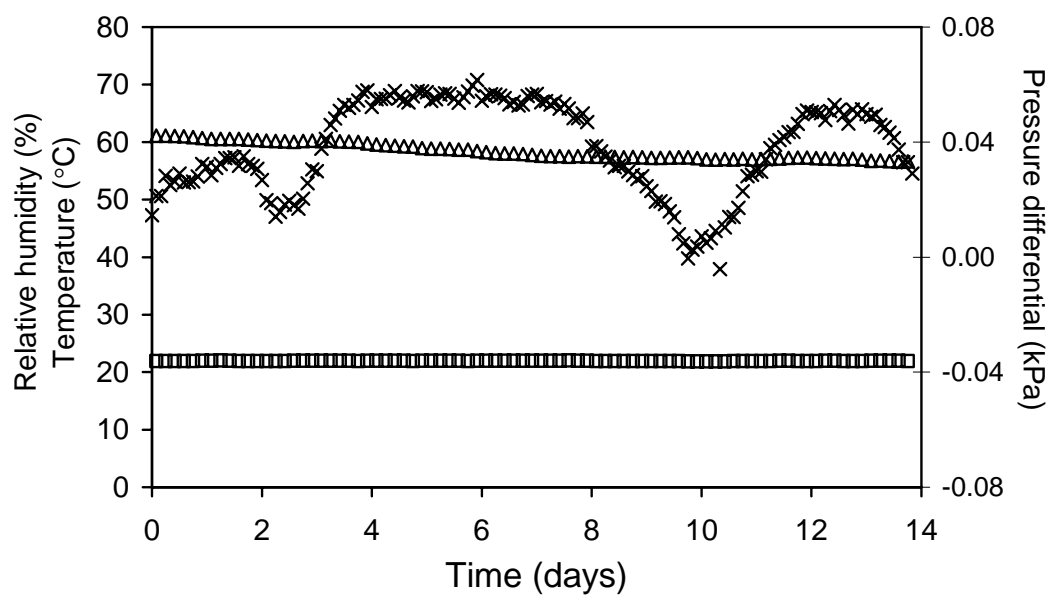


Figure 3-1.3. Temperature (°C) (□), relative humidity (%) (△), and pressure differential between inside and outside (kPa) (×) in reactor 1 during test with inactivated *Pseudomonas fluorescens* bioaerosol. Two-minute data recording interval. Every 60th pressure differential reading and every 120th temperature and humidity reading collected are presented.

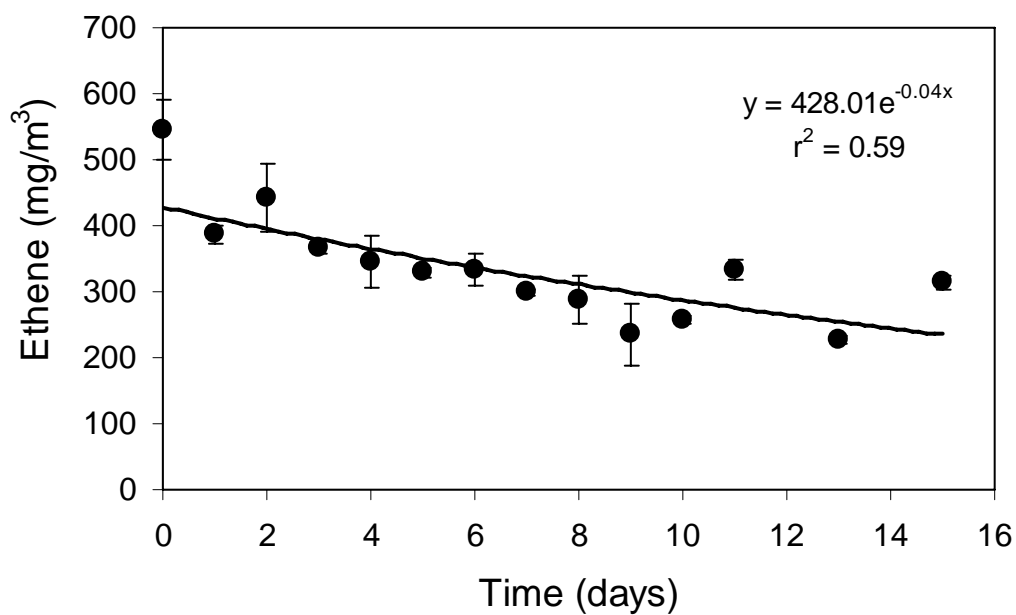


Figure 3-1.4. Time course of ethene (mg/m³) in reactor 1 with inactivated *Pseudomonas fluorescens* bioaerosols (●). Symbols are averages of triplicate measurements and error bars are one standard deviation.

4.1.3. Particle number increase with concentrator (Krumins et al., 2008)

The results of the particle concentrator test are shown in **Table 3-1.3**. The reactor particle concentrations were increased over that of ambient air for all particle sizes when the reactor was filled with air enriched by the particle concentrator. Particles greater than or equal to the size of a common bacterial cell, approximately 0.5 μm , were increased in concentration in the reactor at least 3- to 7-fold over ambient air concentrations.

4.1.4. Retention of airborne inactivated bacteria (Krumins et al., 2008)

The particle size distribution of *P. fluorescens* bioaerosols generated for the two inactivated particle suspension runs is shown in **Figure 3-1.5**. The reactor filling procedure produced a particle size distribution with a peak in the 0.5 to 0.7 μm size range, which is the approximate size of singlet *P. fluorescens* cells. The alternating filling procedure described in 3.2.5 yielded highly reproducible results (**Figure 3-1.5**), with the difference between particle counts in the two reactors for each particle size range less than 5 μm being less than approximately 5 %.

The particle retention over 14 days averaged from two control tests in each of the two reactors (four tests total) is presented in **Figure 3-1.6**. All particle size ranges demonstrated first-order (exponential) loss ($r^2 > 0.97$ for all cases). The half-lives for the given particle ranges are listed in **Table 3-1.4** and presented graphically in **Figure 3-1.7**. The theoretical retention of 1 μm particles based on (Equation (3-1.4)), and corrected by the amount removed by sampling is also shown in **Figure 3-1.6**. The retention of particles larger than 2 μm was underestimated by (Equation (3-1.4)), while the retention of particles smaller than 2 μm was overestimated (data not shown).

Table 3-1.3. Particle counts in rotating reactor after one hour of filling using the particle concentrator and one hour of rotation. Ambient concentrations are average (number of particles/L \pm standard deviation) ambient readings taken at the start of filling, after one hour of filling and after one hour of rotation. The reactor concentration was measured after one hour of rotation.

Particle size range (μm)	Particle Count (#/L)		Factor of Increase
	Ambient	Reactor	
0.3 – 0.5	8140 \pm 1968	19504	2.4
0.5 – 0.7	850 \pm 308	2570	3.0
0.7 – 1.0	227 \pm 127	1075	4.7
1.0 – 2.0	196 \pm 74	1228	6.3
2.0 – 5.0	163 \pm 43	1260	7.7
> 5.0	23 \pm 7	104	4.5

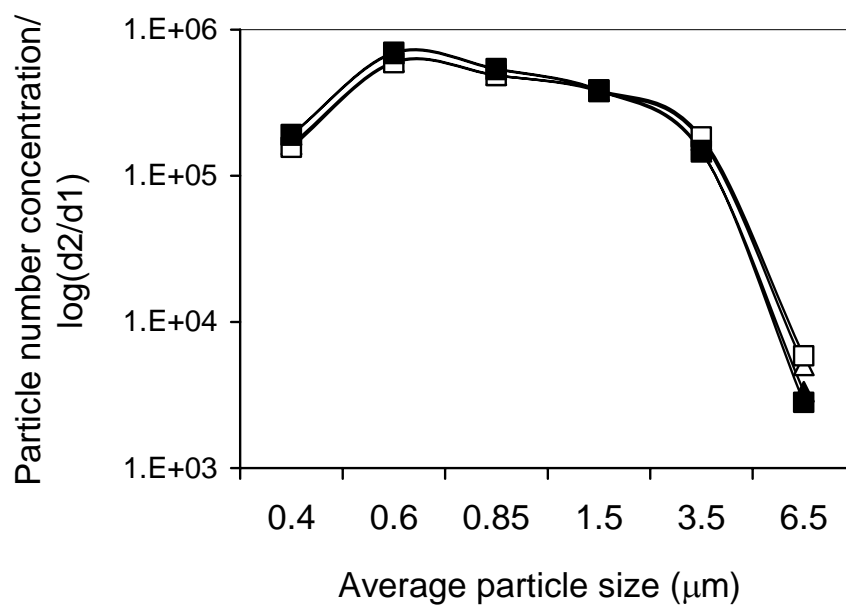


Figure 3-1.5. Initial particle size distribution [(particle number per liter) / (log (ratio of upper (d2) to lower (d1) boundary in size range))] versus particle size during duplicate control tests with inactivated *Pseudomonas fluorescens* bioaerosol in reactor 1 (△, ▲) and reactor 2 (□, ■).

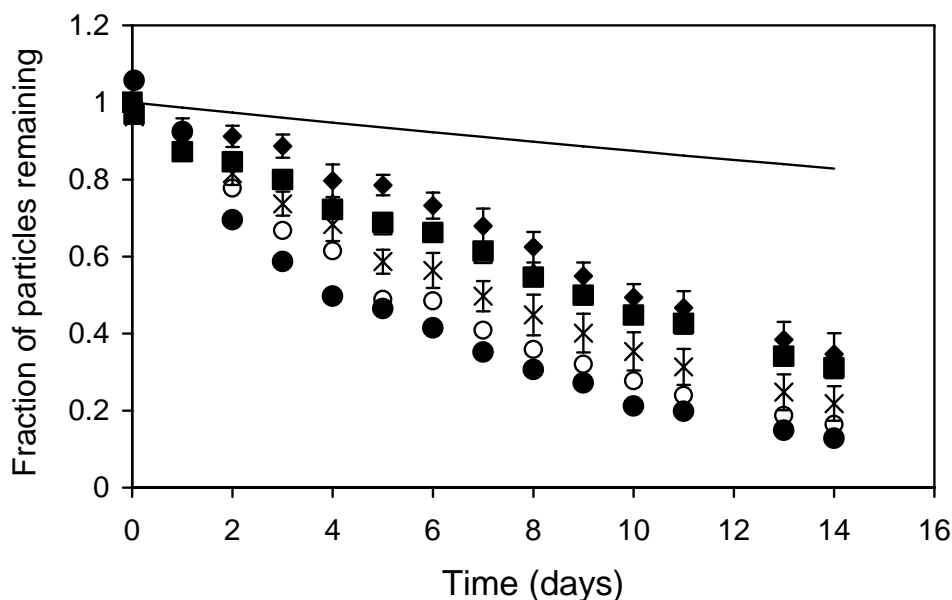


Figure 3-1.6. Average retention of different particle size ranges of inactivated *Pseudomonas fluorescens* bioaerosol for duplicate control tests in reactor 1 and reactor 2, Particle size range 0.3 to 0.5 μm (♦); 0.5 to 0.7 μm (■); 0.7 to 1.0 μm (△); 1.0 to 2.0 μm , (×); 2.0 to 5.0 μm (○); > 5.0 μm (●). The solid line indicates the theoretical retention of 1 μm particles based on Equation 3, and corrected for the amount removed by sampling. Data were collected immediately after filling the drum, after one hour of rotation, and daily thereafter. Symbols are average results from reactor 1 and 2. For clarity, error bars representing one standard deviation are included for the 0.3 to 0.5 μm and 1.0 to 2.0 μm particle size ranges only.

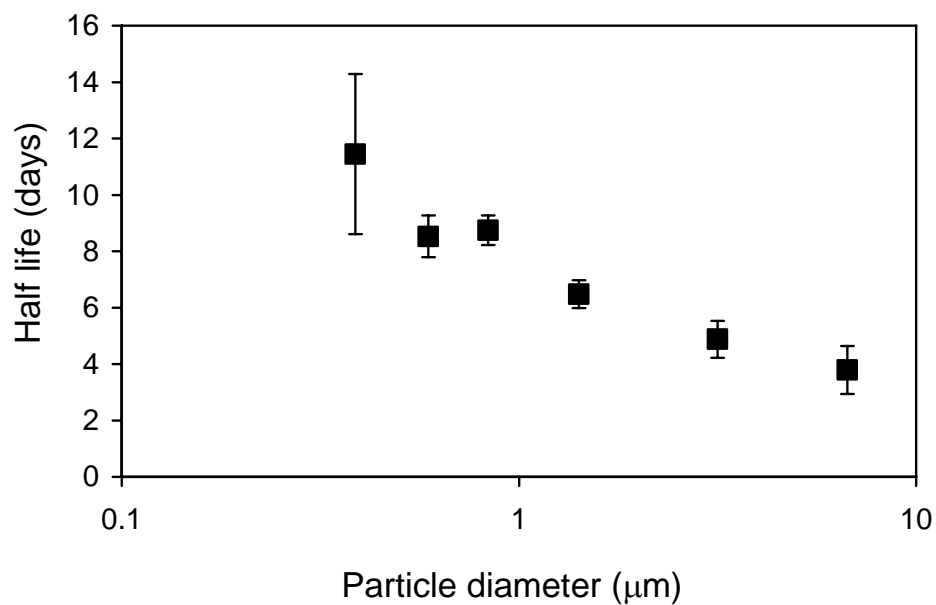


Figure 3-1.7. Half-lives of particles in rotating reactors (0.66 m diameter drum, rotating at 1.3 rpm) during control tests with inactivated bioaerosol of *Pseudomonas fluorescens*. Symbols represent the average of four half-lives calculated from the time series of two runs in each of two reactors. The symbols are averages of results from two reactors and error bars represent one standard deviation.

Table 3-1.4. Half life of particle retention in rotating reactors (days) for different particle size ranges during control tests with inactivated *Pseudomonas fluorescens* bioaerosol. Average (\pm standard deviation) for two runs in each of two reactors.

Particle size range (μm)	Half life (days)
0.3 to 0.5	11.5 ± 2.84
0.5 to 0.7	8.5 ± 0.74
0.7 to 1.0	8.8 ± 0.53
1.0 to 2.0	6.5 ± 0.49
2.0 to 5.0	4.9 ± 0.65
> 5.0	3.8 ± 0.85

4.2. Development of a Quantitative Polymerase Chain Reaction (qPCR) Method

A qPCR method was established using clones which contained a PCR amplified segment of the V3 region of the 16S rRNA gene of *P. fluorescens* of 200 bp. The detection limit of the qPCR method using the primers was determined by amplifying serial dilutions of 10^2 to 10^9 copies of the clones. The minimal detection limit of the method was defined as the amount of the template DNA at which the relationship between the threshold cycle (C_T) and starting template DNA concentration became nonlinear. Serial dilutions of DNA were added to PCR reactions as described in materials and methods. Data analysis was performed using iCycler iQTM Real-Time detection system software (Bio-Rad, Hercules, CA).

The results are shown in **Table 3-1.5** and **Figure 3-1.8**. The 16S rRNA gene copy numbers were converted to the corresponding cell concentrations. The C_T value versus DNA concentrations from 10^3 cells to 10^9 cells per PCR reaction was linear. At DNA concentrations below 10^3 cells per reaction, this relationship became nonlinear, and the resulting C_{TS} were similar to the C_T observed in the no template control (NTC) to which no template DNA was added. This suggested the presence of contaminating bacterial DNA in the NTC. The minimum detection limit of the assay was thus determined to be 10^3 cells per reaction.

The amplified products were electrophorized on a 1.5 percent agarose gel using a 100 bp DNA ladder (Promega, Madison, WI) as a size standard. The products were visualized under UV and the amplicon size was approximately 190 bp, as expected. In addition, all qPCR reactions were followed by melt curve analysis which resulted in a single thermal point at which the *P. fluorescens* amplicon was denatured, 85.5°C.

The efficiency (E) of the qPCR amplification was calculated by the iQ software according to Equation 3-1.7.

$$E = -1 + 10^{\left(\frac{-1}{slope}\right)} \quad \text{Equation (3-1.7)}$$

where

$$slope = \frac{C_T}{\log_{10}[DNA_{initial}]} \quad \text{Equation (3-1.8)}$$

where $DNA_{initial}$ is the starting DNA concentration.

Theoretically when the DNA template is doubled at each amplification reaction, E should attain a value of 100% and $slope$ should be -3.32 for 1:10 serial dilutions (Ibekwe and Grieve, 2003). In practice, slopes between -3.1 and -3.6 yielding reaction efficiencies between 90 and 110% are typically acceptable. In our qPCR experiments, the slopes were between -3.31 and -3.62. The coefficient of determination, R^2 , was approximately 0.99 for each analysis. The qPCR data from each analytical run were examined to determine whether E and the R^2 value were in acceptable ranges.

Table 3-1.5. Results of the qPCR assay using a standard curve developed from a PCR amplified gene containing theV3 region of *P. fluorescens* 16S rRNA gene of approximately 200 bp.

C _T for template bacterial cell number per 25 µL PCR reaction ^a								
NTC ^b	1.33 x 10 ⁹	1.33 x 10 ⁸	1.33 x 10 ⁷	1.33 x 10 ⁶	1.33 x 10 ⁵	1.33 x 10 ⁴	1.33 x 10 ³	1.33 x 10 ²
27.68	6.48	9.76	12.59	15.89	19.96	23.89	26.39	27.06
(± 0.22)	(± 0.34)	(± 0.14)	(± 0.27)	(± 0.71)	(± 0.78)	(± 1.21)	(± 0.60)	(±0.39)

^a Mean values, based on triplicate samples (± standard deviation)

^b NTC = no template control

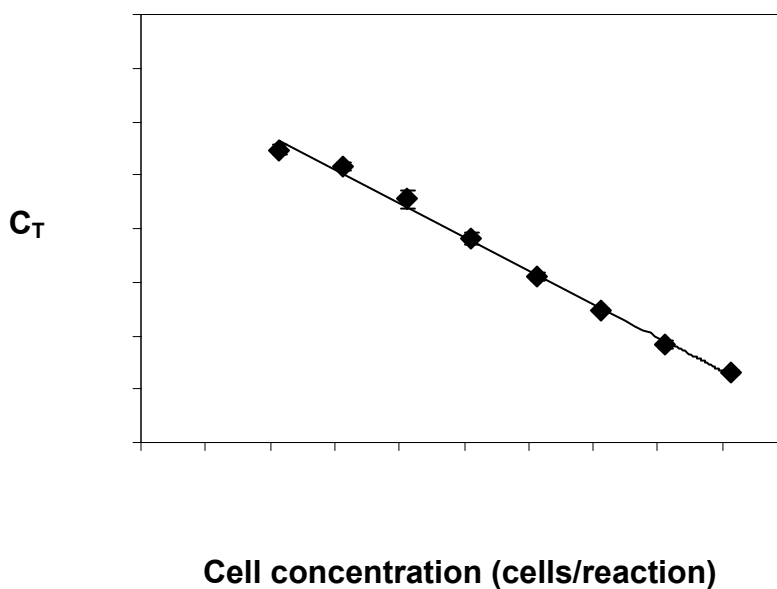


Figure 3-1.8. A standard curve based on the inverse linear relationship of C_T versus starting bacterial concentration as determined by the qPCR method (correlation coefficient = 0.99). C_{TS} were generated from PCR reactions with the template *P. fluorescens* DNA serially diluted 1:10 from 10⁹ cells to 10² cells per 25 μ L reaction. C_T is the cycle number when the threshold fluorescence is reached. Standard deviations from three measurements are shown as error bars.

4.3. Activity and growth of airborne bacteria

4.3.1. Culturability of aerosolized cells

The effect of aerosolization into the rotating bioreactors and the presence of substrate on the culturability of *X. autotrophicus* and *B. subtilis* were determined.

In tests performed with *X. autotrophicus* aerosolized into the gas-phase bioreactors with or without the gaseous substrate, ethene, no culturable cells were recovered from the bioreactors under any condition. This occurred even though the cells were maintained in the reactors for periods of 7 to 9 days, as detected by recovery of DNA (see section 4.3.1).

We aerosolized live *B. subtilis* cell suspensions into the bioreactors under two different conditions: (1) *B. subtilis* cells resuspended in distilled water containing no substrate and (2) *B. subtilis* cells resuspended in dilute TSB medium which contains various growth substrates. In both cases *B. subtilis* was cultured and cells were harvested and washed as described in section 3.2.5 before aerosolization. The aerosolized cells were collected after aerosolization and prior to introduction into the reactor and were further recovered from the reactor at various time points as described in section 3.2.5. The concentrations of airborne *B. subtilis* in the samples were measured using three different methods, qPCR, epifluorescence microscopy and CFUs, as described in section 3.3. *B. subtilis* is an endospore-forming bacterium. Endospores were highly refractile under light microscopy and resistant to basic aniline dye staining. In our experiments, no endospores were detected.

Figure 3-1.9 shows the concentrations of airborne *B. subtilis* cells from the two cell suspensions at different sampling points. Total bacterial numbers were determined by

qPCR assay and epifluorescence microscopy. These two assay methods yielded similar results for bacterial concentrations of approximately 10^5 cells/L aerosol sample. Note that CFUs indicate only the numbers of culturable cells recovered and not the total number of cells. Little difference was observed between total cell concentrations in samples recovered prior to introduction to the reactors and samples recovered directly from the reactors one hour after filling for condition (1) where *B. subtilis* bioaerosols were produced from distilled water with no substrate. In contrast, for condition (2) where *B. subtilis* bioaerosols were produced from dilute TSB with substrate, the cell concentrations in the bioaerosol samples recovered from the reactors after one hour were slightly decreased (less than 1 order of magnitude) when compared to cell concentrations in the samples taken prior to introduction to the reactors. This could be explained by the presence of the TSB medium (containing salts, etc.) aerosolized with the cells. The presence of TSB may have made the bioaerosols heavier, and thus the TSB produced bioaerosols could have had a more rapid settling rate than those produced from distilled water.

Aerosols from distilled water with no TSB substrate yielded 0.6 to 2 percent culturability and aerosols from dilute TSB with substrate had a culturability of 3.3 to 9.3 percent. A *t*-test ($p < 0.05$) indicated that the difference between two sets, condition (1) and condition (2), was statistically significant at the 95% confidence level.

Table 3-1.6. Culturability test of airborne *B. subtilis* in air samples collected before and after the gas-phase bioreactors. Cell numbers are per 1L of aerosol samples.

		Aerosols prior to introduction to bioreactor		Aerosols collected from bioreactor after one hr	
		<i>Bacillus subtilis</i> no substrate	<i>Bacillus subtilis</i> with substrate	<i>Bacillus subtilis</i> no substrate	<i>Bacillus subtilis</i> with substrate
qPCR		$(4.57 \pm 0.13) \times 10^4$	$(1.15 \pm 0.11) \times 10^5$	$(5.52 \pm 0.44) \times 10^4$	$(2.22 \pm 0.13) \times 10^4$
	Microscopic assay	$(6.06 \pm 2.21) \times 10^4$	$(7.84 \pm 3.03) \times 10^4$	$(6.40 \pm 1.68) \times 10^4$	$(4.53 \pm 1.90) \times 10^4$
	CFUs	$(8.87 \pm 3.36) \times 10^2$	$(3.78 \pm 0.95) \times 10^3$	$(3.87 \pm 1.37) \times 10^2$	$(2.06 \pm 0.19) \times 10^3$
% Culturability	qPCR	1.94	3.29	0.70	9.29
	Microscopy	1.46	4.82	0.60	4.55

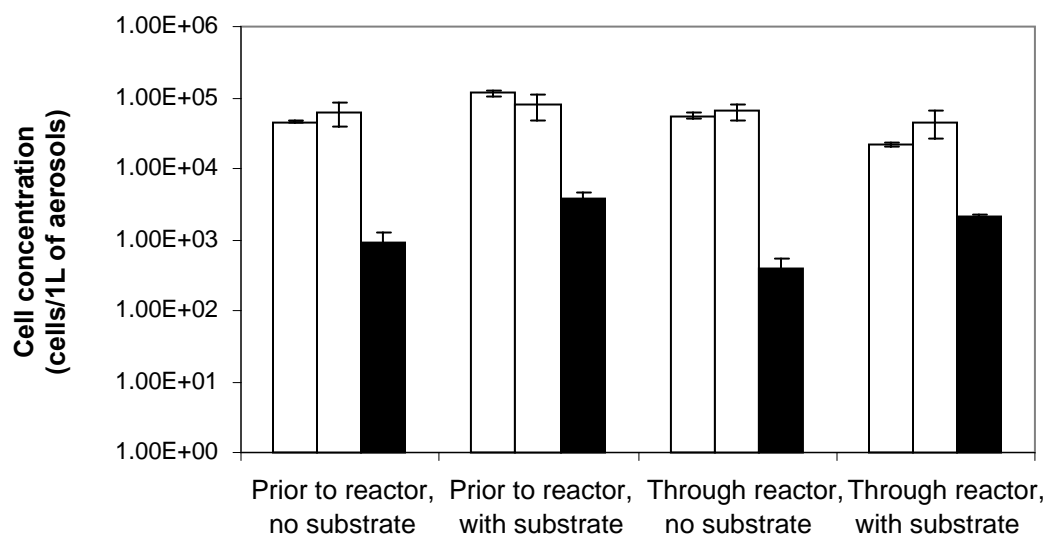


Figure 3-1.9. Concentrations of airborne *B. subtilis* recovered prior to entry into gas-phase bioreactors and after recovery from gas-phase bioreactors where cells were (1) aerosolized from suspensions containing no substrate (distilled water) or (2) with substrate (dilute TSB). Total bacterial numbers were determined by qPCR (open bars) epifluorescence microscopy (diagonally lined bars) and CFU analysis (black bars).

4.3.2. Airborne bacterial growth tests

To test airborne bacterial growth, *X. autotrophicus*, and *B. subtilis* were used as model microorganisms for Gram negative bacteria and Gram positive bacteria, respectively. The reported doubling times of these microorganisms were 20 hrs for ethene-degrading *Xanthobacter* strains (Ginkel and Bont, 1986) and two hrs for *Bacillus* (Burdett et al., 1986). The half-life of bacterial sized particles (0.5 to 1 μm) was eight to nine days in the bioreactors during the characterization tests described in section 4.1.4. We ran the tests with live bacteria for up to nine days by operating the bioreactors under the conditions described in section 3.2.5 of the Materials and Methods and this time could have accommodated approximately ten generations of *X. autotrophicus* or 100 generations of *B. subtilis*.

Aerosolized *X. autotrophicus* was grown on ethene, added to the bioreactors and air samples were recovered over time and analyzed for ethene, cells and ATP as described in the Materials and Methods. Two separate tests were performed with *X. autotrophicus*.

Results from the first test with *X. autotrophicus* are shown in **Figures 3-1.10** and **3-1.11**. **Figure 3-1.10** shows the recovery of DNA as enumerated by qPCR and bacterial activity as ATP in the presence or absence of the substrate, ethene. **Figure 3-1.11** shows the loss of ethene over time in the ethene-amended reactor. The cell numbers and ATP levels first decreased over the first four to six days, then increased until the last day of the test, Day 9. These changes were observed regardless of the presence or absence of ethene. The ethene content decreased over the nine day period with a first order rate loss coefficient of 0.032 d^{-1} . This rate of loss was slower than that observed during the

inactivated bioaerosol control tests ($0.051 \pm 0.010 \text{ d}^{-1}$) (see section 4.1.2 and **Figure 3-1.4**), although the starting ethene concentration was approximately one order of magnitude higher (9000 mg m^{-3}) than the concentrations utilized during the control tests (500 mg m^{-3}).

It is not known why the cell concentrations and ATP levels first decreased and then increased during this test. One possibility is that cells that made an impact with the reactor surface later dissociated from the surface and were reaerosolized. A second possibility is that initially, cell loss occurred then later, cell growth occurred in the reactors in the aerosolized phase. Because the phenomenon was observed in both reactors with and without the added growth substrate, ethene, this possibility seems remote.

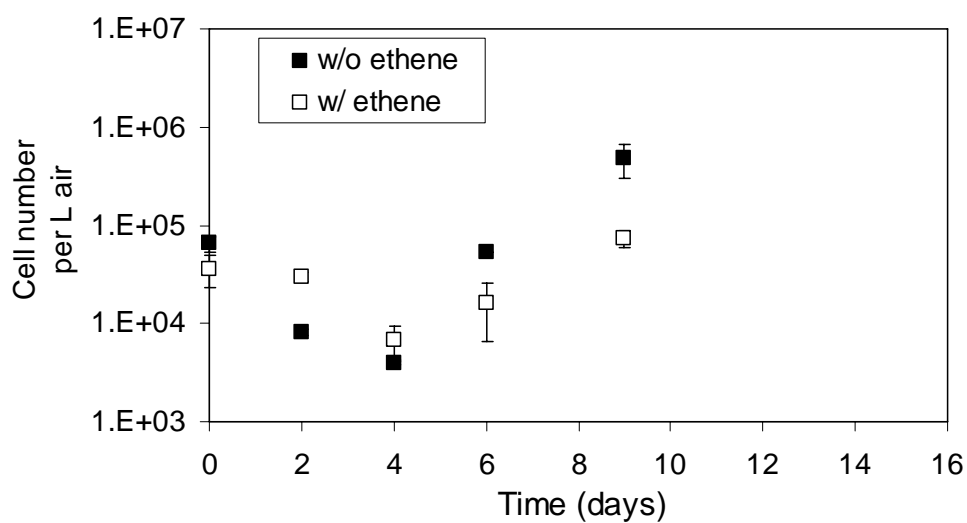
The aerosolization tests performed with *X. autotrophicus* were therefore repeated using two separate qPCR procedures to improve the rapidity and reliability of the quantification method. ATP concentrations were not measured during this test. **Figure 3-1.12** shows the change in *X. autotrophicus* concentrations in the bioreactors over the seven day incubation period. Regardless of the presence of the substrate, ethene, *X. autotrophicus* concentrations decreased with incubation time. Comparison of bacterial cell numbers in bioaerosols revealed that after seven days of incubation approximately 10 %, when analyzed by whole cell lysate method, or 30 to 40 %, when analyzed by DNA extraction method, of the aerosolized *X. autotrophicus* remained in the aerosolized state. For quantification, we recovered five liters of air from each reactor. However, over time the bacterial load in the air samples recovered from the bioreactors evidently became too low to obtain precise cell concentrations and on Day 5 and Day 7 the cell

concentrations were only slightly higher or comparable to the detection limit of the qPCR method (10^3 cells L^{-1}). The ethene concentration in the reactor to which ethene was added is shown in **Figure 3-1.13**. The average rate loss constant for ethene was estimated by fitting the data with a first-order kinetic model. The rate loss constant for ethene was $0.096 \pm 0.003 \text{ d}^{-1}$ which is statistically significantly higher than the rate of loss of ethene during the inactivated bioaerosol control tests ($0.051 \pm 0.010 \text{ d}^{-1}$) (see section 4.1.2 and **Figure 3-1.4**). Assuming that biotic loss was the difference between the ethene loss observed during the live test, 0.096 d^{-1} , and the abiotic ethene loss observed during the control, 0.051 d^{-1} , the corresponding ethene loss per cell is represented by a kinetic rate loss coefficient of 0.043 d^{-1} . After seven days, the average loss of ethene related to biological activity could thus be approximately 270 mg m^{-3} . Assuming the presence of 10^3 to 10^4 cells L^{-1} of reactor (**Figure 3-1.12**), the ethene uptake rate would be 3.9×10^{-6} to $3.9 \times 10^{-5} \text{ mg ethene cell}^{-1} \text{ d}^{-1}$. However, because the bacterial cell concentration data indicate a steady loss of organisms, and were similar in treatments both with and without amended ethene, it is not possible to determine whether growth occurred. Further, note that culturable *X. autotrophicus* cells were at no time recovered from the bioreactors (see section 4.3.1).

It is possible that cells which achieve impact with the reactor wall during the test colonize and grow, and are responsible for ethene loss. To determine the effect of the settled cells on ethene concentration, a blank control test followed the live bioaerosol test. This test was initiated immediately upon conclusion of the live test by flushing the reactors with 10 volumes of filtered air until the cumulative particle counts ($< 5 \mu\text{m}$ in particle size) reached less than approximately 1000, and then re-amending the reactors

with ethene. Ethene concentration monitored over an additional 5 days indicated there was no change in ethene concentration by microbial activity (data not shown).

(A)



(B)

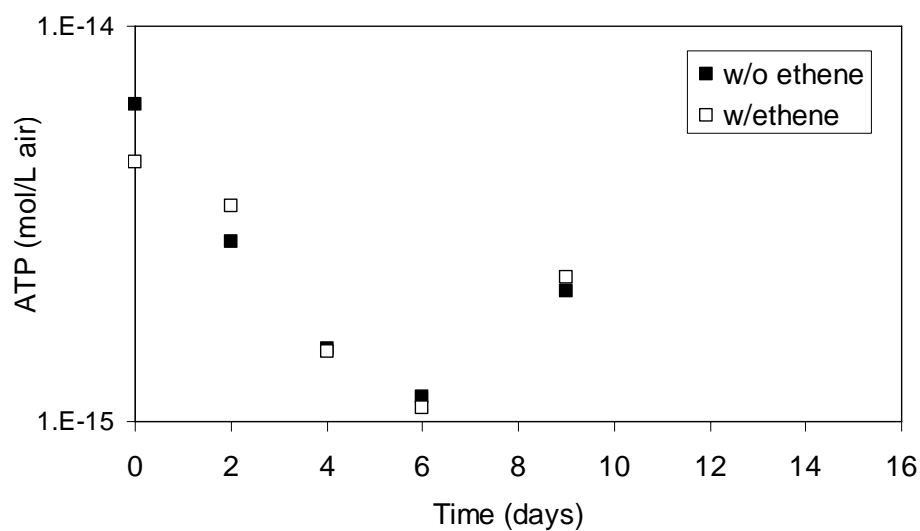


Figure 3-1.10. (A) Change in bacterial cell concentration in the bioreactor determined using qPCR method and (B) ATP measured in bioreactors over 9 days. The solid square and the open square represent *X. autotrophicus* in the absence of substrate (ethene gas) and *X. autotrophicus* in the presence of substrate (ethene gas). Symbols are averages of triplicate measurements and error bars are standard deviation.

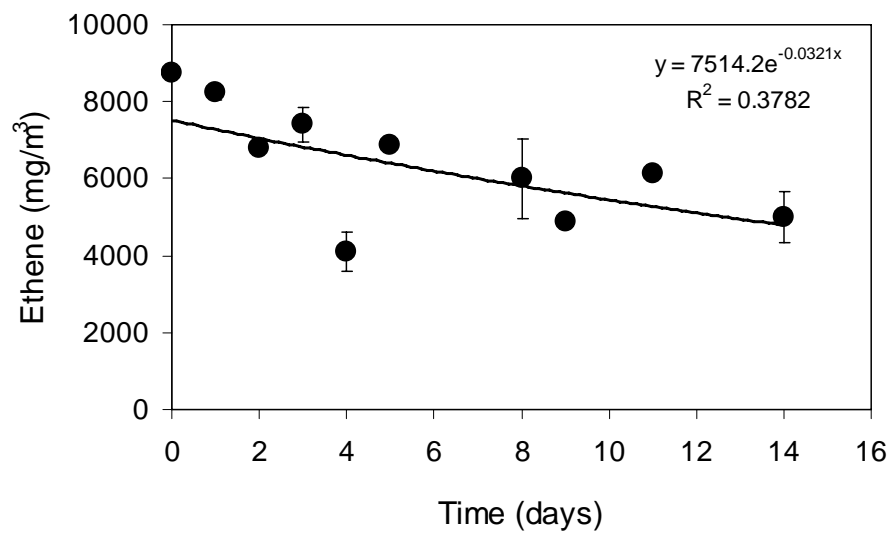
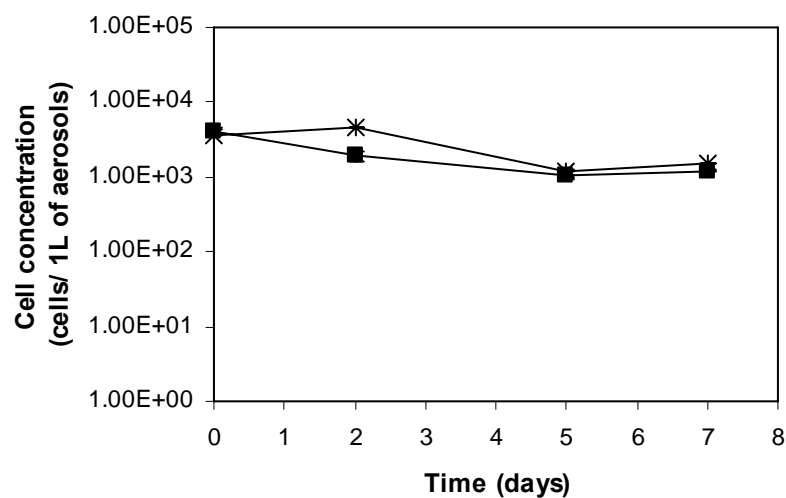


Figure 3-1.11. Time course of ethene (mg/m³) with live *Xanthobacter autotrophicus* bioaerosols (●). Symbols are averages of triplicate measurements and error bars are one standard deviation.

(A)



(B)

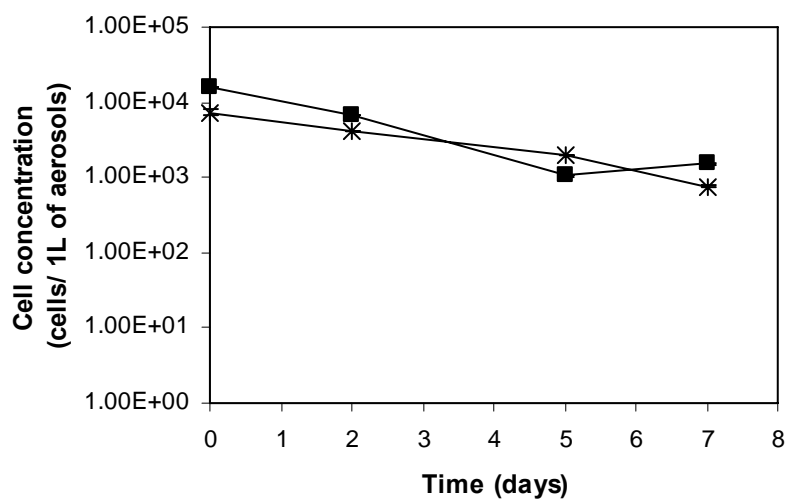


Figure 3-1.12. Change in bacterial cell concentration in the bioreactor for 7 days determined using qPCR method. The solid square and the star represent *X. autotrophicus* in the absence of substrate (ethene gas) and *X. autotrophicus* in the presence of substrate (ethene gas). (A) qPCR using DNA extract, (B) Whole cell qPCR. Symbols are averages of triplicate measurements and error bars are one standard deviation.

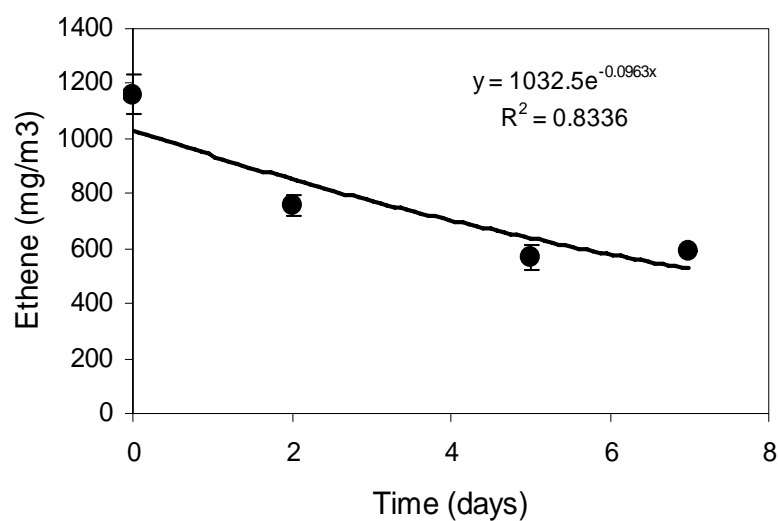


Figure 3-1.13. Time course of ethene (mg/m³) with live *Xanthobacter autotrophicus* bioaerosols (●). Symbols are averages of triplicate measurements and error bars are one standard deviation.

Freshly cultured *B. subtilis* was aerosolized with 10 X dilute TSB medium in the reactor, incubated, and collected for 5 days. The change in cell concentration is shown in **Figure 3-1.12**. During incubation in the rotating bioreactors, the cell concentrations decreased. The starting cell concentration was slightly higher than for the experiments with *X. autotrophicus* and precipitation of bacterial particles seemed to be faster than that observed for *X. autotrophicus*. The difference in precipitation rate may be ascribed to the difference in size of two strains of bacteria. *B. subtilis* is 1.0 to 3.0 μm in diameter whereas *X. autotrophicus* is 0.4 to 1.0 μm in diameter (Van Ginkel et al., 1987). Further for the *B. subtilis* aerosolization test, the bacterial suspension was aerosolized in the presence of dilute TSB medium which contained a variety of mineral salts. These mineral salts could be incorporated into particles with the bacteria, thus increasing the particle size and leading to more rapid removal by centrifugal force.

The concentration of culturable bacteria was determined as described in section 3.4.5. Culturable cells (CFUs) of *B. subtilis* were recovered from the air samples collected from the reactors one hour after filling, but at no other time point during this experiment (see section 4.3.1).

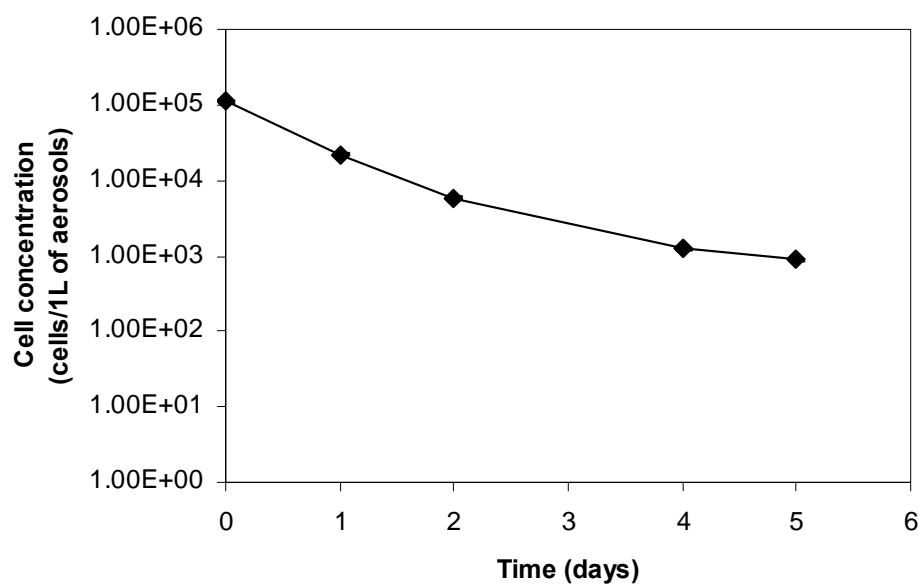


Figure 3-1.14. Change in *B. subtilis* cell concentration in the bioreactor for 5 days determined using qPCR method. The solid diamond represent *B. subtilis* in the presence of substrate (dilute TSB). Symbols are averages of triplicate analyses and error bars represent one standard deviation.

5. Discussion

This study concerned examination of the growth and activity of bacterial bioaerosols. As first steps in this project, methods were developed and tested to enable measurement of growth and/or activity of live bioaerosols. To investigate whether airborne bacteria are able to reproduce under selected conditions, first, we designed, manufactured and tested rotating gas-phase bioreactors which can maintain bacterial cells suspended as aerosols over periods of days. The objective was to attempt to determine whether substrate loss, increasing bacterial populations, or increasing ATP levels could be observed over time.

Through the time course study of settling of inactivated bacterial aerosols, the half lives of particles with the size from 0.5 to 5.0 μm ranged from five to ten days (**Table 3-1.3**). These times are theoretically long enough for viable cells to reproduce (i.e. accommodate several doubling times for some bacterial cells). Particles the size of single bacteria exhibited half lives of 8 to 9 days in the reactors. These half lives are near the doubling time of 9.8 d for the ethene degrader *Pseudomonas aeruginosa* strain DL1 (Verce et al., 2001) and far in excess of the doubling times of 8 hrs for ethene-degrading mixed culture K20 containing *Mycobacterium* and *Corynebacterium* strains (Koziollek et al., 1999), and 20 hrs for ethene-degrading *Xanthobacter* strains (Ginkel and Bont, 1986). The half lives are also similar to some reported atmospheric residence times of bacteria, for example, during transport between continents (Griffin et al., 2001).

The patterns of difference between the theoretical retention based on **Equation (3-1.4)**, and corrected by the amount removed by sampling is similar to findings of Dimmick and Wang (Dimmick and Wang, 1969) who observed less efficient retention of

particles in rotating drums than theoretically predicted. It is possible that the model correctly predicts the behavior of individual particles, but that smaller particles coalesce into larger ones, thus decreasing the half life of the smallest particle size ranges and increasing the apparent half life of larger particles.

These reactors also showed good efficiency in retention of volatile gas (e.g. ethene gas) with a daily loss less than 5%. Possible causes of ethene loss in the absence of biological activity include leakage around the fittings inserted through the lid and drum bottom, through sampling septa, around the gasket at the interface of the lid and drum body, or by sorption to the relatively small amount of organic materials (the gasket material) in contact with the gas inside the drum. A loss of 0.3 % per day was attributable to the removal of 1 L of reactor volume each day for determination of the particle counts.

Throughout the reactor performance tests, pressure inside the reactor was maintained positive. We speculate that the cyclical nature of the pressure differential readings could have been caused by weather patterns or the idiosyncratic nature of the overall operation of the facilities where the controlled-environment chamber was housed.

The results of the concentrator efficiency test indicate that the use of the particle concentrator during experiments with live bioaerosols from natural air could increase the concentration of bioaerosols collected significantly enabling us to better distinguish the quantitative difference in bacterial populations.

Using the bioreactors described above, we performed growth tests of lab-generated live bioaerosols from Gram-negative bacteria, *X. autotrophicus*, and Gram-positive bacteria, *B. subtilis*. The incubation times of seven to nine days could have theoretically accommodated approximately ten generations of *X. autotrophicus* or 100

generations of *B. subtilis*. In general, we observed similar trends in cell numbers both in bioreactors containing bacteria in the presence of substrate and in the absence of substrate, regardless of the strains used (**Figure 3-1.10, 3-1.12 and 3-1.14**). The number of viable or culturable, airborne cells, as well as particles, should theoretically decrease with time in the reactors because of cell death and gravitational deposition of particles. If the rate of biological decay were found to be less than that of mass loss, then new cells were being formed (Hatch, 1969; Rosebury, 1947); if the rates were identical, then there was no net death; and if the biological decay were greater than mass loss, then death of cells was occurring. The disappearance of particles aerosolized from bacterial suspension in water represents the actual particulate precipitation rate.

Based on the half lives observed in the control tests, bioaerosolized singlets of *X. autotrophicus* should remain in air with a half-life of 8 to 9 days, assuming no growth is occurring. Over a period of 9 days at least half of the starting airborne bacteria should remain in suspended in the reactor. In the first aerosolization test of *X. autotrophicus* cell numbers as measured by qPCR indicated an initial decrease followed by an increase in cell numbers suspended in the reactors so that after 9 days approximately 100 % of the bacteria remained in suspension. This phenomenon might have been thought related to growth, however this was observed both in the presence and absence of the growth substrate, ethene. Thus, the increase in cell numbers is likely not explained by growth but perhaps by resuspension of the precipitated bacterial cells from the reactor wall.

During the second test with *X. autotrophicus*, only 10 to 40 % of the initial *X. autotrophicus* concentration was detected in the airborne fraction after 7 days.

For *B. subtilis*, a decrease of nearly two orders of magnitude larger cell loss was observed over the course of the experiment. However, it should be noted that the precipitation rate of bacterial cells in the presence of buffer is likely larger than in distilled water, which is caused by increased particle size of aerosols in the presence of buffer.

Early studies by Dimmick et al. on the ability of a selected microorganism, *Serratia marcescens*, to survive when suspended in the aerosol state have described that airborne bacterial cells could maintain metabolic functions (Dimmick et al., 1975), could produce new DNA (Straat et al., 1977) and reproduce (Dimmick et al., 1979b). We speculate that the reasons for different findings between these 1970s studies and our experiment are different experimental conditions such as short aerosolization time (5 minutes), higher humidity and sampling over a far shorter incubation period of six to ten hrs by Dimmick et al. (Dimmick et al., 1979b) versus seven to nine days in our study, which could provide the higher possibility for bacteria of airborne state to survive. In measurement of culturable bacteria recovered from our reactors, we observed significant maintenance of culturability only after one hr, and only with *B. subtilis*.

In all the experiments with the aerosols from Gram-negative bacterium, *X. autotrophicus*, we could not detect any CFUs from the reactor at any time points even though DNA and ATP were recovered throughout the duration of the tests. A study performed using three different Gram-negative bacterial aerosols (Heidelberg et al., 1997) described the effect of aerosolization on the culturability and the viability of the strains. It was observed that the culturability of the bacterial strains tested rapidly decreased following aerosolization. This can be explained because bacteria exposed to

the air may experience environmental stress mainly by desiccation. Microbial growth is highly variable as a function of environmental conditions. Metabolism may occur in the aerosol state at moderate temperature and high humidity (An et al., 2004; Dimmick et al., 1975). In many previous studies, indirect or direct evidence for microbial growth and activity were reported in very humid atmospheric condition, such as fog and clouds (Amato et al., 2007a; Amato et al., 2005; Sattler et al., 2001).

Here the number of bacteria in the airborne state was determined using a standard curve based on the concentration of serially diluted plasmid DNA containing partial 16S rRNA gene fragments. However, previously it was demonstrated that standard curves using DNA from different preparation methods could result in significant differences in quantification of airborne bacteria (An et al., 2006). For example, quantification using a standard curve based on CFUs significantly underestimated bacterial numbers when compared to enumeration by an optical particle counter. Improved quantification was achieved by using a DNA-based standard curve where the standard DNA was prepared or recovered in the same way as the DNA from the air samples to be quantified (An et al., 2006). Also, not knowing the exact number of 16S rRNA genes in any given species at the time of sampling is a substantial limitation in use of universal bacterial primers targeting the 16S rRNA gene. Further, PCR inhibition by co-extracted substances from air samples, differential amplification, and formation of artifactual products would lead to biased results in qPCR-based quantification (Stetzenbach et al., 2004; von Wintzingerode et al., 1997). Thus, the validation of qPCR at different concentrations for the quantification of airborne microorganisms should be determined by adopting a total cell counting method with relevant airborne bacterial samples.

Although bacteria were apparently rendered nonculturable in the reactors, it is still possible that they could be active. Use of a more direct, sensitive indicator of activity may be needed to conclusively determine this. For example, use of labeled substrates (^{14}C or ^{13}C) could be used to allow detection of minute quantities transformation products (e.g., $^{14}\text{CO}_2$) or detection of labeled carbon in cells via stable isotope probing (Radajewski et al., 2000) through detection of ^{13}C labeled DNA produced as an organism metabolizes a specific labeled substrate. Further, a choice of a substrate that produces a definitive, traceable reaction product during transformation would also be useful. Also alternative bacterial cell detection methods can be developed. Rolling-circle replication cycle can increase bacterial DNA amplification efficiency resulting in providing higher sensitivity in the detection (Gusev et al., 2001).

To ensure greater activity of cells in the bioreactors, the optimization of experimental conditions by varying environmental factors (e.g., RH, temperature, etc.), should be the objective of future studies. Further, in natural air the organisms are likely in association with other particulate matter, e.g. mineral dust or biogenic material, and this association should provide some “protective” mechanisms. The effect of additional aerosolized material will be investigated in future studies. Finally, use of natural, concentrated bioaerosols as starting material in the reactors and measurement of their activity must be investigated.

3-2. Comparison of Denaturing Gradient Gel Electrophoresis and Clone Library Analyses to Characterize Bacterial Diversity in a Pooled Atmospheric Water Sample

1. Introduction

Although the atmosphere is a very harsh environment for microorganisms (Jones and Harrison, 2004), microorganisms are ubiquitous there and may play crucial roles in atmospheric chemistry and physics (Ahern, 2006; Amato, 2007; Deguillaume, 2008; Junge, 2008). Microorganisms have been widely detected and characterized in air (Brodie et al., 2007; Lighthart, 2000), cloud water (Sattler et al., 2001), fog droplets (Fuzzi et al., 1997), hailstones (Harrison, 1898; Mandrioli P., 1973), snow (Calderon et al., 2002a) and rain (Carpenter et al., 2000; Casareto et al.; Sattler et al., 2001). The total bacterial concentration in clouds was estimated to range from about 10^3 to 10^5 cells mL⁻¹ of cloud water (Amato et al., 2005; Bauer et al., 2002; Sattler et al., 2001). Cultivable bacteria and fungi were reported to be present high in the stratosphere (Imshenetzky et al., 1978). Atmospheric transport is theorized to be one possible route for global distribution of even very specialized extremophiles outside their niches to the broader environment (Marchant et al., 2008)

Under atmospheric conditions, cloud water and fog droplets could provide a more favorable temporary habitat for living airborne cells than air, where desiccation and dehydration would be limiting factors for survival or growth. Microbial cell aggregation and incorporation into cloud or fog droplets could decrease the sensitivity to desiccation and microbial cells could have an increased possibility of being transported in a viable state over greater distances. Clouds and fog may also provide a medium in which these

cells can divide, as suggested by the findings of Dimmick et al. (Dimmick et al., 1979a; Dimmick et al., 1979b), Fuzzi et al. (Fuzzi et al., 1997), and Sattler et al. (Sattler et al., 2001).

Fuzzi et al. (Fuzzi et al., 1997) detected greater numbers of culturable bacteria and yeasts in fog droplets when compared to ambient air at the same location, San Pietro Capofiume in the Po Valley in northern Italy. The authors theorized that fog water droplets were a growth medium for microbes and showed that fog water environmental conditions such as pH and presence of lead had an effect on the numbers of cultivable organisms recovered (Fuzzi et al., 1997). Bacterial growth was also observed by Sattler et al. in supercooled cloud droplets collected at Mount Sonnblick, Austria. Doubling times of 3.6 to 19.5 days were estimated for bacteria collected from the cloud water, based on uptake of labeled thymidine at 0°C (Sattler et al., 2001). Amato et al. (Amato et al., 2007c) characterized cloud droplet microbial communities collected at Puy de Dôme in France. In addition to identify specific organisms that were present, the quantification of ATP in cloud water samples indicated that cloud microbial populations were metabolically active (Amato et al., 2007c). Amato et al. (Amato et al., 2007a) also showed that cloud populations could actively transform important atmospheric organic compounds.

Until recently, it has been assumed that transformation of compounds in the atmosphere occurred primarily via chemical and physical processes. Thus, research on atmospheric chemistry has been focused primarily on abiotic photochemical or hydroxyl radical reactions. Recently, Ariya et al. (Ariya et al., 2002) reported evidence that microorganisms collected from air can efficiently transform dicarboxylic acids (DCA), an

important class of organic aerosols. Microbial activities in cloud water could have an effect on the whole composition of cloud water via uptake and transformation of chemical compounds present in the atmosphere (Amato et al., 2007a). Thus, a new paradigm is emerging that microbiological and photochemical processes could act concurrently or simultaneously in controlling the chemistry of cloud water and the atmosphere at large (Deguillaume et al., 2008a).

To fully define the role of bacteria in the atmospheric processes, it is necessary to obtain knowledge of bacterial diversity and activity in the atmosphere (Deguillaume, 2008). However, to date there has been only minimal and fragmentary information about the diversity and structure of microbial communities in atmospheric water, in particular. Fuzzi et al. described cultivable bacteria in fog water at low altitude and detected only three genera, *Pseudomonas*, *Bacillus*, and *Acinetobacter* (Fuzzi et al., 1997). Amato et al. detected a much more diverse population in tropospheric clouds and determined that the cultivable fraction was only approximately 1% of the total bacterial population (Amato et al., 2005). During more intensive investigation of the cultivable microorganisms present in clouds, Amato et al. successfully cultivated and identified 71 bacterial, 42 fungal and 15 yeast strains from seven separate cloud sampling events (Amato et al., 2007b). Most of the fungi isolated were of *Cladosporium* or *Trametes* affiliation, yeasts were of *Cryptococcus* affiliation, and dominant genera of bacteria belonged *Pseudomonas*, *Sphingomonas*, *Streptomyces*, *Staphylococcus* and *Arthrobacter* (Amato, 2007).

Detection and identification of microorganisms in the atmosphere have until relatively recently relied on conventional methods such as microscopy and culturing methods (Buttner, 2002). Further, collection of atmospheric water samples may require

prohibitively expensive aircraft time and there are substantial limits on the microbial density in those samples and on the sample volume that can be collected. For example, Fleishauer et al. measured a peak liquid water content of 0.35 g m^{-3} in midlevel mixed-phase (ice and liquid) non-precipitating clouds over the Great Plains of the United States and reported a literature review of estimates of the liquid water content of similar clouds that ranged from nondetectable to 1.2 g m^{-3} (Fleishauer, 2002).

Beginning more than a decade ago, however, molecular techniques have been applied in aerobiology to overcome limitations on use of culture-based methods to characterize the microbial populations in atmospheric samples. The use of nucleic acids recovered from bioaerosols avoids the need for microscopy or culturing and enables the detection of individual target organisms or genetic changes in microbial populations (Angenent et al., 2005; Brodie et al., 2007; Calderon et al., 2002b; Stetzenbach et al., 2004; Williams et al., 2001). Universal or gene specific primers are used for amplification, identification and quantification of target microorganisms. Because microbial genomes often contain more than one copy of specific genes, DNA-based methods can be sensitive, which is important in atmospheric bioaerosol studies because the microbial quantities sampled are often small (Stetzenbach et al., 2004).

Here we analyzed the bacterial components of a pooled sample of atmospheric water using two different molecular methods—denaturing gradient gel electrophoresis (DGGE) and clone library analysis—in which 16S rRNA genes were used for identification. This study compared the bacterial phylotypes detected using these two methods and compared the results to those from previous studies.

2. Materials and Methods

2.1. Atmospheric sampling

Samples were collected from June 19 to June 25, 2007 during the Cumulus Humilis Aerosol Processing Study (CHAPS) Summer 2007 Atmospheric Science Program Field Campaign which was funded by the Department of Energy. The primary goal of the CHAPS campaign was “to characterize and contrast freshly emitted aerosols above, within and below fields of fair-weather cumulus clouds. These observations will be used to examine the aerosol optical properties and cloud nucleating properties from both below-cloud and above-cloud, and how they differ downwind of a mid-size city relative to similar aerosols in air less affected by emissions...” [Accessed on the internet at <http://asp.labworks.org/> June 2009].

Samples were obtained in the vicinity of Oklahoma City, OK, USA by Mr. John Hubbe and Dr. Nels Laulainen of the Pacific Northwest National Laboratory (PNNL) from an instrumented Grumman Gulfstream 159 (G-1) aircraft flying at elevations up to 8000 ft. The aircraft was equipped with a Mohnen slotted cloud water droplet collector that was designed and machined at The State University of New York at Albany (**Figure 3-2.1**). The collector consisted of two 27.3 cm long Delrin® rods with a width of 0.94 cm and a depth of 2.10 cm which extended upward from the top hatch of the aircraft. The rods had a U-shaped notch through the entire length and these were positioned toward the direction of flight. The rods extended through an aluminum bulkhead fitting through the aircraft hatch and into the cabin. During flight, droplets that impacted the interior of the notch on the exterior of the aircraft coalesced and ran down the notch where they were

collected in a sterile 200 mL polyethylene bottle inside the cabin. The droplet impactor was expected to collect only water droplet sizes of 5 to 50 μm .

The sampling campaign was carried out over several weeks in June and July. The objective of the field campaign was sampling of cumulus-humilis clouds, however, a series of rain events occurred during the sampling period. Thus rain clouds were sampled, along with any rain droplets that may have entered the sampler. Further, the sampling device was placed through the aircraft hatch prior to take-off. Thus, it is possible that during sampling runs, the sampling device collected cloud droplets, particles from air that were deposited on the impactor and rain droplets of the correct size.

Before sampling, all sample bottles were autoclaved and the droplet collector components were rinsed with 70 % alcohol and dried completely in a sterile hood. Then sterilized sampling bottles and the equipment were connected and covered to prevent contamination, prior to overnight shipment to the sampling location. Each day, a sterile sampling bottle was connected to the end of the sampling device prior to departure from the aircraft hanger. After a day of sampling, the sample bottle was removed from the collector and sealed with a sterile cap. Samples were shipped to Rutgers University on ice and stored at 4 °C prior to DNA extraction.



Figure 3-2.1. Photographs of the cloud water droplet sampler (photographs courtesy of John Hubbe, PNNL).

2.2. DNA extraction from cloud water

It was estimated that each sample bottle from cloud water collection contained less than 1 mL liquid. Because of concern that an individual sample might not contain enough biomass to accomplish DNA extraction, the 10 sample bottles containing cloud water were pooled together in one bottle. The total liquid volume was approximately 8 mL. The sample bottles were additionally rinsed with approximately 10 mL of sterile distilled water and the rinseate was added to the samples. Sterile distilled water was also used to rinse sterile sample bottles that had not been used for sample collection and this rinseate was use simultaneously as a negative control throughout the DNA extraction and PCR-DGGE procedures to ensure that false positive bands were not produced during the experiment.

Cells were harvested by filtration onto a 0.2 μm membrane filter (SUPOR 200, Pall Gelman, East Hills, NY). A Mo-Bio Power Soil kit (Mo-Bio Inc., Solana Beach, CA) was used to extract total genomic DNA from the cells collected on the membrane filter. The procedure was slightly modified from that provided by the manufacturer in the following ways. The cells collected onto the membrane filter were resuspended with a bead-beating solution and transferred to a bead-beating tube. Cell lysis was initiated by addition of lysozyme solution to a final concentration of 5 mg/mL. The bead-beating tubes containing bacterial cells and lysozyme were incubated for 30 min at 37 °C. Proteinase K (final concentration, 2 mg/mL) and the C1 solution (SDS solution) from the Mo-Bio Power Soil kit were added to the tubes. The tubes were incubated for 30 min at 50 °C. For further cell lysis, samples were subjected to three cycles of freezing at -80 °C, and thawing at 65 °C. After the final cycle, the bead-beating tubes containing samples

were subjected to horizontal vortexing for 10 min. Nucleic acids were cleaned and concentrated in accordance with the Mo-Bio DNA kit protocols. Final DNA was eluted in 30 μ L of nuclease-free water.

2.3. PCR-denaturing gradient gel electrophoresis (PCR-DGGE)

Universal bacterial 16S rRNA gene fragments were amplified using the Bac338f-GC clamp and Univ519r primer set listed in **Table 3-2.1**. Each 50 μ L PCR reaction contained the following (all reagents from USB Corp., Cleveland, OH): 1x PCR buffer, 2.5 mM of $MgCl_2$, 20 nmol of deoxynucleoside triphosphate, 10 pmol of each primer, 1.5 U of Taq polymerase, and 2 to 4 μ L of template DNA. The thermocycling program was as follows: initial denaturation for 5 min at 94°C; 25 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; and a 7-min final extension at 72°C. The expected PCR amplicon size was verified with agarose gel electrophoresis on a 1.2 % gel prior to DGGE. DGGE of PCR-amplified fragments was performed on an 8 % polyacrylamide gel with a 20 to 60% urea-formamide gradient for 4 h at 150 V and 60°C with the DCode mutation detection system (Bio-Rad Laboratories, Hercules, CA).

The gel was stained in 0.1 % ethidium bromide solution and visualized using UV on a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA). Each prominent band detected in the DGGE gel was excised using a sterile razor blade. Using the excised gel fragments as DNA template, a second PCR-DGGE was performed under the same conditions described above and the purity of each band was confirmed by noting a single DNA band after repeating the DGGE using the same conditions.

The bands resulting from the second round DGGE were then excised and the DNA was reamplified using the Bac338f and Univ519r primer set (**Table 3-2.1**). The resulting amplicons were purified using an UltraClean PCR Clean-Up kit (Mo-Bio Inc., Solana Beach, CA) and then sequenced by Genewiz (Genewiz Inc. South Plainfield, NJ).

2.4. Cloning of bacterial 16S rRNA genes from atmospheric water

16S rRNA gene fragment cloning was performed on the pooled atmospheric water DNA samples using universal primers Bac27f and Bac907r (**Table 3-2.1**). The PCR conditions were same as those described for PCR-DGGE in section 2.3 but with a constant annealing temperature of 53°C and a total of 30 cycles. The PCR amplicons were inserted into pCR®2.1 vectors (Invitrogen Corp., Carlsbad, CA) and the inserted vectors were transformed into chemically competent *Escherichia coli*, TOP10 cells (Invitrogen Corp., Carlsbad, CA), using a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions. Transformed *E. coli* were grown on Luria-Bertani agar plates at 37°C overnight and total of 100 clones containing the inserted plasmid were picked. The picked clones were cultured in 5 mL of Luria-Bertani broth and after 18 hrs of incubation at 37°C, plasmid vectors were recovered from the transformed cells using a plasmid purification kit (QIAGEN Inc., Valencia, CA).

Clones were amplified with vector-specific primers M13f-20 and M13r (**Table 3-2.1**). PCR was performed under the same conditions as those described above but with a constant annealing temperature of 55°C and a total of 30 cycles. PCR products were digested with a restriction enzyme, Hha I. The 14 µL restriction digests contained 2.8 U Hha I (New England Biolabs Inc., Ipswich, MA), 1.4 µL NE buffer 4TM (England Biolabs

Inc., Ipswich, MA), 0.14 μ L BSA solution (England Biolabs Inc., Ipswich, MA), 7 μ L PCR product and 5.3 μ L sterile water. The reaction mixture was incubated for 10 hrs at 37°C. After incubation, restriction patterns of PCR products were visualized on a 1% agarose gel under UV. Seventeen distinct digestion patterns were detected and 24 clones (including representatives of the 17 unique clones) were selected for DNA sequencing. The amplified inserts from the selected clones were purified using an UltraClean PCR Clean-Up kit (Mo-Bio Inc., Solana Beach, CA) and then sequenced by Genewiz (Genewiz Inc. South Plainfield, NJ).

Table 3-2.1. Primer sequences used in this study.

Primer	Sequence	Target gene size (bp)	Ref.
Bac338f	5' -CTCCTACGGGAGGCAGCAG-3'	200	(Nakatsu et al., 2000b)
Bac338f-GC Clamp	5' -CGCCCGCCGCGCCCCGCGCCCGTCCCGC-CGCCCCCGCCCTCCTACGGGAGGCAGCAG-3'		
Univ519r	5' -ATTACCGCGGCTGCTGG-3'		
Bac27f	5'-AGAGTTTGATC(C/A)TGGCTCAG-3'	900	(Lane, 1991)
Bac907r	5'-CCGTCAATTCMTTGTGAGTTT-3'		(Lane et al., 1985)
M13f-20	5'-GTAAAACGACGGCCAGT-3'	Varied by insert size	Invitrogen Corp. (Carlsbad, CA)
M13r	5'-TTCACACAGGAAACAG-3'		

3. Results and Discussion

3.1. Bacterial communities in atmospheric water identified using PCR-DGGE analysis

The DGGE profile of 16S rRNA bacterial gene fragments amplified from DNA extracted from the pooled atmospheric water sample is shown in **Figure 3-2.1**.

For the DGGE profile of the pooled atmospheric sample, approximately 13 distinct bands were observed visually in the DGGE image. This low number of phylotypes suggests low community complexity; however, detection of only a few bacterial strains could be caused by the expected low number of bacteria in the samples, and by the relative insensitivity of DGGE analysis. If each < 1 mL sample contained a bacterial content of 10^3 to 10^5 cells mL⁻¹ as reported for cloud droplets (Sattler et al., 2001), the number of cells in the pooled sample (8 mL) could be as low as 10^4 or as high as 10^6 , while the corresponding mass of DNA could be 0.1 to 10 ng (Neidhardt and Umbarger, 1996).

A small portion of the nine most prominent DNA bands observed in the denaturing gradient gel (see labeled bands in **Figure 3-2.1**.) was excised and the reamplified DNA fragments were sequenced and compared to known sequences in Genbank using BLASTN [<http://blast.ncbi.nlm.nih.gov.ezproxy.lib.utexas.edu/Blast.cgi>] (**Table 3-2.2**). Nine bands detected in the DGGE gel were shown to be closest matches with eight different bacterial strains. Most of these phylotypes were associated with soil or plant origin. The low identity percentile for these different phylotypes may be attributed to the short sequence length (approximately 200 bp) analyzed for identification.

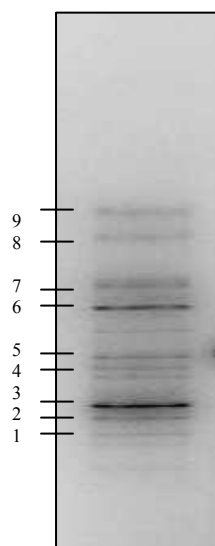


Figure 3-2.2. DGGE profile of amplified 16S rRNA gene sequences of bacteria from a pooled atmospheric water sample.

Table 3-2.2. Summary and comparison of 16S rRNA gene sequence identities from PCR-DGGE analysis

DGGE band number	Closest isolate/clone (accession no.)	% identity	Phylum (genus)	Source	Ref.
1-1	<i>Rhizobium huautlense</i> (AM237359.1)	95%	<i>Alphaproteobacteria</i> (<i>Rhizobium</i>)	plant legume	(Wang et al., 1998)
1-2	<i>Rhizobium tropici</i> strain ISP59 (AY117618.1)	96%	<i>Alphaproteobacteria</i> (<i>Rhizobium</i>)	plant legume	(Diouf et al., 2000)
2	<i>Curtobacterium</i> sp. N321 (AY371408.1)	94%	<i>Actinobacteria</i> (<i>Curtobacterium</i>)	deep water marine invertebrates	not available
3	<i>Sphingopyxis</i> sp. KYH-1 (AB235163.1)	99%	<i>Alphaproteobacteria</i> (<i>Sphingopyxis</i>)	not available	not available
4	No match		not available	not available	not available
5	<i>Exiguobacterium acetylicum</i> strain KSC (DQ870703.1)	98%	<i>Firmicutes</i> (<i>Exiguobacterium</i>)	clean room environments	(La Duc et al., 2007)
6	<i>Rhizosphere</i> soil bacterium BE-9 (AM110778.1)	94%	Unclassified	rhizosphere	not available
7	<i>Pedobacter rhizospharae</i> (AM279214.1)	94%	<i>Bacterioidetes</i> (<i>Pedobacter</i>)	soil	not available
8 and 9	Uncultured soil bacterium clone T1IF3 16S ribosomal RNA gene, partial sequence (DQ297957.1)	97%	not available	not available	not available

3.2. Identification of the bacterial communities in atmospheric water using clonal library analysis

The 16S rRNA genes contained in the genomic DNA extracted from pooled atmospheric water sample were amplified and cloned. Initially, 86 clones were subject to restriction enzyme digestion and 17 unique digestion patterns were observed. A total of 24 samples, including one or more clones from each unique pattern, were selected for DNA sequencing. Sequencing results were successful for 20 clones of the 24 clones and the resulting sequences represented 12 of the 17 unique clones. In total, sequences were obtained for 78 of the original 86 clones. The closest matching phylotype for each sequence was obtained using BLASTN [<http://blast.ncbi.nlm.nih.gov.ezproxy.lib.utexas.edu/Blast.cgi>; accessed on the internet June 2009].

Table 3-2.3 shows the results of the clonal library analysis of the atmospheric water sample. Strains detected in the atmospheric water were associated with phylotypes originally detected in environments including soil, vegetation (grass and plants), marine environments, extreme environments (frozen soil, clean room) and the air itself. A total of 12 bacterial phylotypes were identified. The most abundant phylotypes (35.9 percent of the clones) were related to *Sphingomonas* (*Alphaproteobacteria*). The most highly detected phylotype was 99% similar over 900 bp to *Sphingomonas aerolata* strain NW12 an orange pigmented, psychrotolerant bacterial strain, isolated from room air (Busse et al.). The next most common phylotypes were related to *Betaproteobacterium* strain HJ12 (13 clones 16.7 %) and *Pedobacter koreensis* strain PB92 (11 clones, 14.1 %).

Table 3-2.3. Summary and comparison of unique clones and the 16S rRNA sequence identities from clonal library analysis.

Clone count ^a	Closest isolate/clone (accession no.)	% identity	Phylum (genus)	Source	Ref.
24	<i>Sphingomonas aerolata</i> strain NW12 (AJ429240.1)	99%	<i>Alphaproteobacteria</i> (<i>Sphingomonas</i>),	air	(Busse et al., 2003)
4	<i>Sphingomonas</i> sp. TSBY-49 (DQ151834.2)	99%	<i>Alphaproteobacteria</i> (<i>Sphingomonas</i>),	alpine permafrost	(Bai et al., 2006)
13	Beta <i>proteobacterium</i> HJ12 (AY237409.1)	99%	<i>Betaproteobacteria</i>	soil	(Rintala et al., 2008)
11	<i>Pedobacter koreensis</i> strain PB92 (EF660751.1)	97%	<i>Bacterioidetes</i> (<i>Pedobacter</i>)	soil	(Roh et al., 2008)
7	<i>Curtobacterium flaccumfaciens</i> strain Y6 (EU236753.1)	98%	<i>Actinobacteria</i> (<i>Curtobacterium</i>)	plant	not available
6	<i>Pseudomonas</i> sp. Fa20 (AY131224.1)	99%	<i>Gammaproteobacteria</i> (<i>Pseudomonas</i>)	plant	(Ursula et al., 2005)
4	<i>Exiguobacterium acetylicum</i> strain CS5 (AM887698.1)	99%	<i>Firmicutes</i> (<i>Exiguobacterium</i>)	not available	(Farrow et al., 1994)
4	<i>Variovorax paradoxus</i> strain CAI-26 (DQ257419.1)	98%	<i>Betaproteobacteria</i> (<i>Variovorax</i>)	not available	not available
2	<i>Microbacterium oxydans</i> , isolate OS-36 (AM237353.1)	99%	<i>Actinobacteria</i> (<i>Microbacterium</i>)	not available	available (Schumann et al., 1999)
1	<i>Rhizobium huautlense</i> isolate OS-49.b (AM237359.1)	99%	<i>Alphaproteobacteria</i> (<i>Rhizobium</i>)	plant legume	(Wang et al., 1998)
1	<i>Subtercola pratensis</i> strain DSM 14226T (AJ310412.1)	99%	<i>Actinobacteria</i> (<i>Agreia</i>)	plant	(Behrendt et al., 2002)
1	<i>Frigoribacterium</i> sp. ULA1 (AM180731.1)	99%	<i>Actinobacteria</i> (<i>Frigoribacterium</i>)	marine macroalgae	(Marshall et al., 2006)

^a : A total of 78 clones were used for identification.

3.3. Description of bacterial population in the atmospheric water sample

The results presented in **Table 3-2.2** and **Table 3-2.3** show that using bacteria-specific primers only bacterial strains were detected using both methods; however, the number and diversity of bacteria identified varied depending upon the detection method. DGGE analysis detected fewer bacterial strains and with a lower percentage match to the closest relative in Genbank, when compared to the clonal library method. Some strains were detected using both methods, however, the results from the clonal library analysis showed a broader spectrum of bacterial strains. A negative control (described in **2.2**) was employed to test whether false positive bands were produced through DNA extraction and PCR processes. Results from PCR targeting the bacterial 16S rRNA gene showed no band from the negative control.

Four genera, *Rhizobium*, *Exiguobacterium*, *Pedobacter* and *Curtobacterium* were identified in common between the DGGE and clonal library methods. *Pedobacter* (Amato, 2007) and *Curtobacterium* (Amato, 2007; Shaffer and Lighthart, 1997) have been also identified in the previous studies of bacterial structure in atmospheric samples. The most highly detected phylotype was 99% similar to *Sphingomonas aerolata* strain NW12, an orange pigmented, psychrotolerant bacterial strain, isolated from room air (Busse et al., 2003). The second most common phylotypes were related to Betaproteobacterium strain HJ12 (AY237409.1), a phylotype from Chinese soil (13 clones 16.7 %) and *Pedobacter koreensis* strain PB92 isolated from Korean soil (11 clones, 14.1 %) (Roh et al., 2008). Interestingly, *Exiguobacterium acetylicum* strain KSC_Ak2F, detected using both methods, was reported to be a alkalophile which was isolated from a clean room environment (La Duc et al., 2007) by a cultivation method at

pH 9 and thus, may not be retrieved using typical cultivation methods under neutral pH conditions.

Alphaproteobacteria and *Actinobacteria* were the most abundant phyla (77%, 60 of 78 clones) in terms of number of clones identified and of genera represented, similar to information reported in the previous studies (Ahern, 2006; Amato, 2007; Fuzzi et al., 1997).

Interestingly, unlike previous reports (Ahern, 2006; Amato, 2007; Fuzzi et al., 1997; Maron et al., 2005; Radosevich et al., 2002; Sattler et al., 2001) on microbial composition in fog, cloud or rain water, the genus, *Pseudomonas*, was not dominant in our results. This genus was not detected by the DGGE method and only 6 clones matching this phylotype were observed by clonal library analysis. *Gammaproteobacteria* have been reported in many airborne microorganism studies (Amato et al., 2005; Radosevich et al., 2002). Particularly, *Pseudomonas* were found in many air samples, which reflect the ubiquitous nature of this microorganism. Another finding different from the previous reports is that the predominance of Gram-negative strains. From the DGGE method, four bands out of six bands on the gel were identified to be related to Gram-negative strains and in results from clone library analysis indicated that 63 clones among a total of 78 clones (81 %) were matched to 16S rRNA genes from Gram-negative strains. These results are in contrast to the previous studies detecting microorganisms in atmospheric samples using cultivation methods, and could be explained by seasonal selection of airborne bacteria. During the summer it was reported that Gram negative bacteria may be more abundant in the air because they are more resistant to UV radiation damage (Amato, 2007; Shaffer and Lighthart, 1997)

In an extensive study of cultivable microorganisms in cloud water (Amato, 2007), Amato et al. reported that a total of 71 bacterial strains (3 to 19 strains at each sampling event) were isolated from seven sampling events. From a one pooled sample of which the maximum bacterial quantity would be estimated to be 10^6 cells, in total, we retrieved eight to 12 different bacterial strains. While the number of phylotypes identified was relatively low in our first attempt at characterization of cloud bacteria, this proof of concept for using the Mohnen slotted cloud water droplet collector to collect cloud or rain borne bacteria at elevation may increase the possibility of obtaining more *in situ* information related to microbial communities present in the atmosphere. Future work should include repeated sampling of clouds and molecular characterization of cloud microbes coupled to extensive cloud chemical and physical characterization performed simultaneously during the Cumulus Humilis Aerosol Processing Study (CHAPS). Further, detection of other informative molecular targets including ribosomal and message RNA, proteins and ATP would better define activity in these microbial systems. If molecular methods are used along with cultivation methods in the investigation of the microbial structure of the atmospheric samples, more comprehensive information could be obtained.

Chapter 4

Summary and Conclusions

This dissertation describes research that sought to expand understanding of bacterially-mediated biotransformation in two microbially challenging environments, subsurface groundwater and the atmosphere. These environments are both of critical importance to human populations and are linked by the hydrologic and biogeochemical cycles that characterize planetary processes.

The first research area, described in **Chapter 2**, addressed characterization of dehalorespiring bacteria from a PCE-contaminated groundwater aquifer in New Jersey. As a results of this work over a four year period, a highly tetrachloroethene (PCE)-enriched culture—designated as RU11/PCE—which dehalorespires PCE, TCE, *cis*-1,2-DCE and VC to ethene was developed. Quantitative PCR results showed that the RU11/PCE culture is able to grow on all four chlorinated ethenes and has similar yields on PCE, TCE, *cis*-1,2-DCE and VC (0.6×10^8 to 1×10^8 16S rRNA gene copies/ $\mu\text{mol Cl}^-$) as other *Dehalococcoides* strains.

PCR-denaturing gradient gel electrophoresis (PCR-DGGE), 16S rRNA clonal library analysis and direct DNA sequence analysis of 16S rRNA gene and the superoxide dismutase gene (*sod*) and the DNA gyrase subunit B gene (*gryB*) revealed only one bacterial genus, *Dehalococcoides*, in the RU11/PCE culture. The 16S rRNA sequence (~1.5 kb) of the culture is identical to that of *Dehalococcoides* sp. strain CBDB1, strain

FL2, strain GT and highly enriched culture KB-1/VC. The RU11/PCE culture contained ten RDase genes highly similar to those found in the KB-1 culture, five highly similar to those found in strain FL2, four highly similar to those found in strain CBDB1, three highly similar to those found in strain 195 and one highly similar to those found in strain BAV1. Of the well-characterized reductive dehalogenase genes (*bvcA*, *tceA* and *vcrA*) only the *vcrA* gene, involved in VC dechlorination in *Dehalococcoides* strain VS, was detected in the RU11/PCE culture. These findings open the exciting possibility that a new and novel strain of *Dehalococcoides* has been isolated—one that dehalorespires all the chloroethenes and has a different functional gene assembly than current isolates.

However, since the *Dehalococcoides* spp. have high similarity not only in 16S rRNA genes, but also in the *sod*, and *gyrB* genes between strains, it is remotely possible that this culture could be composed of more than one *Dehalococcoides* strains and that they have not been differentiated using these conventional, PCR-based phylogenetic analysis methods.

In **Chapter 3-1**, to address the hypothesis that the air contains an active microbial ecosystem, we developed compact rotating bioaerosol bioreactors which were manufactured to keep bacteria suspended for 5 to 10 days while measuring their activity. The reactor design is a rotating drum system similar to that described by Goldberg et al. with the modification of being rolled in a rack, rather than being mounted on a rotating shaft. This adaptation allowed that the bioreactors in this study were relatively inexpensive.

The bioreactor system was equipped with a virtual impactor positioned at the reactor inlet to have the capability to increase the particle concentration when filling the

reactor. Particles greater than or equal to the size of a common bacterial cell, approximately 0.5 to 1.0 μm , were increased in concentration in the reactor at least 3- to 7-fold over ambient air concentrations. Concentrating the bioaerosol particles from ambient air could increase the concentration of bacteria inside the reactor and would thus increase the sensitivity of activity tests with ambient airborne bacteria.

These bioreactors also showed good efficiency in retention of volatile gas (e.g. ethene gas) with a daily loss less than 5% and a bioaerosol of inactivated (killed) *Pseudomonas fluorescens* bacteria.

A quantitative polymerase chain reaction (qPCR) method using bacterial 16S rRNA gene fragments was developed and used along with an ATP assay, acridine orange staining and microscopy, and traditional plate counts to enumerate bacteria in the airborne state within the reactors under different conditions.

The effect of aerosolization into the rotating bioreactors and the presence of substrate on the culturability of *X. autotrophicus* and *B. subtilis* were determined. In tests performed with *X. autotrophicus*, no culturable cells were recovered from the bioreactors under any condition. *B. subtilis* aerosols from dilute TSB with substrate yielded statistically higher culturability than aerosols from distilled water with no TSB substrate at the 95% confidence level.

We performed growth tests of lab-generated live bioaerosols from Gram-negative bacteria, *X. autotrophicus*, and Gram-positive bacteria, *B. subtilis*. In the first aerosolization test of *X. autotrophicus* cell numbers as measured by qPCR indicated an initial decrease followed by an increase in cell numbers suspended in the reactors so that after nine days approximately 100% of the bacteria remained in suspension. During the

second test with *X. autotrophicus*, only 10 to 40% of the initial *X. autotrophicus* concentration was detected in the airborne fraction after seven days. For *B. subtilis*, a decrease of nearly two orders of magnitude larger cell loss was observed over the course of the experiment.

Although bacteria were apparently rendered nonculturable in the reactors, it is still possible that they could be active. Here we measured bulk loss of a primary substrate (ethene) as a measure of activity. Use of a more sensitive indicator of activity is needed to conclusively determine substrate use. For example, use of labeled substrates (^{14}C or ^{13}C) could be used to allow detection of minute quantities transformation products (e.g., $^{14}\text{CO}_2$) or detection of labeled carbon in cells via stable isotope probing through detection of ^{13}C labeled DNA. Further, a choice of a substrate that produces a definitive, traceable reaction product during transformation would also be useful.

To ensure greater activity of cells in the bioreactors, the optimization of experimental conditions by varying environmental factors (e.g., RH, temperature, etc.), should be the objective of future studies. Further, in natural air the organisms are likely in association with other particulate matter, e.g. mineral dust or biogenic material, and this association should provide some protective mechanisms. The effect of additional aerosolized material will be investigated in future studies. Finally, use of natural, concentrated bioaerosols as starting material in the reactors and measurement of their activity will be investigated.

In **Chapter 3-2**, we analyzed the bacterial components of a pooled sample of atmospheric water collected in the vicinity of Oklahoma City, OK, USA using two different molecular methods—denaturing gradient gel electrophoresis (DGGE) and clone

library analysis—in which 16S rRNA genes were used for identification. This study compared the bacterial strains from these two methods and compared the results from previous studies.

From DGGE analysis, eight strains were detected and six of them belonged to the phyla *Actinobacteria*, *Firmicutes*, *Proteobacteria* and *Bacterioidetes*. In clone library analysis, 12 bacterial strains were identified with dominant occurrence of the genera, *Sphingomonas*, *Betaproteobacterium*, *Curtobacterium*, and *Pseudomonas*. The most highly detected phylotype (24 of 78 clones) was 99% similar to *Sphingomonas aerolata* strain NW12 (AJ429240.1), an orange pigmented, psychrotolerant bacterial strain, isolated from room air (Busse et al., 2003). Interestingly, highly similar strains have been isolated from cold environments. Strain Ant 20 (AF184221.1) was cultured from fuel contaminated soils around Scott Base, Antarctica (Aislabie et al., 2000) and strain M3C203B-B (AF184221.1) was isolated from a 4200 year old ice core from Taylor Dome in Antarctica (Christner et al., 2001). Other organisms closely related to this clone were from clean room environments (Christine et al., 2007).

Interest regarding the potential role of microorganisms in atmospheric chemistry is increasing. The results of this study, though preliminary, suggest that bacteria adapted to the extreme environments of ice, clean room environments and air, such as the orange pigmented *Sphingomonas aerolata* making up 30% of the clones recovered from the Oklahoma atmospheric water sample, might be a good candidate for further pure culture studies of activity of bacteria in air. This species might be found in Antarctic ice because it has some adaption to survive and be transported long distances via the atmosphere.

Such organisms might be good candidates for further testing for activity in the airborne state.

Until relatively recently, detection of microorganisms in atmospheric samples are focused on cultivable aerobes. If culture - independent, molecular methods are used in combination with cultivation methods in the investigation of the microbial structure of atmospheric samples, more comprehensive information of the microbial biota could be obtained. Such information might allow for a detailed understanding of the role of microorganisms in atmospheric environments.

Taken together, the results of these studies have highlighted the importance of the study of bacteria in challenging environments. Continued understanding of environmental processes mediated by bacteria could improve the chances for environmental restoration or allow a greater understanding and prediction of atmospheric processes.

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Curriculum Vitae

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EDUCATION

- Oct. 2009 Ph.D., Environmental Sciences, Rutgers, the State University of New Jersey
- Dissertation "Characterization of Bacterial Processes in the Subsurface and the Atmosphere" directed by Professor Donna E. Fennell.
- Feb. 1999 M.S., Agricultural Chemistry, Seoul National University, Seoul, South Korea
- Dissertation "Novel Auxin-Binding Protein from Soluble Fractions of Rice (*Oryza sativa* L.) Roots"
- Feb. 1997 B.S., Agricultural Chemistry, Seoul National university, Seoul, South Korea

RESEARCH EXPERIENCE

- May 2003- Jun 2008 Graduate assistant, Rutgers University, New Brunswick, NJ
Conducted research work towards a Ph.D. in Environmental Sciences.
- My dissertation work focused on
- 1) characterization and identification of enrichment cultures developed from the groundwater and sediments which were drawn from a tetrachloroethylene (PCE)-contaminated aquifer
 - 2) determination of whether bacteria are capable of growing in while in bioaerosols.

TEACHING EXPERIENCE

- Jan.-May 2006 Teaching assistant for Air Sampling and Analysis Techniques, Rutgers University, New Brunswick, NJ

GRANTS AND AWARDS

Jun.2008	Corporate Activities Program Student Travel Grants, American Society for Microbiology 108 th General Meeting, Boston, MA
2005-2006	Graduate Student Grants-In-Aid (\$5,000), New Jersey Water Resources Research Institute
May 2004	Graduate Student Poster Competition 3 rd Prize, New Jersey Water Environment Association 89 th Annual Conference and Exposition, Atlantic City, NJ

PUBLICATIONS

Son, E.-K.; Fennell, D. F. Characterization of a Novel PCE-Enrichment Culture from PCE-contaminated Groundwater and Sediments. In preparation.

Krumins, V., **E.-K. Son**, G. Mainelis, and D. E. Fennell. 2008. Retention of Inactivated Bioaerosols and Ethene in a Rotating Bioreactor Constructed for Bioaerosol Activity Studies. *CLEAN - Soil, Air, Water*, 36, 593-600.

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