ABSTRACT

Title of Document: THE KINETICS OF TWO HETEROTROPHIC TETRACHLOROETHENE-RESPIRING POPULATIONS AND THEIR EFFECTS ON THE SUBSTRATE INTERACTIONS WITH *DEHALOCOCCOIDES* STRAINS

Deyang Huang, Doctor of Philosophy, 2009

Directed By: Associate Professor, Jennifer G. Becker, Department of Environmental Science and Technology

This study focused on evaluating how interactions between the hydrogenotroph *Dehalococcoides ethenogenes* strain 195, which is able to completely dechlorinate tetrachloroethene (PCE) to ethene, and the two heterotrophs *Desulfuromonas michiganensis* strain BB1 and *Desulfitobacterium* sp. strain PCE1, which dechlorinate PCE to either *cis*-dichloroethene (*cis*-DCE) or trichloroethene (TCE), on the fate of PCE under common in situ bioremediation scenarios. Meaningful kinetic parameter estimates were obtained for the heterotrophic dehalorespirers under a wide range of conditions. Batch culture assays and numerical experiments were conducted with *Desulfuromonas michiganensis* to evaluate the effect of the initial conditions including the ratio of the initial substrate concentration (S_0) to the initial biomass concentration (X_0) and the ratio of S_0 to the half-saturation constant (K_S) on parameter correlation. Most importantly, S_0/K_S, but not S_0/X_0, strongly influenced parameter

correlation. Correlation between the Monod kinetic parameters could be minimized by maximizing S_0/K_S.

In the present study, dechlorination of high PCE concentrations by *Desulfuromonas michiganensis* and *Desulfitobacterium* sp. strain PCE1 was monitored. The maximum level of PCE that could be dechlorinated by each strain was not constant, and varied with X_0. This phenomenon could not be described using conventional Monod kinetics; therefore, a new model that incorporated an inactivation term into the biomass growth equation was developed to describe dechlorination at high PCE concentrations.

The interactions among *Dehalococcoides ethenogenes* and heterotrophic dehalorespirer in continuous-flow stirred tank reactors (CSTRs) were performed under two conditions that reflect either a natural attenuation or engineered bioremediation treatment scenario. Extant kinetic estimates accurately predicted the steady-state chlorinated ethene concentrations in the CSTRs. However, intrinsic kinetic parameter estimates better described the CSTR start-up phase. The modeling and experimental results suggested that the ability of *Dehalococcoides ethenogenes* to utilize PCE and TCE is limited by the presence of a PCE-to-TCE/*cis*-DCE dehalorespirer, which forces *Dehalococcoides ethenogenes* to function primarily as a *cis*-DCE-respiring population.

This study provides insight into how the activities of different dehalorespiring cultures are interrelated and will aid in the design of engineered bioremediation approaches that optimize the potential benefits associated with different

dehalorespiring populations to achieve efficient and effective clean-up of PCE- and TCE-contaminated sites.

THE KINETICS OF TWO HETEROTROPHIC TETRACHLOROETHENE-RESPIRING POPULATIONS AND THEIR EFFECTS ON THE SUBSTRATE INTERACTIONS WITH *DEHALOCOCCOIDES* STRAINS

By

Deyang Huang

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2009

Advisory Committee:
Associate Professor Jennifer G. Becker, Chair
Professor Allen P. Davis
Professor Bruce R. James
Associate Professor Eric A. Seagren
Professor Alba Torrents

© Copyright by
Deyang Huang
2009 Doctor of Philosophy

Acknowledgements

I would like to express my sincere gratitude to my Ph.D. advisor Dr. Jennifer G. Becker, for her valuable guidance, advice, and encouragement during my study at University of Maryland. I have learned a great deal from her and it was a great pleasure to work under her direction. Without her, I would not have accomplished this work.

My appreciation also goes to my other committee members as well. To Dr. Eric A. Seagren, Dr. Allen P. Davis, Dr. Bruce R. James, and Dr. Alba Torrents, for serving on doctoral committee and spending time in reviewing this work.

I would like to acknowledge my fellow graduate students Emily N. Devillier, Supida Piwkhow and Yenjung Lai for their assistance and cooperation on this project. I am also thankful to Dr. Hubert Montas, for his advice on the MATLAB program.

I am grateful to Gary Seibel and Ali Djamshidi for not only assisting me to setup my reactor systems, but also the other equipments I used in our lab. Their valuable work helps to overcome lot difficulties.

I would also thank to the National Science Foundation (NSF) for kindly providing research funding on this project.

Finally, my give my deepest appreciation to my parents, Qinlan Guan and Ji'an Huang, my sister, Fang Huang and my girlfriend, Bin Zhang, for their love, understanding and support.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

The chlorinated ethenes tetrachloroethene (PCE) and trichloroethene (TCE) are commercially important chlorinated hydrocarbon solvents and chemical intermediates. They are widely used for dry cleaning and textile processing, for vapor degreasing in metal-cleaning operations (McCarty, 1997), and in semiconductor manufacture (Vogel et al., 1987). Improper handling and disposal of PCE and TCE have contributed to the contamination of groundwater with these contaminants. According to the report of U.S. Environmental Protection Agency (2005), both PCE and TCE are among the most frequently detected chemical contaminants in groundwater at Superfund sites.

PCE and TCE are highly oxidized compounds and thus readily undergo reductive dechlorination under anaerobic conditions (Vogel et al., 1987), and complete dechlorination to ethene is feasible, as first shown by Freedman and Gossett (1989). Therefore, engineered bioremediation approaches and natural attenuation based on reductive dechlorination are promising for the *in-situ* cleanup of chlorinated ethene contamination. However, at many contaminated sites, the potential of *in-situ* bioremediation is not realized because complete dechlorination of chlorinated ethenes does not occur. Incomplete dechlorination is generally attributed to insufficient electron donors, slow dechlorination rates, low numbers of dichloroethene (DCE)- or vinyl chloride (VC)-dechlorinating bacteria, which are thought to be less common than PCE-to-DCE-dechlorinators (Fennell et al., 2001), a high redox potential, or unfavorable environmental factors (Bradley, 2003; Christ et al., 2005; Interstate

Technology Regulatory Council, 2005; Mohn and Tiedje., 1992; Stuart et al., 1999). Thus, improved understanding of the factors that influence the extent and rate of PCE biotransformation in contaminated environments is necessary to improve our ability to predict contaminant fate and transport, protect human health, and develop effective bioremediation strategies.

The availability of electron donors is of particular concern because many contaminated sites are electron donor-limited (McCarty and Semprini, 1994; Gossett and Zinder, 1996). As discussed below, in some cases, organic substrates, such as lactate can be used directly as electron donors in reductive dechlorination reactions. Often, however, organic compounds are thought to undergo fermentation, which produces hydrogen that is used as the electron in reductive dechlorination. In anoxic environments, hydrogen in particular can be oxidized in some other anaerobic respiratory processes, such as methanogenesis, sulfate reduction and denitrification. Therefore, competition for electron donors, especially hydrogen, between chlorinated ethene-reducing bacteria and other microorganisms can greatly exaggerate the problem of electron donor availability at contaminated sites. Although, in theory, it should be possible to minimize competition from other hydrogenotrophic populations by maintaining low concentrations of hydrogen, e.g., by providing hydrogen through the fermentation of organic substrates that are fermented only at extremely low concentrations. This technique should direct the reducing equivalents to the dechlorinating populations because they have lower hydrogen thresholds compared with other hydrogenotrophic groups (Fennell and Gossett, 1998).

There is also increasing evidence that multiple dechlorinating populations are present at many chlorinated ethene contaminated sites (Lendvay et al., 2003; Löffler et al. 2000; Yang et al., 2005). At these sites, competition among different dechlorinating populations for electron donors and/or electron acceptors, i.e., chlorinated ethenes may also conceivably arise. These organisms vary with respect to the chlorinated ethenes that are transformed, electron donors used in reductive dechlorination, and kinetic characteristics. Substrate interactions among different dechlorinating bacteria involving electron donors or acceptors could potentially play an important role in determining the extent and rate of chlorinated ethene transformation at a site (Becker, 2006). The goal of the proposed research project is to improve our understanding of the factors that control interactions among key chlorinated ethene-dechlorinating organisms and the impact of these interactions on contaminant fate so that the full potential of *in situ* bioremediation at chlorinated ethene-contaminated sites can be realized.

The central hypothesis of the proposed research is that if multiple dechlorinating populations are present in either natural environments or man-made systems, such as reactors, substrate interactions involving electron acceptors, i.e., chlorinated ethenes, and/or electron donors, may arise between the dechlorinating populations and could affect the fate of chlorinated ethenes and the individual populations of dechlorinators in the system. For example, if *Dehalococcoides* species, the only organisms that are known to dehalorespire beyond *cis*-DCE, are unable to compete with other dehalorespirers for growth substrates, incomplete dechlorination of PCE may result. On the other hand, there is evidence suggesting that dehalorespiring populations in

other genera have faster specific utilization rates compared with *Dehalococcoides* species. Thus, if these organisms cannot compete with *Dehalococcoides* species, the overall rate of PCE removal may be relatively slow. Therefore, conditions that allow *Dehalococcoides* species to co-exist with other dehalorespiring populations may ideal by promoting relatively fast PCE-to-cDCE-respiring kinetics and by taking advantages of the ability of *Dehalococcoides* species to dechlorinate beyond *cis*-DCE.

A number of studies employing both molecular analyses and microcosm experiments have demonstrated the diversity of dechlorinating populations in both natural environments and laboratory systems (Duhamel and Edwards, 2006; Fennell et al., 2001; Loffler et al., 1999; Nijenhuis et al., 2006; Yang et al., 2005). Two recent studies suggested that substrate interactions among dehalorespiring populations can significantly affect the fate of chlorinated compounds in environments (Becker, 2006; Grostern and Edwards. 2006). However, currently the lack of reliable estimates of kinetic parameters for key dehalorespiring populations under the broad range of substrate concentration that exist in contamination plumes limits our ability to theoretically evaluate the outcome of these interactions using mathematical modeling. Further, systematic experimental evaluations of the interactions among dehalorespiring populations have not been conducted.

Therefore, the specific objectives of the proposed research are to:

(1) Obtain accurate and reliable estimates of the growth and substrate utilization kinetic parameters for two PCE dehalorespiring strains (*Desulfuromonas michiganensis* strain BB1 and *Desulfitobacterium* sp. strain PCE1) under both

intrinsic and extant conditions.

(2) Develop mathematical models to describe the dechlorination of high aqueous concentrations of PCE that develop in the vicinity of non-aqueous phase liquid (DNAPL) source zone by these dehalorespirers and estimate the parameter values in the models.

(3) Predict the outcome of interactions among dehalorespiring populations in three treatment scenarios involving *Dehalococcoides ethenogenes* and *Desulfuromonas michiganensis* or *Desulfitobacterium* sp. strain PCE1 using model simulations and the independently estimated extant kinetic parameters.

(4) Experimentally validate the predicted substrate interaction for three treatment scenarios involving *Dehalococcoides ethenogenes* and *Desulfuromonas michiganensis* or *Desulfitobacterium* sp. strain PCE1 in continuous-flow stirred tank reactor (CSTRs).

Six chapters follow Chapter 2 provides detailed background information on key topics related to the overall research project, including reductive dechlorination and dehalorespiration processes, microbial substrate utilization kinetics, and microbial competition. Key results and discussion of the major phases of this research project are described in chapters 3 through 6. These are written as drafts of individual journal manuscripts. Chapter 3 has already been submitted to Biotechnology and Bioengineering. Thus, each of these chapters also contains some background information as well as the materials and methods used in conducting that portion of the research.

Chapter 2: Literature review

In this chapter, an overview of the negative health effects of PCE and TCE as well as key physical and chemical characteristics of these compounds is given. The major microbial reactions that lead to biodegradation of PCE are described along with the organisms known to carry out these reactions. Finally, the available information on the kinetic characteristics of these organisms and factors affecting kinetic parameter estimates are also discussed.

2.1 Toxicological effects and physical/chemical properties of PCE and TCE

Both PCE and TCE are hazardous, toxic to animals and plants, and destructive to the ozone layer (Holliger et al., 1999). They are also suspected human carcinogens (U.S. Environmental Protection Agency, 1988a and 1999). However, vinyl chloride (VC), a lightly chlorinated ethene that can form through reductive dechlorination of PCE and TCE is a known carcinogen. PCE and TCE may also damage developing fetuses. Exposure to high concentrations of chlorinated ethenes in air, particularly in poorly ventilated areas, can cause dizziness, light-headedness, nausea, confusion, difficulty in speaking and walking, possibly unconsciousness, and death. Overexposure can cause the heart to beat irregularly or stop. Tetrachloroethene can also damage the liver and kidneys enough to cause death. Breathing the vapor may irritate the lungs, causing coughing and/or shortness of breath. Higher exposure can cause a build-up of fluid in the lungs, which can cause death. Some adverse neurological and reproductive effects caused by PCE, such as sensory symptoms, menstrual disorders, altered sperm structure, and reduced fertility, have been reported

from exposure to PCE (ATSDR, 1997; Calabrese et al., 1991; U.S. Environmental Protection Agency, 1988b).

When PCE is released to the environment, several different fates are possible. If it is released to the unsaturated zone, volatilization may occur. In addition, PCE is slightly adsorbed on sand and clay minerals. Based on the reported K_{OC} values for PCE, which vary from 209 to 1685, PCE is expected to exhibit low to medium mobility in soil and therefore may leach slowly to the groundwater (Otson et al., 1982). Due to its higher vapor pressure (18.47 mm Hg at 25^{o}C), TCE is more readily volatized and transported through soil in comparison with PCE. Because of the low aqueous solubilities (1.459 mM for PCE and 10.78 mM for TCE at 22^{o}C, Knauss et al., 2000) and high hydrophobicities of PCE and TCE, these chlorinated ethenes tend to accumulate in environments like sediments and sludges and form dense non-aqueous phase liquids (DNAPLs) in subsurface environments when sufficient quantities are present. The resulting DNAPLs can penetrate through permeable groundwater aquifers and pool at a non-permeable zone where they can serve as a long term contamination source to groundwater.

2.2 Overview of microbial reductive dechlorination of chlorinated ethenes

2.2.1 Microbial dechlorination reactions

In general, the biodegradation of chlorinated ethenes can occur via oxidative or reductive dechlorination reactions (El Fantroussi et al., 1998; Janssen et al., 2001). Oxidative dechlorination of lightly chlorinated ethenes, i.e., VC and DCEs, has been demonstrated in both aerobic and anaerobic environments (Bradley and Chappelle,

1996; Bradley et al., 1998; Coleman et al., 2002a, 2002b; Hartmans and deBont, 1992; Singh et al., 2004; Verce et al., 2000, 2001). Aerobic oxidation of TCE can be catalyzed by many microorganisms through cometabolic reactions, a process, in which the compound of interest (e.g. PCE) is dechlorinated by a biological enzyme system or cofactor and does not serve as a source of carbon or energy (Kan and Deshusses, 2006; Kuo et al., 2004; McCarty et al., 1998; Newman and Wackett, 1997). As discussed further below, the energy yielded by cometabolic transformations cannot be conserved by the organisms carrying out the reactions. Anaerobic oxidation of chlorinated ethenes was first reported by Vogel and McCarty (1985) for transformation of VC to CO_2 under methanogenic conditions. Because PCE is highly oxidized, it is generally not thought to undergo oxidation reactions. However, it is amenable to reductive dechlorination. Reductive dechlorination is a process in which the chlorine substituents are removed through the addition of electrons. Under certain conditions, abiotic reductive dechlorination may be mediated by metallocofactors or other catalysts (Vogel and McCarty, 1987), but the focus here is on reactions carried out by whole microorganisms.

Two types of microbial reductive dechlorination reactions, hydrogenolysis and dihaloelimination, are commonly observed (Mohn and Tiedje, 1992), but dihaloelimination, which involves the removal of chlorines from the adjacent carbons in unsaturated bond is not feasible with chlorinated ethenes. Hydrogenolysis involves the replacement of a chlorine substituent with a hydrogen atom. Both types of reactions require reducing equivalents. A wide variety of organic compounds, including lactate, acetate, ethanol, methanol, pyruvate, butyrate, formate, succinate,

and benzoate (Gerritse et al, 1996; 1999; Löffler et al, 1998; Sung et al, 2003; Zinder and Gossett, 1995), as well as complex organic substrates such as chitin and vegetable oil (Brennan et al., 2006a, b; Zawtocki et al., 2004; Zenker et al., 2000) have been shown to support reductive dechlorination by mixed cultures or in environmental samples. It is thought that H_2 often serves as the ultimate electron donor for reductive dechlorination and the organic compounds indirectly supply reducing equivalents by undergoing fermentation to H_2 (Distefano et al., 1992; Fennell et al., 1997; Yang and McCarty, 1998). However, as discussed below, a number of dehalorespiring organisms cannot utilize H_2 as electron donor, and instead require the organic substrates as electron donors in the reductive dechlorination reactions.

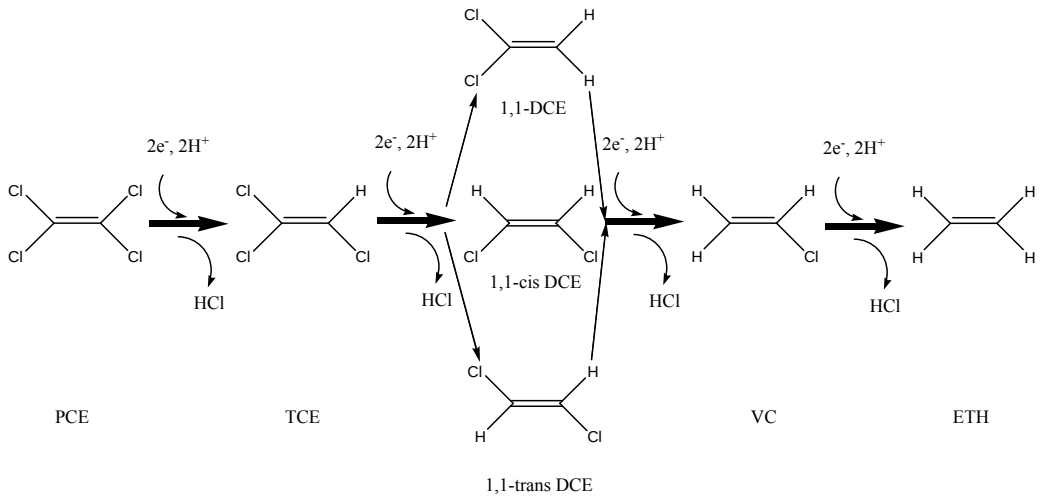

Figure 2.1 Sequential microbial reductive dechlorination of chlorinated ethenes

As first shown by Freedman and Gossett (1989), complete dechlorination of PCE to ethene (ETH) is feasible (Figure 2.1). PCE is dechlorinated mainly to *cis*-DCE

through TCE, although the isomers, 1,1-DCE and 1,1-*trans* DCE are also sometimes produced in less amounts and may be present as biodegradation products at some contaminated sites (Christiansen et al., 1997; Griffin et al., 2004; Maymó-Gatell et al., 1999; Miller et al., 2005). The formation of the 1,1-DCE was first believed to be a result of abiotic dechlorination of TCE in the presence of sulfide (Kästner, 1991). However, a recent study showed that 1,1-DCE can be the predominant intermediate of microbial TCE reduction mediated by a *Dehalococcoides* species (Zhang et al., 2006).

2.2.2 Thermodynamics of reductive dechlorination

The balanced reactions for reductive dechlorination of the chlorinated ethenes using hydrogen as the electron donor are shown in Table 2.1 along with the change in the Gibbs free energy ($\Delta G^{o\prime}$) and redox potentials ($E^{o\prime}$) at standard conditions (pH 7). The favorable thermodynamics of these reactions led to speculation that the microorganisms carrying out these reactions might be able to grow as a result of reductive dechlorination.

In fact, as discussed below, a growing number of organisms have been isolated that can conserve energy via the reductive dechlorination of chlorinated ethenes through a process known as halorespiration, chloridogenesis, or dehalorespiration, the latter of which is used here. In other words, the chlorinated ethenes are used as terminal electron acceptors in an anaerobic form of respiration. The redox potentials for PCE, TCE, DCE, and VC range between 420 and 574 mV (Table 2.1), and thus are considerably higher than that of the SO_4^{2-}/H_2S couple ($E^{o\prime}$= −217 mV),

comparable to that of the NO_3^-/NO_2^- couple ($E^{o'}$= +433 mV), but substantially lower than that of the O_2/H_2O couple ($E^{o'}$= +818 mV).

Table 2.1 Standard Gibbs free energy changes and redox potentials for the reductive dechlorination of chlorinated ethenes using H_2 as electron donor (adapted from Dolfing, 2000)

Chlorinated ethene	Reaction	$\Delta G^{o'}$ (kJ/mol)	$E^{o'}$ (mV)
PCE	$C_2Cl_4+H_2 \rightarrow C_2HCl_3+H^++Cl^-$	-171.8	574
TCE	$C_2HCl_3+H_2 \rightarrow C_2H_2Cl_2+H^++Cl^-$	-166.1	550 to 527
DCE isomers	$C_2H_2Cl_2+H_2 \rightarrow C_2H_3Cl+H^++Cl^-$	-144.8	420 to 397
VC	$C_2H_3Cl+H_2 \rightarrow C_2H_4+H^++Cl^-$	-154.5	450

2.2.3 Cometabolic transformations

In cometabolic reductive dechlorination, the chlorinated ethenes are not utilized as a terminal electron acceptor and therefore the energy released by the transformation cannot be conserved by the microorganisms carrying out the transformation (Middeldorp et al., 1999). The ability to carry out cometabolic reductive dechlorination of PCE, unlike dehalorespiration, is observed in a wide variety of microorganisms including methanogens (Fathepure and Boyd, 1988a; 1988b; Cabirol et al., 1998), acetogens (Terzenbach and Blaut, 1994), and sulfate-reducing bacteria (Cole et al., 1995). Studies conducted with boiled cell extracts of methanogens and acetogens demonstrated that metallocofactors, rather than enzymes are responsible for the cometabolic reductive dechlorination (Cabirol et al., 1998;

Fathepure and Boyd, 1988a; 1988b; Gantzer and Wackett, 1991). Some of the metallocofactors that have been shown to play a key role in chlorinated ethene cometabolism in the organisms include vitamin B_{12} (cyanocobalamin), which contains cobalt and is abundant in methanogens, acetogens, and sulfate reducers; cofactor F_{430}, which contains nickel and is present in methanogens; and iron-containing hematin, which is present in sulfate-reducers. These metallocofactors can also be used to mediate abiotic reductive dechlorination of chlorinated ethenes and other organic compounds in the presence of a reducing agent (Gantzer and Wackett, 1991). The cofactors involved in cometabolism are also present in the reductive dehalogenases that catalyze reductive dechlorination in dehalorespiring bacteria (Smidt and de Vos, 2004).

In addition to cometabolism carried out by methanogens, acetogens, and sulfate reducers, dehalorespiring bacteria can sometimes cometablize lesser chlorinated ethenes while utilizing more chlorinated ethenes as the primary electron acceptor. For example, the dechlorination of VC by *Dehalococcoides ethenogenes* strain 195 has been shown to be a cometabolic process, and the presence of PCE was required for VC dechlorination (Maymó-Gatell et al., 2001). Another example of cometabolic reductive dechlorination of VC that is dependent on PCE was observed within a VC-degrading enrichment culture (Distefano, 1999). The rates of VC cometabolism in methanogenic sludge are four or more orders of magnitude slower compared with the rates of VC dehalorespiration. This highlights another important difference between cometabolic reductive dechlorination reactions and dehalorespiration. Cometabolic transformations tend to be much slower than metabolic reactions. In some cases,

organisms that can dehalorespire lesser chlorinated ethenes can cometabolize more highly chlorinated ethenes. For example, *Dehalococcoides* sp. strain BAV1 can cometabolize TCE, as well as PCE, in the presence of a DCE isomer or VC, which serve as growth substrates for strain BAV1 (He et al., 2003).

2.2.4 Survey of key dehalorespiring bacteria

The first dehalorespiring bacterial species discovered was *Desulfomonile tiedjei* (DeWeerd et al., 1990). It is able to couple the reductive dehalogenation of 3-chlorobenzoate to energy conservation and hence to microbial growth. Later, a strict anaerobic culture, *Dehalobacter restricus*, capable of metabolic dechlorination of PCE coupled to the oxidation of H_2 was purified by Holliger et al. (1993). Since then, numerous dehalorespiring bacteria have been isolated and identified from very different pristine and polluted environments. The dehalorespiring bacteria currently available in pure cultures are affiliated with several distinct phyla, as shown in Figure 2.2.

Furthermore, among the dehalorespiring pure cultures, some are metabolically versatile with respect to their spectrum of electron donors. Electron acceptors other than chlorinated ethenes can be used by some dehalorespiring bacteria. The ability to use nitrate or ferric iron may prevent reductive dechlorination of DCE or VC from occurring in some natural environments due to the lower redox potential of these lesser chlorinated ethenes. In contrast, several *Dehalococcoides* and *Dehalobacter* strains are highly specialized bacteria that strictly depend on halorespiration for growth and use hydrogen as sole electron donor (Holliger et al., 1998; Maymó-Gatell

et al., 1997). The metabolic characteristics of some key dehalorespiring cultures are summarized in Table 2.2. Dehalorespiring bacteria affiliated with the *Dehalococcoides*, *Desulfitobacterium*, *Dehalobacter*, and *Desulfuromonas* genera are described in greater detail below, because representives of these genera are being used in this research.

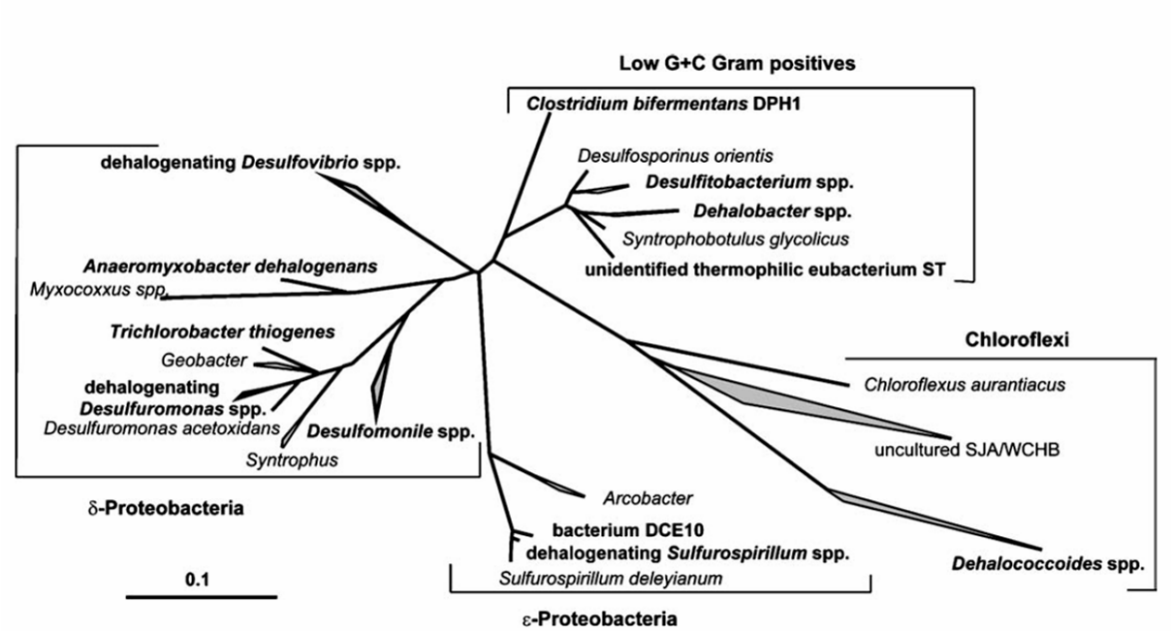

Figure 2.2 Phylogenetic tree of dehalorespiring isolates (in bold) based on bacterial SSU rRNA sequences (Smidt et al., 2000)

Table 2.2 Characteristics of key chlorinated ethene-respiring strains

Organism	Chlorinated ethene electron acceptors	Other terminal electron acceptors	Electron donors	Dechlorination products	Reference
Desulfomonile tiedjei	TCE	3-chlorobenzoate	H_2, formate, and pyruvate	*cis*-DCE	DeWeerd et al., 1990
Dehalococcoides ethenogenes strain 195	PCE, TCE, *cis*-DCE, 1,1-DCE, *trans*-DCE	1,2-dichloroethane, 1,2-dibromoethane	H_2	VC, ethene	Maymó-Gatell et al., 1997; 1999
Desulfitobacterium sp. strain PCE1	PCE	2-chlorophenol, 2,4,6-trichlorophenol, 3-chloro-4-hydroxy-phenylactate, sulfite, thiosulfate, fumarate	Lactate, pyruvate, butyrate, formate, succinate, ethanol	TCE	Gerritse et al., 1996
Desulfitobacterium strain PCE-S	PCE, TCE	Fumarate, sulfite	Pyruvate, yeast extract	*cis*-DCE	Miller et al., 1997
Dehalobacter restrictus	PCE, TCE	None	H_2	*cis*-DCE	Holliger et al., 1993
Desulfuromonas michiganensis strain BB1	PCE, TCE	Fumarate, Fe(III), S^0	Acetate, lactate, pyruvate, succinate, malate, and fumarate	*cis*-DCE	Sung et al., 2003
Desulfuromonas chloroethenica	PCE, TCE	Fumarate, Fe(III), nitriloacetate, polysulfide	Acetate, pyruvate	*cis*-DCE	Krumholz, 1997
Dehalospirillum multivorans	PCE, TCE	Fumarate, nitrate	H_2, pyruvate, lactate, ethanol, formate, glycerol	*cis*-DCE	Scholz-Muramatsu et al., 1995

As a group, *Dehalococcoides* species are able to grow by using a variety of chlorinated hydrocarbons as terminal electron acceptors, including chlorinated alkanes and alkenes, and chlorinated benzenes, biphenyls, and dioxins (Maymó-Gatell et al., 1999). *Dehalococcoides ethenogenes* strain 195 is the only dehalorespiring bacterium that has been shown to completely dechlorinate PCE to ethene, although the last step, dechlorination of VC, is a relatively slow cometabolic process (Maymó-Gatell et al., 1999**)**. However, *Dehalococcoides* strains that can respire DCE isomers or VC at high rates have been isolated. As previously mentioned, *Dehalococcoides* strain BAV1 can reductively dechlorinate DCE isomers and VC to ethene at relative high rates, but can only cometabolize PCE and TCE in the presence of DCE or VC (He et al., 2003).

Dehalococcoides strain VS is another bacterium that can metabolize DCE isomers and VC (Cupples et al., 2003; Müller et al., 2004). Unlike strain BAV1 and strain VS, strain FL2 uses only TCE and DCE as electron acceptors (He et al., 2005). PCE and VC are slowly and cometabolically dechlorinated by strain FL2. Strain GT was described recently and can dechlorinate TCE to ethene (Sung et al., 2006). To date, the ability to use DCE isomers and VC as electron acceptors has been observed only in *Dehalococcoides* species.

As their name suggests, members of the genus *Desulfitobacterium* are able to use sulfite as an electron acceptor. Some of these Gram-positive strains are also capable of dehalorespiring a variety of chlorinated hydrocarbons, including chlorinated ethenes, haloalkanes, and/or haloaromatics. *Desulfitobacterium dehalogenans* (Utkin et al., 1994; Mackiewicz and Wiegel, 1998), *Desulfitobacterium* sp. strain PCE1

(Gerritse et al., 1996), and *Desulfitobacterium* strain PCE-S (Miller et al., 1997) are able to utilize both PCE and chlorinated phenols as electron acceptors. PCE is transformed to TCE by *Desulfitobacterium dehalogenans* and strain PCE1. Trace amounts of *cis*-DCE, 1,1-DCE, or *trans*-DCE are also formed from PCE by strain PCE1. The PCE reductive dehalogenase of strain PCE-S has been purified and shown to mediate the reduction of PCE via TCE to *cis*-DCE as the end product. *Desulfitobacterium* strain Y51 can dehalorespire PCE as well as polychloroethanes (Suyama et al., 2001). Unlike the other *Desulfitobacterium* species, strain Y51 cannot utilize haloaromatic compounds as terminal electron acceptors (Furukawa et al., 2005).

Based on 16S rRNA analysis, *Dehalobacter* species are affiliated with the same group of Gram-positive bacteria as the *Desulfitobacterium* species (Figure 2.2). As previously mentioned, *Dehalobacter restrictus* is restricted to the utilization of hydrogen as an electron donor, and it can only use PCE or TCE as an electron acceptor (Holliger et al., 1998). *Dehalobacter restrictus* cannot dechlorinate chlorinated ethenes beyond *cis*-DCE and requires fermented yeast extract or growth factors (iron, thiamine, cyanocobalamin, arginine, histidine and threonine) for growth (Damborsky, 1999).

Desulfuromonas species are Gram-negative, sulfur-reducing δ-Proteobacteria (Figure 2.2). Several strains are also able to respire chlorinated ethenes. Several of the chlorinated ethene-respiring *Desulfuromonas* strains are frequently detected in anaerobic environments (Löffler et al., 2000; Sung et al., 2003), and their ability to use several organic electron donors like lactate that are added to biostimulate

reductive dechlorination at chlorinated ethene-contaminated sites and acetate, which is produced through the fermentation of lactate and other organic compounds *in-situ*, suggests that these organisms may play a significant role in *in-situ* bioremediation (Löffler et al., 2000). *Desulfuromonas chloroethenica* utilizes pyruvate or acetate as the electron donor to dehalorespire PCE and TCE to *cis*-DCE (Krumholz,1997). *Desulfuromonas michiganensis* strains BB1 and BRS1 were isolated from pristine river sediment and chlorinated ethene-contaminated aquifer material, respectively. Both *Desulfuromonas michiganensis* strains convert PCE to *cis*-DCE and can use a wide range of organic electron donors, including acetate, lactate, pyruvate, succinate, malate, and fumarate (Table 2.2, Sung et al., 2003).

2.2.5 In situ bioremediation approaches employing dehalorespiration

In-situ bioremediation based on dehalorespiration has been demonstrated to be effective for the clean-up of chlorinated ethene-contaminated sites (Aulenta et al., 2005; Lendvay et al., 2003; Major et al., 2002). Based on the degree of human involvement, *in-situ* bioremediation can be classified in two categories: natural attenuation and engineered bioremediation.

In natural attenuation, a variety of physical, chemical, and/or biological processes reduce the mass, toxicity, mobility, volume, or concentration of the contaminants in soil or groundwater without the involvement of human activities (Interstate Technology Regulatory Council, 2005). Because the sites undergoing natural attenuation are not engineered, indigenous microorganisms are utilized to degrade the contaminants under the existing geochemical conditions. In contrast, engineered

bioremediation approaches increase the desired activities of microorganisms in the subsurface environments to enhance the destruction or transformation of the contaminants.

Compared with engineered bioremediation, natural attenuation is less costly. However, the accumulation of *cis*-DCE or VC is often observed at chlorinated ethene-contaminated sites where natural attenuation are being applied (Aulenta et al., 2005; Fennell et al., 2001; Henrickson et al., 2002; Lendvay et al., 2003). This is unacceptable because of the toxicity of these daughter products. The incomplete detoxification of PCE and TCE at sties undergoing natural attenuation could be due to a number of factors including conditions that are toxic to key populations or slow kinetics of the DCE- and VC-dechlorinating populations, but it is often attributed to limitations in the availability of electron donors and/or dehalorespiring populations. Therefore, common engineered bioremediation strategies for chlorinated ethene-contaminated sites include the addition of electron donors and/or other nutrients, i.e., biostimulation, to enhance the activity of dehalorespirers and/or the introduction of the dehalorespiring populations themselves, i.e., bioaugmentation.

2.3 Kinetics of metabolic reductive dechlorination

2.3.1 Models of substrate utilization and dehalorespiring bacterial growth

2.3.1.1 Dual substrate Monod kinetics

During the dehalorespiration process, either the electron donor or acceptor or both can limit the rate of substrate utilization. Therefore, dechlorination kinetics is usually

described with the dual substrate Monod equation (Equation 2.1, Bagley, 1998; Fennell and Gossett, 1998; Haston and McCarty, 1999) according to:

$$\frac{dS_a}{dt} = -q_{\max} X \left(\frac{S_d}{K_{S,d} + S_d} \right) \left(\frac{S_a}{K_{S,a} + S_a} \right) \tag{2.1}$$

where q_{max} [$M_S \cdot M_X^{-1} \cdot T^{-1}$] is the maximum specific substrate utilization rate; X [$M_X \cdot L^{-3}$] is the biomass concentration; S_d and S_a [$M_S \cdot L^{-3}$] are the aqueous concentrations of electron donor and acceptor, respectively; and $K_{S,d}$ and $K_{S,a}$ [$M_S \cdot L^{-3}$] are the half-maximum rate constants for the electron donor and acceptor, respectively.

The net growth rate of a dehalorespiring organism during the reductive dechlorination process can be expressed according to Equation 2.2,

$$\frac{dX}{dt} = -Y \frac{dS_a}{dt} - k_d X \tag{2.2}$$

where Y [$M_X \cdot M_S^{-1}$] is the yield coefficient and k_d [T^{-1}] is the decay coefficient. It is clear that when one of the substrate concentrations is much higher than the corresponding K_S, the term $S/(K_S + S)$ approaches unity, and the specific growth rate will be controlled by the second substrate. Under these conditions, the dual Monod equation can be simplified into a single Monod equation.

2.3.1.2 Inhibition kinetics

The dual Monod model (Equation 2.1) assumes that the substrates are non-inhibitory. While the Monod model has been shown to be useful at describing the

dechlorination of chlorinated ethenes over a range of conditions (Fennell and Gossett, 1998), other studies have shown that Monod kinetics did not adequately describe chlorinated ethene removal data. For example, Tandoi et al. (1994) found that conversion of VC to ETH was inhibited by other chlorinated ethenes during the sequential reductive dechlorination of PCE by a methanol-enriched anaerobic mixed culture. When competitive inhibition coefficients of 10 μM for each of the higher chlorinated ethenes were incorporated into the dechlorination term for VC, the simulation was qualitatively similar to the experimental data. However, it is not clear whether the inhibition was due to competitive or non-competitive processes. In another study of reductive dechlorination kinetics (Garant and Lynd, 1998), competitive inhibition (K_I [$M_S \cdot L^{-3}$) was incorporated into the Monod kinetic expression for dechlorination of the chlorinated ethenes as shown for PCE in Equations 2.3,

$$\frac{dS_{PCE}}{dt} = -\frac{q_{\max} X S_{PCE}}{K_{S,PCE}\left(1 + \frac{S_{TCE}}{K_{I,TCE}} + \frac{S_{DCE}}{K_{I,DCE}} + \frac{S_{VC}}{K_{I,VC}}\right) + S_{PCE}} \tag{2.3}$$

This competitive inhibition expression as well as non-competitive Monod kinetics was fit to the dechlorination data of Tandoi et al. (1994). The competitive inhibition model fit the reductive dechlorination data better than the non-competitive model. However, the usefulness of this analysis was limited because it was assumed that the biomass remained constant during the dechlorination process. As a result, true inhibition coefficients could not be estimated and the study yielded only apparent Monod coefficients.

More recent studies included experiments designed specifically to evaluate inhibition effects and determine inhibition constants, rather than fitting mathematical models to existing data. Cupples et al. (2004) observed competitive inhibition between *cis*-DCE and VC within a *cis*-DCE- and VC-respiring culture. Inhibition coefficients of 3.6±1.1 μM and 7.8±1.5 μM were estimated for *cis*-DCE and VC, respectively, indicating that *cis*-DCE has more of an inhibitory effect on VC dechlorination than vice versa. Inhibition kinetics have also been observed for the highly chlorinated ethenes. Yu and Semprini (2004) studied the kinetics for anaerobic reductive dechlorination of high concentrations of PCE and TCE (up to their aqueous solubility limits) by two mixed cultures. Competitive inhibition was modeled by setting the K_I terms in Equation 2.3 equal to the respective K_S terms. Competitive inhibition occurred among the chlorinated ethenes when the concentrations were relatively high (~300 μM) and both competitive and self inhibition effects occurred when the concentrations were near the solubility limits (1260 μM at 30^{o}C). The more highly chlorinated ethenes strongly inhibited the reductive dechlorination of the lesser chlorinated ethenes. The lesser chlorinated ethenes caused only weak inhibition of the reductive dechlorination of PCE and TCE (Yu et al., 2005). These results are consistent with the findings of Cupples et al. (2004).

However, it is not yet known whether these inhibition effects are widely distributed among dechlorinating cultures. In addition, as noted above, there are few accurate estimates of inhibition coefficients for reductive dechlorination of chlorinated ethenes. Therefore, more information is needed on the inhibition effects

that occur at different chlorinated ethene concentrations for different dehalorespiring cultures.

In addition to competitive inhibition, self-inhibition effects have been observed when high concentrations of PCE were supplied to *Desulfuromonas michiganensis* strain BB1 and *Desulfitobacterium* sp. strain Viet1 (Amos et al., 2007). The Andrews kinetic model (Equation 2.4) predicts decreasing rates of dechlorination with increasing substrate concentration,

$$\frac{dS_{PCE}}{dt} = -q_{\max} X \frac{S_{PCE}}{K_S + S_{PCE} + \frac{S_{PCE}^2}{K_I}} \tag{2.4}$$

However, in the study by Amos et al., (2007), when PCE reached a maximum tolerance concentration ($S_{a\text{-max}}$ [Ms·L^{-3}]), dechlorination stopped completely. This could be described by a model (Equation 2.5) developed by Luong (1987),

$$\frac{dS_{PCE}}{dt} = -q_{\max} X \left(\frac{S_{PCE}}{K_{S,PCE} + S_{PCE}} \right) \left(1 - \frac{S_{PCE}}{S_{a-\max}} \right)^n \tag{2.5}$$

2.3.2 Factors affecting parameters estimates

Accurate estimates of the kinetics of dehalorespiration are needed to successfully assess and predict the bioremediation of chlorinated ethene-contaminated sites. However, as summarized in Table 2.3, there is considerable variability in the reported kinetic values for chlorinated ethene reductive dechlorination. Some of this variability may truly reflect differences in the kinetic characteristics of different dehalorespiring organisms. In addition, culture history, parameter identifiability, and the nature of the

assay employed to measure the parameters can have a major impact on microbial kinetics parameter estimation (Grady et al., 1996). These interrelated factors are described below.

2.3.2.1 Culture history

Culture history refers to "the type of environmental conditions imposed and the duration of their imposition" (Grady et al., 1996). The culture history of the inoculum used for kinetic assays can greatly influence the parameters estimated for mixed and pure cultures (Ellis et al., 1996a; Ellis and Anselm, 1999; Grady et al., 1996). There are several ways in which culture history can have effect on parameter estimates. For example, in mixed cultures maintained at low substrate concentrations, the composition of a mixed culture can be changed by selecting for members with higher affinity enzymes can occur. This selection could result in an underestimation of the K_S values, especially in mixed culture systems with long continuous-flow operation (Chiu et al., 1972). For example, in a study of the biodegradation of an aromatic compound and TCE in a mixed culture, Monod kinetic parameters obtained in batch culture assays varied up to 50% over a 5-month period using a semi-continuous culture as the source culture (Bielefeldt and Stensel, 1999). For pure cultures, a similar selection mechanism could result in the replacement of low affinity enzymes with high affinity enzymes (Dijkhuizen and Hartle, 1983). For example, the concentration of glucose in a pure culture maintained in a chemostat system decreased after the biomass concentration reached a steady state (Rutgers et al., 1987). These findings may be explained by increased substrate affinity and increased substrate utilization with increased culture age (Rutgers et al., 1987).

In addition, when batch kinetic assays are conducted under non-growth conditions, an organism's physiological state will be influenced by its culture history, as experimentally and theoretically demonstrated in several studies (Daigger and Grady, 1982; Templeton and Grady, 1988; Ellis et al., 1996a). Thus, the kinetic parameters measured under non-growth (or extant) conditions reflect the previous growth conditions used to maintain the inoculum for the batch assay. This is discussed further below.

2.3.2.2 Parameter identifiability

Parameter identifiability refers to "the ability of the mathematical routine used for parameter estimation to uniquely estimate the values of the individual parameters" (Grady et al., 1996). In other words, to be meaningful and useful, estimates of individual kinetic parameters must be independent. Previous studies have demonstrated that parameter identifiability in batch experiments depends on the initial conditions in the assays, i.e. the ratio of the initial substrate concentration (S_0) to the initial biomass concentration (X_0) or the half-saturation coefficient (K_S) (Holmberg, 1982; Robinson and Tiedje, 1983; Simkins and Alexander, 1984; Ellis et al., 1996a; Liu and Zachara, 2001; Liu et al., 2005). In other words, if the value of S_0/X_0 or S_0/K_S chosen for the kinetic assay results in a high degree of correlation between the two parameters, any combination of the two parameters that results in the same "lumped" parameter value will be fit equally well by the parameter estimation routine.

Table 2.3 Summary of Monod kinetics parameters for chlorinated ethene-respiring cultures

Organisms	Y (mg VSS/2 μmol e^-) [a]	q_{max} (μmol/ mg VSS·h) [b]		$K_{S,acceptor}$ (μM) [b]	$K_{S,donor}$ (nM)	k_d (h^{-1})	Reference
Mixed culture containing *Dhc. ethenogenes* strain 195	0.00612	PCE TCE DCE VC	1.8 3 3 3	0.54 0.54 0.54 290	H_2 100	0.001[c]	Fennell and Gossett., 1998
Mixed culture PM containing *Dhc*.-like microorganisms		PCE TCE DCE VC	0.30 (0.04) 2.84 (0.39) 0.51 (0.04) 0.06 (0.01)	3.9 (1.4) 2.8 (0.3) 1.9 (0.5) 602 (7)	N/A		Yu et al., 2005
Mixed culture EV containing *Dhc*.-like microorganisms		PCE TCE DCE VC	0.29 (0.02) 2.87 (0.32) 0.32 (0.03) 0.19 (0.02)	1.6 (0.2) 1.8 (0.4) 1.8 (0.3) 62.6 (2.4)	N/A		Yu et al., 2005
Mixed culture containing *Dhc*. sp. strain VS		PCE TCE DCE VC	0.08 (0.01) 0.07 (0.01) 0.015 (0.003) 0.014 (0.003)	0.11 (0.04) 1.4 (0.9) 3.3 (2.2) 2.6 (1.9)	N/A		Haston and McCarty, 1999
Bachman culture containing *Dhc*. sp.		*c*-DCE *t*-DCE VC	2.27 (0.15) 0.96 (0.03) 1.09 (0.03)[b]	8.9 (0.4) 8.5 (0.3) 5.8 (0.4)	N/A		He et al., 2003a
Dhc.sp. strain VS	0.0082	DCE VC	1.9 1.9	3.3 (2.2) 2.6 (1.9)	H_2 7±2	0.0038	Cupples et al., 2003; 2004
Dhb. restrictus	0.0035	PCE	7.3				Holliger et al., 1998
Dsf. frappieri TCE1	PCE 0.0067 TCE 0.0078	PCE	18.1	N/A	N/A	N/A	Gerritse et al., 1999
Dsf. sp. strain PCE1	0.0076	PCE	4.62 to 10.23	N/A	N/A		Gerritse et al., 1999
Dfm.michiganensis strain BB1		PCE TCE	5.37 (0.09) 13.96 (1.53)	14.2 (2.1) 23.4 (5.2)	N/A		Amos et al., 2007

[a] Where necessary, Y values given in terms of protein were converted to VSS by assuming protein is 55% of total biomass. [b] Values in parenthesis represent 95% confidence region. [c] Expressed as kX, in which X is not determined. [d] N/A=not available

Sensitivity analyses can be used to evaluate the degree of correlation between Monod kinetic parameter estimates. For example, Robinson and Tiedje (1983) used sensitivity coefficients, which are the first derivatives of substrate concentration with respect to the Monod parameters (dS/dK_S, dS/dq_{max}, and dS/dY), to study the effects of the S_0/K_S ratio on Monod parameter correlation and identifiability. They found that poor estimates of Monod parameters will be obtained when the initial substrate concentration is very low (S_0/X_0=0.1 and S_0/K_S=0.02) or very high (S_0/X_0=250 and S_0/K_S=50). The least correlation was found for S_0/X_0=20 and S_0/K_S=4. Using a similar method, Ellis et al. (1996a) studied the degradation of organic chemicals by activated sludge and indicated that the parameters were identifiable when S_0/K_S was higher than 1.0.

2.3.2.3 Kinetic assays

Finally, the nature of the kinetic assays themselves contributes to the variability in parameter estimates. Batch culture assays are typically used for kinetic estimates (Grady et al., 1999). However, as discussed above, the outcomes of the experiments will be significantly influenced by the initial conditions of batch cultures. Therefore, it is very important to choose appropriate initial conditions (S_0/X_0 and S_0/K_S ratios) in order to achieve the desired objectives. For example, to determine kinetics that reflect conditions in the source environment, S_0/X_0 or S_0/K_S should be very small so that the physiological characteristics of the cells do not change during the batch experiments. Estimated parameters that represent the kinetics of the culture in the source environment are referred to as “extant”. Previous studies suggest that when the

limiting substrate is the electron donor, extant kinetics can be obtained when the value of S_0/X_0 is lower than 0.025, when both S_0 and X_0 are expressed on a chemical oxygen demand (COD) basis. Extant kinetics are particularly useful for describing substrate removal in a reactor or predicting performance in some other treatment environment (Grady et al., 1999). For example, in order to study the biodegradation kinetics of organic compounds in fed-batch or continuous flow reactors for real full scale applications, low S_0/X_0 values were used for batch culture assays, under which the characteristics of source cultures were not changed (Ellis et al., 1996b; Hu et al., 2005; Noutsopoulos et al., 2006; Sahinkaya and Dilek, 2007).

On the other hand, if the objective is to determine the maximum metabolic capabilities of a culture, very large values of S_0/X_0 and S_0/K_S should be chosen. The physiological changes that must occur to allow an organisms' activity to increase until it reaches the maximum rate can only occur in a batch assay if S_0/X_0 is high enough to allow several cell divisions to occur and S_0 is greater than K_S (Grady et al., 1996). Specifically, if the value of S_0/X_0 is higher than 20 (on a COD basis), bacteria can grow unrestricted and the parameters should have good identifiability. The kinetic parameters measured under these conditions are referred as "intrinsic", because theoretically they are not influenced by culture history and represent the inherent nature of the bacteria. Generally, intrinsic parameters are most useful for comparing the biodegradability of organic compounds (Grady et al., 1999).

To evaluate the impact of different kinetic assay methods on Monod kinetic estimates, Stasinakis et al. (2003) compared q_{max} values obtained under high S_0/X_0 ratio (=20) and low S_0/X_0 ratio (=1.5) conditions. They found that the maximum

substrate utilization rate estimates obtained at high S_0/X_0 ratios were higher than those obtained at low S_0/X_0 ratios. Further, the difference in the kinetic estimates obtained at high and low S_0/X_0 ratios decreased as the SRT of the continuous flow reactor from which the inoculum for batch assays was obtained decreased, which highlights the conditions under which culture history has an impact on kinetic parameter estimates. At low SRTs in the source culture, the cells were already growing at a high rate. Thus, increasing S_0/X_0 in the batch assay had relatively little impact on the rate of biodegradation.

Unfortunately, most reports of kinetic parameter estimates do not describe the initial experimental conditions, which make the significance of these estimates unclear. Consequently it is difficult to utilize these estimates in bioremediation applications and model simulations.

2.3.3 Previous work on estimating dehalorespiration kinetics

A variety of methods have been used to estimate the Monod kinetic parameters for various dehalorespiring cultures, as summarized in Table 2.3. Batch cultures have been widely used in the dehalorespiration kinetic assays. In some cases, parameter estimates were obtained in batch assays by fitting single substrate batch depletion curves to the Monod equation (Cupples et al., 2003; Smatlak and Gossett, 1996). In other cases, multiple batch cultures with different initial substrate concentrations were prepared and employed to estimate Monod kinetic parameters (Haston and McCarty, 1999; Yu et al., 2004). In this method, the initial dechlorination rate is measured before substantial biomass growth occurs and is plotted as a function of substrate

concentration. Chemostat cultures can also be used to estimate kinetic parameters, and this method was used to estimate Monod kinetic parameters for several *Desulfitobacterium* strains under PCE-limiting conditions (Gerritse et al., 1999).

The kinetics of reductive dechlorination are of central importance in this research project due to their role in determining the outcome of competition between dehalorespiring populations for growth substrates. For example, compared with heterotrophic dehalorespirers, dehalorespirers that use H_2 as the ultimate electron donor for dehalorespiration generally have higher yield coefficients. However, organic compounds, such as acetate (8 electron eq per mol) and lactate (12 electron eq per mol) can provide more electrons per mol than H_2 (2 electron eq per mol). Thus, organic electron donors should theoretically have greater reductive dechlorination potential than H_2 on a mol basis.

The reported maximum specific substrate utilization rates for chlorinated ethenes by *Dehalococcoides* species are 3 μmol/mg VSS·h or lower. The maximum specific substrate utilization rates previously reported for PCE-to-*cis*-DCE- or (PCE-to-TCE)-respiring organisms are one or two magnitude higher than those of *Dehalococcoides* cultures. Considering that the yield coefficients of these PCE-to-*cis*-DCE-dehalorespirers are only slightly lower than those of *Dehalococcoides* species, it follows that the former group also has higher maximum specific growth rates than the *Dehalococcoides* cultures. This is important because it suggests the PCE-to-*c*DCE dehalorespirers might outcompete *Dehalococcoides* species for more chlorinated ethenes, i.e., PCE and TCE at contaminated sites and result in accumulation of *cis*-DCE under electron donor limiting conditions (Becker, 2006).

It should be noted that there is considerable variability in the parameter estimates. For examples, for mixed cultures containing *Dehalococcoides* species, the maximum specific substrate utilization rates for PCE, TCE, DCE, and VC ranged from 0.08 to 1.8 μmol/ mg VSS·h for PCE. The variability in reported values may result from the differences in the *Dehalococcoides* species or other populations in the mixed cultures. As discussed above, the kinetic parameter estimates may also be influenced by the initial conditions of the batch cultures that were used in the kinetic assays (Grady et al., 1996).

Because of uncertainties regarding the initial conditions used in these assays and the variabilities in the estimated values because of differences in methodologies and initial conditions, a consistent set of estimated values must be obtained for the dehalorespiring cultures. This information is needed for accurate description and prediction of the concentrations of chlorinated ethenes and dehalorespiring populations at contaminated sites.

2.4 Competition for limiting substrates in mixed cultures

2.4.1 Limiting substrates in the reductive dechlorination process

The electron donor, the electron acceptor, or both substrates can limit the dehalorespiration process according to Equation 2.1. There is no systematic approach for determining whether the electron donor, acceptor, or both substrates limit a biological process. However, Grady et al. (1999) noted that when the dimensionless substrate concentration (S/K_S) of one substrate is in stoichiometric excess compared

to the dimensionless concentration of the other substrate, then the substrate with the smaller dimensionless concentration is limiting.

Under natural conditions, subsurface environments are generally oligotrophic. Thus, the availability of electron donors is often thought to limit *in situ* bioremediation of chlorinated ethene contamination (McCarty and Semprini, 1994; Gossett and Zinder, 1996; Vogel et al., 1994). However, when engineered bioremediation is applied to contaminated sites, electron donors are frequently added in excess compared with electron acceptors. In this type of situation, the electron acceptor(s) will limit the dehalorespiration process.

As discussed below, competition between dechlorinators and other groups of hydrogenotrophic microorganisms, such as methanogens for H_2 has been shown in numerous studies to affect the *in-situ* bioremediation of chlorinated ethene contamination (Fennell et al., 1997; Fennell and Gossett, 1998; Löffler et al., 1999; McCarty, 1997; Smatlak et al., 1996; Yang and McCarty, 1998). However, systematic experimental evaluation of competition among dehalorespiring cultures for electron donors and acceptors has not yet been completed, although Grostern and Edwards (2006) observed competition between *Dehalobacter* and *Dehalococcoides* for electron donors and Becker (2006) used mathematical modeling to theoretically evaluate the competition for electron donors and/or acceptors among two dehalorespirers in three scenarios (as discussed below).

2.4.2 Distribution of chlorinated ethene dechlorinators in environments

Microorganisms capable of dehalorespiring chlorinated ethenes are assumed to be widely distributed in the environment because numerous dehalorespiring microorganisms have been detected and isolated from pristine and chlorinated ethene-contaminated sites, and reductive dechlorination of chlorinated ethenes is frequently observed in subsurface environments (Bradley, 2003). Currently, most methods used to detect and quantify dehalorespiring populations rely on molecular techniques including 16S rRNA gene-based polymerase chain reaction (PCR) amplification and fluorescent in situ hybridization (FISH) (Fennell et al., 2001; Hendrickson et al., 2002; Yang and Zeyer, 2003; Aulenta et al. 2004). In addition, quantification of the activity of specific dehalogenase genes is increasingly being used to accurately assess the dechlorinating potential at the contaminated sites (Holmes et al., 2006; Lee et al., 2006).

Because they are the only microorganisms known to dehalorespire beyond DCE and VC, studies of indigenous dehalorespirers have focused on characterizing the distribution of *Dehaococcoides* strains. For example, Hendrickson and coworkers (2002) tested samples from 24 chlorinated ethene-contaminated sites for the presence of *Dehalococcoides*-like 16S rRNA sequences. The detection of *Dehalococcoides*-like sequences at contaminated sites where complete dechlorination of PCE and TCE occurred, and their absence at sites where dechlorination stopped at the level of *cis*-DCE, suggests that *Dehalococcoides* strains play an important role in the natural attenuation of chlorinated ethenes (i.e., dechlorination of the lesser chlorinated ethenes) and are promising candidates for use in engineered bioremediation

approaches, especially bioaugmentation (Löffler et al., 2000; Fennell et al., 2001; Hendrickson et al., 2002; Richardson et al., 2002).

However, in other studies the accumulation of *cis*-DCE or VC was observed at contaminated sites where *Dehalococcoides* species were detected indicating that the presence of *Dehaococcoides* species does not guarantee complete dechlorination of PCE (Daprato et al., 2007; Fennell et al., 1997; 2001; Lendvay et al., 2003; Lu et al., 2006; Macbeth et al., 2004; Zheng et al., 2001). In fact, a growing number of field and enrichment culture studies have demonstrated that, dehalorespirers that cannot dehalorespire beyond *cis*-DCE may be present together with *Dehaococcoides* species. For example, *Desulfuromonas michiganensis* strain BRS1 was isolated from a PCE-to-ethene-dechlorinating consortium that also contained *Dehalococcoides* species and was derived from a PCE-contaminated site (Bechman Road Residential Wells Site) (Lendvay et al., 2003). It is possible that the *Desulfuromonas michiganensis* strain grew primarily on non-chlorinated electron acceptors (Table 2.3) in the contaminated aquifer.

On the other hand, PCE-to-*c*DCE dechlorinators like *Desulfuromonas michiganensis* tend to have faster substrate utilization kinetics than the known *Dehalococcoides* strains. Becker (2006) showed theoretically that, when sufficient electron donor is provided, the high substrate utilization rates of PCE-to-TCE or PCE-to-*cis*-DCE dehalorespirers allow them to compete successfully for PCE and TCE, while *Dehalococcoides* strains use the remaining electron donor to dechlorinate the lesser chlorinated ethenes (*cis*-DCE and VC). Thus, it is possible that the *Desulfuromonas michiganensis* and *Dehalococcoides* strains coexisted at the

Bachman Road Residential Wells Site by functioning as PCE-to-*c*DCE and *c*DCE-to-ethene dechlorinating specialists, respectively. In another study, *Desulfitobacterium*-like dehalorespirers were detected in an anaerobic culture that also contained *Dehalobacter* strains and was obtained from a PCE-contaminated site where complete PCE dechlorination was observed (Yang et al., 2005). The analysis provided by Becker (2006) again suggests that specialization by two dechlorinating populations could explain their coexistence with the *Desulfitobacterium* strains dechlorinating at least a portion of the PCE to *cis*-DCE and the *Dehalococcoides* strain "cleaning up" the lesser chlorinated ethenes.

Dehalobacter species, like *Dehalococcoides* strains, are restricted to the use of H_2 as the electron donor. Thus these two species will compete for the electron donor, and possibly electron acceptors, if they are present together. *Dehalobacter* species were detected with *Dehalococcoides* species in an anaerobic methanogenic consortium that can completely dechlorinate PCE into ethene (Da Silva et al., 2006; Zheng et al., 2001). In this mixed dehalorespiring culture, the concentration of *Dehalobacter* species is three times that of *Dehalococcoides* species, based on quantification of 16S rRNA gene copies. Because *Dehalobacter* species can only use PCE and TCE as electron acceptors, it is likely that *Dehalococcoides* strains are growing primarily on DCE and possibly VC. Similar findings were obtained by Daprato et al. (2007), who examined three dechlorinating consortia that were enriched with PCE and methanol. In all of the consortia, the concentrations of gene copies of the *Dehalobacter* species were higher than the *Dehalococcoides* species. Interestingly, the consortium with the highest concentration of *Dehalococcoides* had the lowest dechlorination rate of PCE,

which implies the concentration of *Dehalobacter* might determine the dechlorination rate.

In some cases, more than one PCE-to-*cis*-DCE respirer has been found with *Dehalococcoides* in mixed cultures or at contaminated sites. For example, in a sand column reactor that was inoculated with the mixed culture used by Da Silva et al. (2006), *Sulfurospirillum* and *Desulfuromonas* species were detected. These genera contain both dehalorespiring and non-dehalorespiring members. Because these species were not detected in the source culture, one explanation is that the sand used for the column reactor contained these organisms. At a TCE-contaminated site, where TCE was completely dechlorinated only after the addition of lactate, two proteobacterial phylotypes most similar to *Trichlorobacter thiogenes* and *Sulfurospirillum multivorans*, which are known dechlorinators, were detected along with a *Dehalococcoides*-like phylotype (Macbeth et al., 2004). The *Dehalococcoides* strain did not dominate the consortium suggesting that other organisms play an important role in the detoxification of TCE. One possibility is that the *Dehalococcoides* strain was responsible for dehalorespiring *cis*-DCE or VC produced from the dechlorination of TCE by the other dehalorespirers.

A number of diverse species that could potentially carry out dehalorespiration were found in a PCE-contaminated aquifer in Germany (Nijenhuis et al., 2007). Potential dechlorinating organisms including *Dehalococcoides*, *Desulfuromonas*, *Desulfitobacterium* and *Dehalobacter* were demonstrated to be present. Because the sequences of *Dehalococcoides* derived in the field were most closely related to *Dehalococcoides* sp. BAV1, which is capable of dechlorination of DCE and VC (He

et al., 2003), the other dehalorespirers were considered to be responsible for transformation of PCE to *cis*-DCE.

2.4.3 Electron donor thresholds

Competition for H_2 between dehalorespirers and other hydrogenotrophic microorganisms, such as methanogens, homoacetogens, and denitrifiers, has been studied in detail (Fennell et al., 1997; Fennell and Gossett, 1998; Smatlak et al., 1996; Yang and McCarty, 1998). These studies indicated that low H_2 concentrations (2-11 nM) favor growth of dehalorespirers over other hydrogenotrophic organisms, because dehalorespirers can consume H_2 to a lower minimum concentration, i.e., threshold, than the other hydrogenotrophic organisms (Löffler et al., 1999). Reported H_2 thresholds and changes in the standard Gibbs free energy ($\Delta G^{o\prime}$) for several common hydrogen-consuming anaerobic terminal electron accepting processes (TEAPs) are shown in Table 2.4. In general, as the amount of free energy released from a TEAP increases, the H_2 threshold decreases. Dehalorespiration is a highly favorable thermodynamic process, and dehalorespiring bacteria appear to have lower H_2 threshold concentration than acetogens, methanogens, and sulfate reducers, which carry out less favorable forms of anaerobic metabolism. When H_2 concentrations are limiting growth and become low, dehalorespirers may be able to decrease the concentration of H_2 below the thresholds of other populations. When the H_2 concentration drops below an organism's threshold, it will no longer be able to take up H_2, resulting in net decay. This can be understood by recognizing that H_2 thresholds are related to the concept of S_{min} [$M \cdot L^{-3}$], the substrate concentration below which biomass cannot be maintained at steady-state, according to (Rittmann, 1987),

$$S_{\min} = \frac{K_S k_d}{Y q_{\max} - k_d} \tag{2.6}$$

The factors controlling thresholds are probably more complex, but Equation 2.6 helps explain why $\Delta G^{o\prime}$ and the associated threshold concentration are inversely related. As $\Delta G^{o\prime}$ increases, Y increases, and S_{min} (or the threshold concentration) decreases. However, it is also clear from Equation 2.6 that the kinetic characteristics (K_S and q_{max}) of an organism can also affect its threshold. Therefore, while the thresholds of different dehalorespirers are expected to fall within a similar range of values, there might be some differences due to variations in their kinetic characteristics.

The phenomenon of the thresholds is not limited to H_2. Thresholds have been observed for other electron donors, including acetate, in dehalorespirers and other groups (He and Sanford, 2004; Sung et al., 2006). Dehalorespiration has a lower threshold for acetate compared with methanogenesis, as shown in Table 2.4. However, threshold concentrations have not been reported for heterotrophic chlorinated ethene-respirers.

Table 2.4 Electron donor thresholds for different terminal electron accepting processes

Electron donor	TEAPs	Threshold concentration (nM)	$\Delta G^{o\prime}$ (kJ/mol H_2)	$E^{o\prime}$ (V)	Reference
H_2	Acetogenesis	336-3640	-26.1	-0.28	Breznak, 1994; Cord-Ruwisch et al., 1988
	Methanogenesis	5-95	-33.9	-0.24	Conrad, 1996; Cord-Ruwisch et al., 1988; Lovley, 1985; Lovley and Goodwin, 1988
	Sulfate reduction ($SO_4^{2-} \rightarrow HS^-$)	1-15	-38.0	-0.22	Conrad, 1996; Cord-Ruwisch et al., 1988; Lovley and Goodwin, 1988
	Fumarate reduction	0.015	-86.2	+0.032	Cord-Ruwisch et al., 1988
	Ammonification ($NO_3^- \rightarrow NH_4^+$)	0.015-0.025	-149.9	+0.36	Conrad, 1996; Cord-Ruwisch et al., 1988
	Denitrification ($NO_3^- \rightarrow N_2O$, N_2)	<0.05	-240	+0.79	Lovley and Goodwin, 1988; Lovley et al., 1994
	Fe(III) reduction	0.1-0.8	-228.3	+0.77	Conrad, 1996; Lovley and Goodwin, 1988; Lovley et al., 1994
	Dehalorespiration	<0.3	-130 to -187	+0.30 to +0.55	Loffler et al., 1999
Acetate	Methanogenesis	7,000–69,000			Jetten et al., 1990
	Dehalorespiration	3-69			He and Sanford, 2004; Sung et al., 2006
	Denitrification ($NO_3^- \rightarrow N_2O$, N_2)	3.6			Sung et al., 2006
	Fe(III) reduction	1.2-19			He and Sanford, 2004; Sung et al., 2006

Threshold concentrations are expected to affect competition for electron donors only when they are limiting and present at low concentrations. This is likely to occur

at contaminated sites undergoing natural attenuation or some distance downgradient from electron donor delivery locations at sites where biostimulation is being applied. In laboratory systems, thresholds are most important in batch reactors in which the concentration of the limiting substrate can decrease until the threshold is reached or in continuous-flow systems operated with extremely long solid retention times. Under these conditions, differences in threshold concentrations may affect the outcome of competition for electron donors between different groups of organisms. The effect of thresholds on kinetics can be incorporated by including a threshold term into the Monod equation according to Equation 2.7,

$$\frac{dS_a}{dt} = -q_{\max} X \left(\frac{S_d - S_{d,threshold}}{K_{S,d} + S_d - S_{d,threshold}} \right) \left(\frac{S_a}{K_{S,a} + S_a} \right) \tag{2.7}$$

where $S_{d,threshold}$ is the electron donor threshold concentration for the dehalorespirer. Clearly when the aqueous concentration of the electron donor is lower than the threshold, no dechlorination will occur.

2.4.4 Competition in batch culture

In batch cultures, the outcome of competition between two organisms is primarily determined by the magnitude of q_{max} and K_S for each species. When the two species compete for the same limiting substrate, each organism will grow at its maximum rate if the limiting substrate concentration is much higher than the K_S value until the limiting substrate is exhausted (Pirt, 1975). The organism with the higher q_{max} will reach a higher biomass concentration, but will not eliminate the other population from the batch culture. If the limiting substrate concentration is low compared with K_S,

then the growth of both cultures will be lower than the maximum possible rate. The ratio of q_{max}/K_S will determine which organism will be dominant in the mixed culture system.

As discussed in the previous section, if the limiting substrate is electron donor, the outcome of the competition in batch cultures will also be influenced by the threshold concentrations of the competing organisms. For example, dehalorespirers can outcompete other hydrogenotrophic organisms in batch cultures under low H_2 concentrations because they have lower H_2 thresholds (Ballapragada et al., 1997; Fennell et al., 1997; Fennell and Gossett, 1998; Smatlak et al., 1996; Yang and McCarty, 1998).

In batch cultures, the feeding interval can also have a big impact on the outcome of competition between two populations. For example, model simulation of competition for substrates between *Dehalobacter restrictus* and *Dehalococcoides ethenogenes* (Figure 2.3A) showed that the two populations were present at approximately the same concentration at the end of the simulation when the feeding interval was 48 hours (Becker, unpublished data). However, when a feeding interval of 12 hours was used, *Dehalobacter restrictus*, which have high q_{max} and lower K_S values, outcompeted *Dehalococcoides ethenogenes* (as shown in Figure 2.3B). The explanation for the different outcome is most likely the amount of time each organism spends in lag phase under the different feeding regimens. Because *Dehalobacter restrictus* can only utilize PCE and TCE as electron acceptors, whereas *Dehalococcoides ethenogenes* can also respire *cis*-DCE, *Dehalobacter restrictus* spends much more time in lag phase compared with *Dehalococcoides ethenogenes*

when the 48 h feeding interval was used. When a 12 h feeding interval is used, neither population spends much time under substrate depleted conditions, so this effect is minimized.

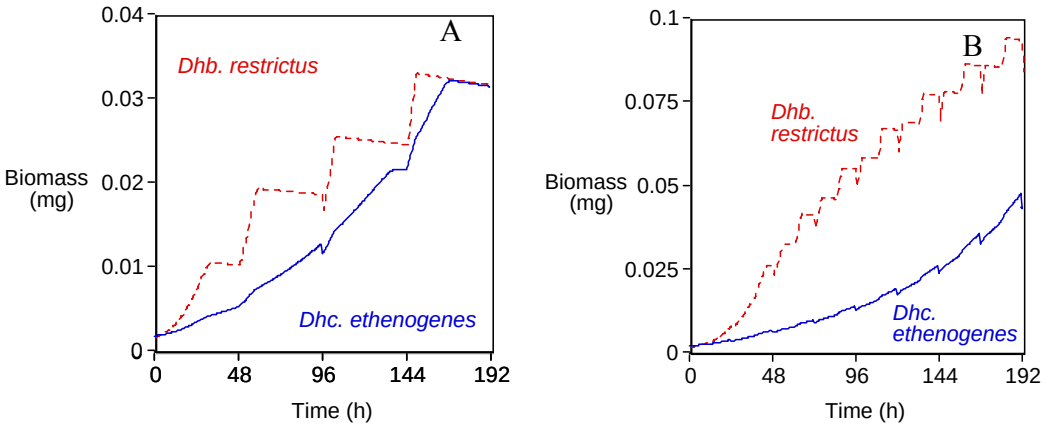

Figure 2.3 Competition between *Dehalobacter restrictus* and *Dehalococcoides ethenogenes* in semi-continuous cultures with (A) 48 hour feeding interval, and (B) 12 hour feeding interval.

2.4.5 Competition in chemostat culture

In batch cultures, substrates become limiting only at the end of a feeding cycle. In contrast, in chemostats, growth in generally limited continuously by substrate availability. The limitation of substrate concentrations on growth make chemostat cultures ideally suited for studying competition between dehalorespirers (Pirt, 1975). In chemostat cultures, the substrate concentrations are not only controlled the by the utilization kinetics, but also by the dilution rate (D, where $D=Q/V$). If D is faster than the rate an organism can grow, which is expressed by μ, then the organism will be washed out. When two species grow on the same limiting substrate, the relationships between S and μ for the two populations generally follow one of the patterns shown in

Figure 2.4. If there is an intersection between the μ-S curves of the two species, as shown in Figure 2.4B, then the dilution rate will determine which population is dominant. S_X is the substrate concentration that results in the same specific growth rate (μ_x) for both populations (Figure 2.4). If the chemostat reactor can be maintained at the critical dilution rate (D_X) equal to μ_x, a steady-state between the two species can be established. For the two populations represented in Figure 2.4, at all $D < \mu_x$, population B will be dominant, and population A will be dominant at all $D > \mu_x$. However, if there is no intersection between the μ-S curves of the two species, as shown in Figure 2.4A, then the species with higher μ_{max} and lower K_S will outcompete the other population. Thus, in this example population a will outcompete population b.

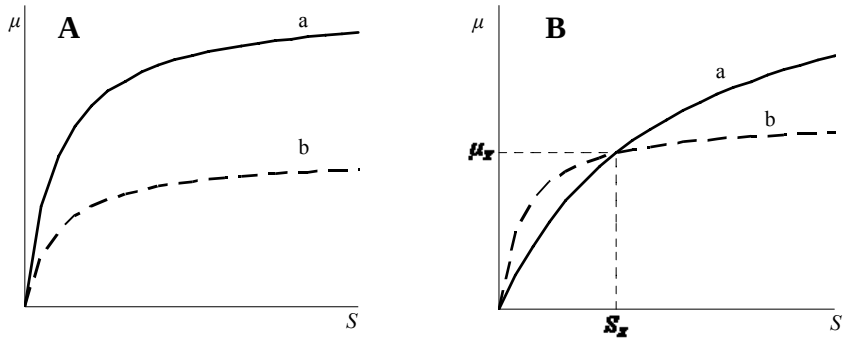

Figure 2.4 The possible relations between the specific growth rate (μ) and growth-limiting substrate concentration (S) of two species (a) and (b): A. without intersection, B. with intersection.

As mentioned above, two populations that compete for the same limiting substrate can be maintained by operating a chemostat reactor at their critical dilution rate. In

addition, two populations can be maintained in a chemostat reactor if one population can grow on the product(s) of the other population. For example, if a *Dehalococcoides* species cannot outcompete a PCE-to-*c*DCE respiring population for PCE and TCE in a chemostat because of its lower q_{max} value (Table 2.3), it may be possible for it to grow by utilizing *cis*-DCE and VC, which are daughter products of PCE, as electron acceptors. In this case, it may be possible to maintain both populations at a steady state (Pirt, 1975). Becker (2006) demonstrated this phenomenon by using mathematical simulations, in which populations of *Dehalococcoides ethenogenes* compete with either *Desulfuromonas michiganensis* strain BB1 or *Desulfitobacterium* sp. strain PCE1 under engineered bioremediation conditions, i.e., electron acceptor-limiting conditions. The model simulation predicted that *Desulfuromonas michiganensis* strain BB1 or *Desulfitobacterium* sp. strain PCE1 could be sustained by respiring most of the PCE and, in the case of *Desulfuromonas michiganensis* strain BB1, most of the TCE. *Dehalococcoides ethenogenes* was sustained on *cis*-DCE. Similar results were found by Grostern and Edwards (2006) experimentally using a mixed 1,1,2-TCA-degrading culture that contained *Dehalobacter* and *Dehalococcoides* species. In the experiments, the *Dehalobacter* strain grew by dechlorinating 1,1,2-TCA to VC only and *Dehalococcoides* grew only on the dechlorination of VC to ethene. If both hydrogenotrophic and heterotrophic dehalorespiring populations can be maintained in mixed cultures, it makes it possible to take advantage of the reducing equivalents available in the form of both H_2 and the organic acids produced from the fermentation of organic electron donors added to biostimulate reductive dechlorination. Therefore, from a bioremediation standpoint,

maintenance of multiple dehalorespiring populations appears ideal because it may result in the fastest and most extensive dechlorination.

2.5 Summary and conclusion

As reviewed above in this chapter, chlorinated ethenes are ubiquitous contaminants in soil and groundwater. Many studies have suggested that *in-situ* bioremediation based on anaerobic reductive dechlorination is an efficient technique to cleanup chlorinated ethene contamination in subsurface environments (Aulenta et al., 2005; Lendvay et al., 2003; Major et al., 2002). However, incomplete dechlorination of PCE or TCE which results in accumulation of *cis*-DCE and VC is frequently observed at contaminated sites (Major et al. 2002). There may be several reasons for the incomplete dechlorination of PCE, including the absence of appropriate dechlorinating populations and insufficient electron donors (Fennell et al., 2001).

So far, *Dehalococcoides* species are the only pure cultures that can dehalorespire beyond *cis*-DCE. They were found at chlorinated ethene-contaminated sites where complete dechlorination occurs. However, the detection of *Dehalococcoides* species does not guarantee complete dechlorination of chlorinated ethenes, in part because it may be difficult for *Dehalococcoides* strains to compete with other dehalorespirers (Becker, 2006) or hydrogenotrophic and acetotrophic populations because of unfavored conditions for *Dehalococcoides* strains (Fennell et al, 1997). This is important because all these microorganisms have been shown to be present in subsurface environments where chlorinated ethene contamination occurs. For

example, Smatlak et al. (1996) studied effect of hydrogen concentration on dechlorination of PCE in a methanogenic consortium and showed that the half-velocity constant for dechlorination of PCE is much lower than that of methanogenesis. Thus dehalorespirers can theoretically out-compete methanogens for hydrogen utilization under low hydrogen concentrations. This conclusion was also confirmed by experimental studies of Ballapragada et al. (1997) and modeling work of Fennell and Gossett (1998).

Heterotrophic dehalorespirers generally have higher specific dechlorination rates than *Dehalococcoides* species. Because chlorinated ethene-respiring bacteria differ with respect to the rate and extent of dechlorination, competition among them for electron donors and acceptors can have a major impact on the fate of chlorinated ethenes in *in-situ* bioremediation. Therefore, understanding the competition among dehalorespiring bacteria is key to predicting the outcome of bioremediation and the development of bioremediation strategies. Unfortunately, the lack of a consistent set of dehalorespiration kinetic parameters currently limits our ability to advance our progress in this area Therefore, it is necessary to estimate meaningful dehalorespiration kinetic parameters under the same, defined sets of conditions, i.e. use these values to mathematically predict the outcome of competition among dehalorespiring populations, and validate these modeling predictions in bench-scale competition experiments.

Chapter 3: Intrinsic Monod kinetics and their implications in model predictions for two heterotrophic tetrachloroethene-respiring strains

3.1 Introduction

Several bacterial strains, including members of the genus *Dehalococcoides* (Cupples et al. 2003; He et al. 2003b; 1997; Sung et al. 2006b), use H_2 as an electron donor in the dehalorespiration of tetrachloroethene (PCE) and trichloroethene (TCE). Other dehalorespirers, such as *Desulfitobacterium* (Gerritse et al. 1999; Gerritse et al. 1996; Miller et al. 1997; Suyama et al. 2001) and *Desulfuromonas* (Krumholz 1997; Sung et al. 2003) strains can use a range of organic electron donors, but not H_2. To predict the activities of these organisms and their effects on contaminant fate in groundwater systems using mathematical models, accurate and meaningful kinetic parameter estimates are needed. Relatively few kinetic parameter estimates have been reported in the literature. Several studies described kinetics of mixed cultures (Haston and McCarty 1999; Smatlak et al. 1996; Yu et al. 2005; Yu and Semprini 2004). However, interpretation of the kinetics of mixed cultures may be complicated by the presence of multiple dehalorespiring cultures. Modeling (Becker 2006) and experimental studies (Bunge et al. 2007; Yang et al. 2005) have shown that under some conditions, biostimulation with an organic electron donor like lactate or benzoate may sustain heterotrophic dehalorespirers as well as *Dehalococcoides* strains. Thus, chlorinated ethene removal kinetics measured with mixed cultures may not reflect the activities of individual dehalorespiring populations, which likely

function as PCE-to-TCE or dichloroethene (DCE)-dechlorinating specialists under some conditions (Becker 2006; Bunge et al. 2007). Under other circumstances, multiple dehalorespiring populations may compete for limiting amounts of the chlorinated ethenes and/or electron donor(s). Thus, good kinetic parameter estimates are particularly important for understanding whether the activities of multiple dehalorespiring populations complement each other or negatively impact contaminant fate.

The reported chlorinated ethene utilization kinetics for pure dehalorespiring cultures vary considerably. Some of the differences in the parameter estimates may reflect true differences in the inherent kinetic characteristics of different organisms. However, several interrelated factors pertaining to the method used to culture the organisms and determine the kinetic parameters including culture history and parameter correlation, can also have a major impact on the estimated values (Grady et al. 1996). When batch culture assays are used, the influence of these factors in the parameter estimates is related to the initial experimental conditions. The ratio of the initial substrate concentration (S_0) [$M_S\ L^{-3}$] to the initial biomass concentration (X_0) [$M_X\ L^{-3}$] is important because it determines how much culture history affects parameter estimates. At very low S_0/X_0 ratios, the cells cannot grow in the batch assay. Thus, the parameters estimated under these conditions reflect the kinetics in the source environment (e.g., a bioreactor or groundwater aquifer) where biodegradation activity may have been restricted by substrate availability. Because culture history controls the kinetic parameter estimated under non-growth or "extant" conditions, they generally are not relevant to other systems. According to Grady et al. (1996),

extant kinetics can be estimated when S_0/X_0 is less than 0.025 for typical heterotrophs and electron donor substrates, when both S_0 and X_0 are expressed on the basis of chemical oxygen demand (COD). In contrast, if a large amount of substrate is provided relative to the biomass concentration, then the cells will be able to grow without restriction. Kinetics measured under these conditions are known as "intrinsic kinetics", and are independent of any limits on substrate availability in the source environment. It is recommended that $S_0/X_0 > 20$ (on a COD basis) be used to determine intrinsic kinetics for typical organisms growing on electron donor substrates. From a practical prospective, intrinsic kinetic parameters may be useful for comparing the inherent or ultimate biodegradation capability of microbial cultures or predicting their performance in applications involving abundant substrates. One such application is the use of biostimulation in engineered in-situ bioremediation applications.

In this study, the guidelines of Grady et al. (1996) were adapted and used to determine intrinsic and identifiable Monod kinetic parameter estimates for two heterotrophic dehalorespirers, *Desulfuromonas michiganensis* strain BB1 and *Desulfitobacterium* sp. strain PCE1, under both electron donor- and acceptor-limiting conditions. We found that the maximum specific substrate utilization rates for PCE and TCE ($q_{max,PCE}$ and $q_{max,TCE}$ [$M_S\ M_X^{-1}\ T^{-1}$]) and half-saturation constants for PCE and TCE ($K_{S,PCE}$ and $K_{S,TCE}$ [$M_S\ L^{-3}$]) estimated for the two heterotrophic strains are higher than the values reported for *Dehalococcoides* cultures. Intrinsic kinetic parameter estimates for the chlorinated ethenes could reliably be obtained at $S_0/X_0 >$ 10, but these values do not accurately describe dechlorination rates at $S_0/X_0 < 10$. This

study also suggests that determination of electron donor utilization kinetic parameters should be made independently of parameters describing dehalorespiration unless reliable estimates of $f_e°$ (the fraction of total electron donor reducing equivalents used in energy generation) are available.

3.2 Materials and Methods

3.2.1 Chemicals

PCE (99%, Spectrum Chemical Mfg. Corp.), TCE (99.9%, Fisher Scientific), and *cis*-DCE (99.9%, Supelco) were used in kinetic experiments and preparation of analytical standards. Sodium acetate (Certified ACS) was obtained from Fisher Scientific and sodium L-lactate (60% wt. solution) was obtained from Acros Organics. [1,2-^{14}C]sodium acetate ([^{14}C]acetate, 98.8%, 110 mCi/mmol) was obtained from Moravek Biochemicals and Radiochemicals (Brea, CA), and [1,2-^{14}C]sodium lactate ([^{14}C]lactate, >95%, 110 mCi/mmol) was obtained from Sigma-Aldrich Co. (St. Louis, MO).

3.2.2 Culture maintenance

Desulfuromonas michiganensis strain BB1 (DSM 15941) was grown on the low chloride medium described by Sung et al. (2003). *Desulfitobacterium* sp. strain PCE1 (DSM 10344) was grown on the basal medium described by Gerritse et al. (1996). To ensure that all cultures used for batch culture kinetics parameter determinations and competition experiments had the same culture history and were always at the same physiological state, the cultures were maintained on a semi-continuous culture basis

in 160-ml serum bottles and incubated statically at 30°C in the dark. Every 4 d: (1) the headspace of each culture was purged with O_2-free N_2 gas (Ultra pure carrier grade, Airgas East) for 15 min, to remove daughter chlorinated ethenes, followed by 5 min with O_2-free CO_2/N_2 (20%/80%, V/V, Airgas East), to equilibrate bicarbonate in the medium; and (2) 20-ml of culture was replaced with fresh media to give a solids retention time (SRT) of 20 d (dilution rate of 0.05 d^{-1}). Strain BB1 was supplied with 100 µM of acetate and 20 µmol of PCE, which results in an aqueous PCE concentration of 100 µM at 30˚C. Strain PCE1 was routinely fed with 250 µM lactate and 50 µmol of PCE, which corresponds to an aqueous PCE concentration of approximately 250 µM at 30˚C.

3.2.3 Reductive dechlorination kinetic model

At non-inhibitory chlorinated ethene concentrations, dehalorespiration of PCE can be described using a modified dual Monod model (Eq. 3.1) that includes a threshold term for the electron donor $S_{d,threshold}$ [M_S L^{-3}], which represents the concentration below which the substrate cannot be utilized (Cupples et al. 2004; Fennell and Gossett 1998).

$$\frac{dS_{PCE}}{dt} = -q_{\max,PCE} X \left(\frac{S_{PCE}}{K_{S,PCE} + S_{PCE}} \right) \left(\frac{S_d - S_{d,threshold}}{K_{S,d} + \left(S_d - S_{d,threshold} \right)} \right) \tag{3.1}$$

where S_{PCE} [M_S L^{-3}] is the aqueous concentration of PCE, X [M_X L^{-3}] is the biomass concentration, S_d [M_S L^{-3}] is the concentration of the electron donor, and $K_{S,d}$ [M_S L^{-3}] is the half-saturation constant for the electron donor.

Recent studies (Cupples et al. 2004; Yu et al. 2005; Yu and Semprini 2004) suggest that dehalorespiration of TCE may be competitively inhibited by PCE according to:

$$\frac{dS_{TCE}}{dt} = \left(\frac{q_{\max,PCE} X S_{PCE}}{K_{S,PCE} + S_{PCE}} - \frac{q_{\max,TCE} X S_{TCE}}{K_{S,TCE}\left(1 + \frac{S_{PCE}}{K_{I,PCE}}\right) + S_{TCE}} \right) \left(\frac{S_d - S_{d,threshold}}{K_{S,d} + \left(S_d - S_{d,threshold}\right)} \right) \quad (3.2)$$

where S_{TCE} [M_S L^{-3}] is the aqueous concentration of TCE, and $K_{I,PCE}$ [M_S L^{-3}] is the competitive inhibition coefficient. Growth of dechlorinating strains that respire PCE and TCE is described according to

$$\frac{dX}{dt} = \frac{q_{\max,PCE} Y X S_{PCE}}{K_{S,PCE} + S_{PCE}} + \frac{q_{\max,TCE} Y X S_{TCE}}{K_{S,TCE}\left(1 + \frac{S_{PCE}}{K_{I,PCE}}\right) + S_{TCE}} - k_d X \quad (3.3)$$

where Y [M_X M_S^{-1}] is the yield coefficient, and k_d [T^{-1}] is the decay coefficient. The second term on the right side of Equation (3) is neglected for strain PCE1, which does not respire TCE.

3.2.4 Determination of Monod kinetic parameters

Intrinsic K_S and q_{max} values for electron donors and electron acceptors were fit to single substrate depletion curves obtained using batch cultures. For determination of electron donor kinetics, an initial acetate concentration of 90 μM (5.8 mg COD/L) was supplied to 0.22 mg VSS/L (0.29 mg COD/L) of strain BB1 biomass to obtain an S_0/X_0 ratio of 20 on a COD basis, as recommended (Grady et al. 1996). PCE was

added at twice the stoichiometric requirement based on the initial acetate concentration (50 μmol of PCE or 320 μM at 30°C) to ensure it was provided in excess. At this PCE concentration, the $\left(\frac{S_{PCE}}{K_{S,PCE}+S_{PCE}}\right)$ term in Equation (3.1) approaches unity and, thus, the effects of S_{PCE} on electron donor kinetics could be neglected. For strain PCE1, the initial lactate and biomass concentrations were 100 μM (10.0 mg COD /L) and 0.35 mg/L VSS (0.5 COD mg/L), respectively. Electron donor-limiting conditions were ensured by adding 60 μmol PCE (or 384 μM at 30°C) to strain PCE1. The initial conditions used to determine electron acceptor kinetics were selected to ensure that the kinetic parameter estimates were intrinsic and independent of the electron donor concentration. For strain BB1, X_0 = 0.12 mg VSS/L (0.17 mg COD/L), $S_{0,donor}$ = 1500 μM acetate (97 mg COD/L), and $S_{0,\,PCE}$ = 120 μM (or $S_{0,TCE}$ = 200 μM). For strain PCE1, X_0 = 0.73 mg VSS/L (1.0 mg COD/L), $S_{0,donor}$ = 5000 μM lactate (486 mg COD/L), and $S_{0,PCE}$ = 336 μM. The competitive inhibition term in Equation (2) could be neglected in the determination of $q_{max,TCE}$ and $K_{S,TCE}$ for strain BB1 because PCE was not present.

Three (for electron donor-limiting conditions) or four (for electron acceptor-limiting conditions) replicate 100-mL batch cultures were used for each kinetic parameter determination experiment and maintained in 160-mL serum bottles sealed with black butyl rubber septa (Geo-Microbial Technologies Inc., Ochelata, OK). PCE (or TCE) was added to the serum bottles at least 1 d before initiating an experiment to allow it to equilibrate between the aqueous and gas phases. Semi-continuous cultures

were maintained for more than 60 d or three SRTs before being used as the inoculum in kinetic assays to ensure that that the cultures were at steady-state.

Y was independently determined by measuring the change in protein and chlorinated ethene concentrations during a finite period of exponential growth in the batch assays according to

$$Y = \frac{\Delta X}{\Delta S} \tag{3.4}$$

The estimates of *Y* made using protein analyses were confirmed by measuring 16S rRNA gene copy numbers using quantitative polymerase chain reaction amplification (qPCR). The following assumptions were made: (1) the average molecular weight for a base pair in double-stranded DNA is 660 g/mol, (2) one 16S rRNA gene operon per genome (He et al. 2003a), (3) the genome size of strain BB1 is similar to the 3.8 Mbp of *Desulfuromonas acetoxidans* (NCBI 2006), and (4) the genome size of strain PCE1 is similar to the 5.7 Mbp of *Desulfitobacterium hafniense* strain Y51 (Nonaka et al. 2006). The initial biomass concentrations (X_0) in the kinetic assays were calculated based on measured biomass concentrations in the source culture.

For each replicate, the estimates of *Y* and X_0 and the measured concentrations of the limiting substrate were fit individually to the integrated form of Monod equation,

$$t = \frac{1}{q_{\max}}\left(\frac{K_S}{YS_0 + X_0}\ln\left(\frac{\left(X_0 + YS_0 - YS\right)S_0}{X_0 S}\right) + \frac{1}{Y}\ln\left(\frac{X_0 + YS_0 - YS}{X_0}\right)\right) \tag{3.5}$$

by non-linear regression analysis (Smith et al. 1998). For determination of electron donor kinetics, the term ($S_d - S_{d,threshold}$) was substituted for S in Equation (3.5). The averages of the individual parameter estimates were calculated and are reported, unless otherwise noted.

k_d was estimated for each strain following the procedure described by Cupples et al. (2003). Briefly, an inoculum was obtained from a semi-continuous culture and incubated without any electron acceptor, i.e. under non-growing conditions, which caused the biomass concentration to decrease over time due to endogenous decay. On days 0, 1, 4, 7, 10, 13, and 16, a 2.5 ml volume of the non-growing culture was removed and divided equally among duplicate batch cultures, each of which contained 97.5 ml of fresh mineral media with PCE and excess electron donor. The initial TCE production rate, $\frac{dM_{TCE}}{dt}$ [M_S T^{-1}], was determined in each of these batch cultures within 10 h. Biomass can be considered constant during this period and thus reflects the biomass concentration in the source culture at the time of sampling (X_t). Because $\frac{dM_{TCE}}{dt}$ is proportional to X_t, k_d could be determined by fitting $\frac{dM_{TCE}}{dt}$ data to Equation (3.6),

$$\left(\frac{dM_{TCE}}{dt}\right)_t = \left(\frac{dM_{TCE}}{dt}\right)_0 e^{-k_d t} \tag{3.6}$$

where $\left(\frac{dM_{TCE}}{dt}\right)_0$ is the TCE production rate measured in the PCE-free medium on day 0.

3.2.5 Identifiability analysis

Sensitivity coefficients for the Monod kinetic parameters were calculated as the first derivatives of S, with respect to q_{max} or K_S ($\frac{dS}{dq_{\max}}$ or $\frac{dS}{dK_S}$) (Ellis et al. 1996; Grady et al. 1996; Robinson and Tiedje 1983). Correlation coefficients (R^2) for q_{max} and K_S were calculated following the method of Liu and Zachara (2001). The linear interdependence of the sensitivity functions was quantified according to the collinearity index, γ_K, which is based on the sensitivity coefficients and was calculated following the method described by Brun et al. (2002). In this study, parameter estimates with a γ_K of less than 10 were considered identifiable, following recommended guidelines (Brockmann et al. 2008; Brun et al. 2002).

3.2.6 Analytical methods

Chlorinated ethenes were analyzed using the headspace gas chromatograph technique described by Gossett (1987). Protein concentrations were analyzed using the NanoOrange protein quantitation kit from Molecular Probes Inc. (Eugene, OR) at low concentrations (<1 mg/L) or the Bradford reagent kit from Sigma-Aldrich (St. Louis, MO) at higher concentrations. Bovine serum albumin was used for protein standards, and a protein-to-volatile suspended solids (VSS) ratio of 0.55 was assumed (Neidhardt et al. 1990).

The concentrations of acetate and lactate in the batch culture assays were determined using ^{14}C-labeled compounds following the method described by He and Sanford (2004). Acetate and lactate were separated from the other components using

a high performance liquid chromatograph (HPLC) equipped with RSpak KC-811 column (8.0 mm ID×300 mm; Shodex, Milford, MA). The acetate and lactate fractions were collected using a Fraction Collector III (Waters Corporation, Milford, MA), and the ^{14}C-activity was quantified by counting samples for 10 min in a PerkinElmer Tri-Carb Liquid Scintillation Counter (Boston, MA).

3.2.7 DNA extraction and qPCR analysis

DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN; Valencia, CA) following the protocol provided by the manufacturer, except that an enzyme lysis buffer with the component of 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 1.2% Triton X-100, was used to resuspend the cell pellets after centrifuging (Ritalahti and Löffler, 2004). The final extracted DNA from 10 ml of liquid culture sample was dissolved in 120 µL Buffer AE of the DNA extraction kit and stored at –20˚C. The concentration of extracted DNA was determined spectrophotometrically (Sambrook and Russell 2001).

Real-time PCR amplification of 16S rRNA gene sequences was performed in a Roche LightCycler® 480 System (Roche Diagnostics Corporation, Indianapolis, IN). The 16S rRNA gene primers used for amplification of each strain are shown in Table 3.1. Calibration curves (log 16S rRNA gene copy concentration versus an arbitrarily set cycle threshold value [C_T]) for each strain were obtained by using duplicate serial dilutions of pure culture genomic DNA. The reaction mixture consisted of (per 15 µL reaction volume): 10 µL of 2X LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics Corporation, Indianapolis, IN), 2 µL of a primer solution containing 5

μM of each primer, 3 μL of PCR-grade water (Roche Diagnostics Corporation, Indianapolis, IN). DNA template (5 μL) was added to each tube. Triplicate reactions were analyzed using the following temperature program: 15 min at 94˚C, followed by 50 cycles of 30 sec at 94˚C, 20 sec at 58˚C, and 30 sec at 72˚C.

Table 3.1 16S rRNA gene primers for *Desulfuromonas michiganensis* strain BB1 and *Desulfitobacterium* sp. strain PCE1

Strain	Primer name[a]	Primer sequence	Reference
Desulfuromonas michiganensis strain BB1	Dsm205F Dsm1015R	5'-AACCTTCGGGTCCTACTGTC-3' 5'- GCCGAACTGACCCCTATGTT-3'	Löffler et al. (2000)
Desulfitobacterium sp. strain PCE1	Dsb406F Dsb619R	5'-GTACGACGAAGGCCTTCGGGT-3' 5'-CCCAGGGTTGAGCCCTAGGT-3'	Smits et al. (2004)

[a] Numbers refer to position in *Escherichia coli* 16S rRNA gene sequence.

3.2.8 Model approach

PCE dechlorination by *Desulfuromonas michiganensis* strain BB1 and *Desulfitobacterium* sp. strain PCE1 was simulated for batch cultures with different initial conditions (S_0/X_0 ratios) by modifying a previously described model (Becker 2006). The model was changed as follows: (1) the influent and effluent flows were eliminated to reflect the batch nature of the experiments; (2) the reactor volume was increased to 160-ml to accommodate a 60-ml headspace, and chlorinated ethenes were allowed to equilibrate between the gas and liquid phases; and (3) the competitive inhibition term in Equation (3.2) was incorporated into the TCE utilization expression for strain BB1. $K_{I,PCE}$ was set equal to $K_{S,PCE}$ (Cupples et al. 2004; Yu et al. 2005; Yu and Semprini 2004). The intrinsic kinetic parameter estimates for strain BB1 and strain PCE1 given in Table II were used along with the

assumed $K_{I,PCE}$ value to predict chlorinated ethene concentrations measured in batch culture assays with different S_0/X_0 ratios.

3.3 Results and Discussion

3.3.1 Electron donor kinetics

The intrinsic q_{max} and K_S values for strains BB1 and PCE1 under electron donor-limiting and electron acceptor-limiting conditions summarized in Table 3.2 were fit using the single substrate depletion curves shown in Figures 3.1 and 3.2, respectively. $K_{S,donor}$ values have not, to our knowledge, been previously been reported for heterotrophic PCE-respiring populations, and there are few reported half-saturation constants for hydrogen utilization by dehalorespirers (Cupples et al. 2004; Löffler et al. 1999). The $K_{S,donor}$ for acetate utilization by strain BB1 (5.77 μM) is similar in magnitude to the minimum concentration of acetate (or other fatty acids) that is considered to be adequate for natural attenuation (Wiedemeier et al. 1999). It is also one-half of the $K_{S,donor}$ for acetate in an Fe(III)-citrate-grown culture (Esteve-Núñez et al. 2005) and orders of magnitude lower than the $K_{S,donor}$ values typically reported for aceticlastic methanogens (Pavlostathis and Giraldo-Gomez 1991). Its relatively low $K_{S,acetate}$ should be advantageous if strain BB1 has to compete with other populations for acetate under the electron donor-limited conditions that prevail in many contaminated subsurface environments. The $K_{S,donor}$ for lactate utilization by strain PCE1 (9.40 μM) is somewhat higher, but should not prevent it from competing for electron donor under natural attenuation conditions. When engineered bioremediation approaches are implemented, lactate or another organic electron donor is typically

added at a concentration of 100 μM or higher, e.g., (Lendvay et al. 2003). Under these conditions, electron donor limitation should not be a concern for either organism.

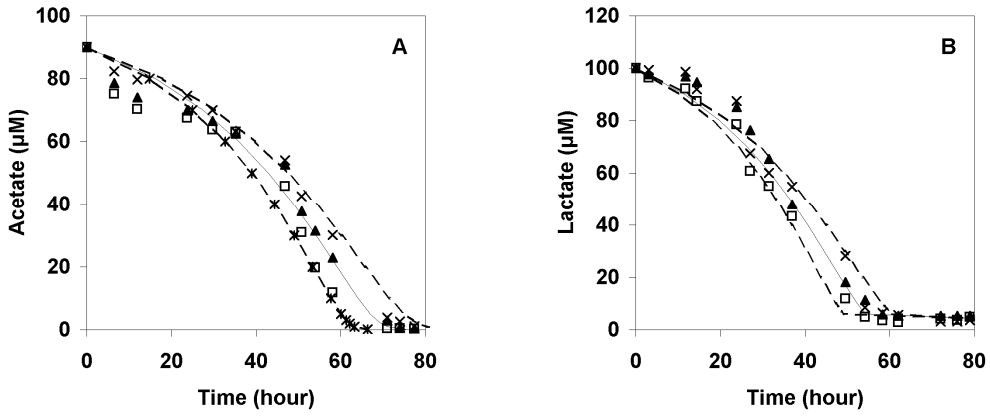

Figure 3.1 Electron donor depletion data used to fit $K_{S,donor}$ and $q_{max,donor}$ for (A) acetate utilization by *Desulfuromonas michiganensis* strain BB1, and (B) lactate utilization by *Desulfitobacterium* strain PCE1. Data points represent experimental results from triplicate batch cultures. Solid lines represent model predictions using average parameter estimates and dashed lines represent 95% confidence intervals.

In anaerobic batch cultures, the level of the electron donor frequently decreases until a threshold concentration is reached below which metabolism apparently is not feasible (Cord-Ruwisch et al. 1988; He and Sanford 2004; Hopkins et al. 1995; Jetten et al. 1990; Löffler et al. 1999; Lovley and Goodwin 1988; Min and Zinder 1989; Sung et al. 2006a). In this study, electron donor thresholds were also observed in the batch assays. The acetate threshold for strain BB1 (0.41 μM) was higher than the acetate thresholds reported for 2-chlorophenol respiration by *Anaeromyxobacter dehalogenans* (He and Sanford 2004) and chlorinated ethene respiration by

Geobacter lovleyi (Sung et al. 2006a), but orders of magnitude lower than the acetate thresholds reported for *Geobacter thiogenes* (Sung et al. 2006a) and several acetotrophic methanogens (Jetten et al. 1990; Min and Zinder 1989; Westermann et al. 1989). A lactate threshold of 4.33 µM was measured for strain PCE1. To our knowledge, thresholds for lactate utilization have not previously been reported.

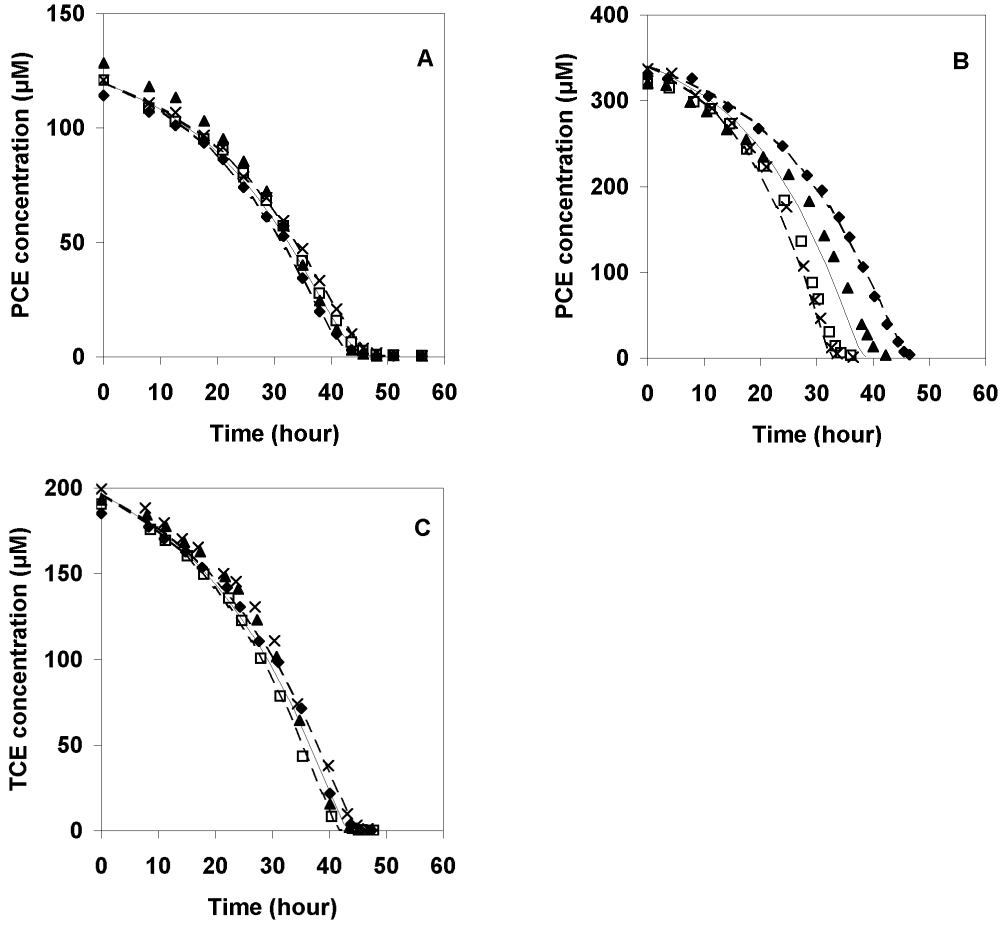

Figure 3.2 Electron acceptor depletion data used to fit K_S and q_{max} for (A) PCE utilization by *Desulfuromonas michiganensis* strain BB1, (B) PCE utilization by *Desulfitobacterium* strain PCE1, and (C) TCE utilization by *Desulfuromonas michiganensis* strain BB1. Data points represent experimental results from four replicate batch cultures. Solid lines represent model predictions using average parameter estimates and dashed lines represent 95% confidence intervals

Most studies of chlorinated ethene dehalorespiration are conducted with excess levels of electron donors and therefore, estimates of $q_{max,donor}$ are not generally determined. However, accurate estimates of $q_{max,donor}$ are needed because these values may play an important role in determining the outcome of competition among dehalorespiring populations (Becker 2006; Becker and Seagren 2009) or between dehalorespirers and other populations in electron donor-limited continuous-flow systems. The $q_{max,donor}$ values determined experimentally are given in Table 3.2 and highlight the importance of independently estimating q_{max} values for utilization of electron donors and acceptors by dehalorespiring bacteria.

For example, when $q_{max,donor}$ values are needed for modeling studies, they can be calculated based on estimates of q_{max} for chlorinated ethenes and the fraction of electron donor equivalents that are used in energy production ($f_e°$), e.g., (Bagley 1998). Following this approach using the $q_{max,PCE}$ values experimentally determined in this study, a stoichiometric ratio of 1 mol acetate/mol PCE respired, and a previously reported $f_e°$ of 0.66 (Löffler et al. 1999), a $q_{max,acetate}$ of 9.55 μmol/mg VSS/h is calculated for strain BB1. This is more than double the value independently estimated in the electron donor kinetic assay. Use of the $f_e°$ value (0.504) calculated based on theoretical considerations of the thermodynamics of the electron donor, electron acceptor, and cell synthesis half-reactions (McCarty 1971) yields an even higher estimate of $q_{max,acetate}$ (12.6 μmol/mg VSS/h). In the present study, $f_e°$ could be estimated from measurements of electron donor and acceptor utilization during the electron donor kinetic assays (Figure 3.3). The $f_e°$ value for strain PCE1 (0.55) is similar to the theoretically estimated value. However, an $f_e°$ of 0.95 was determined

in this study for strain BB1 suggesting that dechlorination became uncoupled from growth, as previously observed (Sung et al. 2003). The $q_{max,donor}$ values calculated using the $f_e°$ values of 0.55 and 0.95 for strains PCE1 and BB1, respectively, closely match and provide confirmation of the $q_{max,donor}$ values that were fit to the substrate depletion data in the electron donor kinetic assays.

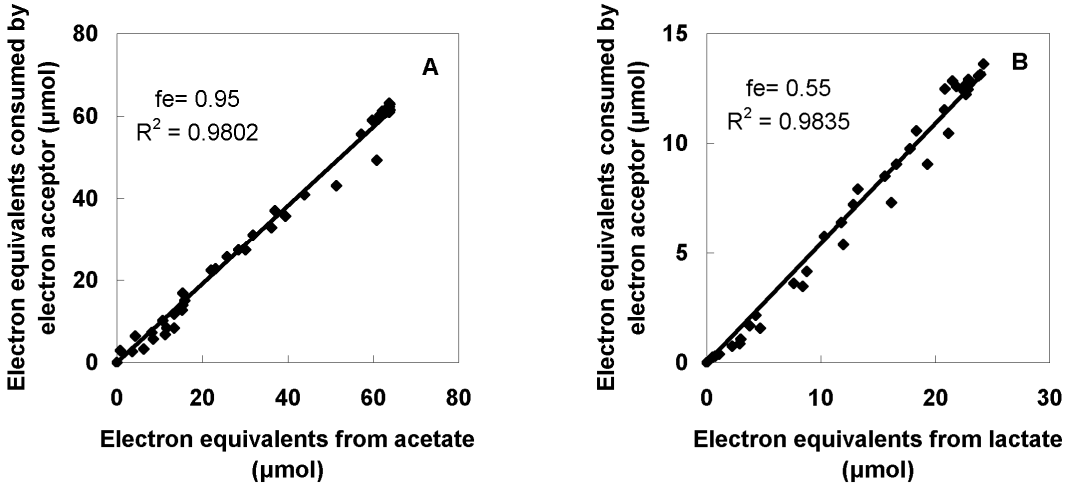

Figure 3.3 Determination of $f_e°$ values for (A) *Desulfuromonas michiganensis* strain BB1 using acetate as electron donor, and (B) *Desulfitobacterium* strain sp. PCE1 using lactate as electron donor. Data points represent individual measurements in triplicate batch cultures. The $f_e°$ values were determined by linear regression analysis by plotting the amount of electron equivalents consumed by reductive dechlorination versus the amount of electron equivalents provided by the electron donor. $f_e°$ values were derived from the slopes of the regression lines.

Table 3.2 Monod kinetic parameter estimates for *Desulfuromonas michiganensis* strain BB1 and *Desulfitobacterium* sp. strain PCE1 and several other chlorinated ethene-respiring cultures

Culture	*Dsm. michiganensis* strain BB1		*Dsf.* sp. strain PCE1	Mixed culture containing *Dhc. ethenogenes*	Mixed culture containing *Dhc.*-like microorganisms (PM)	(EV)
Y (mg VSS/μmol Cl^-)[a]	0.0033 (0.0009)	0.0048	0.0116 (0.0034)	0.00612	N/A[b]	N/A
q_{max} (μmol/mg VSS/h)[a]						
PCE	12.6 (0.46)	5.37 (0.09)	5.36 (0.87)	1.8	0.30 (0.04)	0.29 (0.02)
TCE	17.0 (0.58)	14.0 (1.53)		3	2.84 (0.39)	2.87 (0.32)
DCE				3	0.51 (0.04)	0.32 (0.03)
VC				3	0.06 (0.01)	0.19 (0.02)
Acetate	4.66 (0.38)					
Lactate			3.86 (0.32)			
K_S (μM)[a]						
PCE	9.31 (0.23)	14.2 (2.1)	8.81 (1.68)	0.54	3.9 (1.4)	1.6 (0.2)
TCE	2.83 (0.20)	23.4 (5.2)		0.54	2.8 (0.3)	1.8 (0.4)
DCE				0.54	1.9 (0.5)	1.8 (0.3)
VC				290	602 (7)	62.6 (2.4)
Acetate	5.77 (1.44)	N/A				
Lactate			9.40 (1.29)			
H_2				0.1		
S_0/X_0 (COD/COD)	22	3.6	20	N/A	N/A	N/A
Reference	This study	(Amos et al. 2007)	This study	(Fennell and Gossett 1998)	(Yu et al. 2005)	

[a]. Values in parentheses represent 95% confidence interval

[b] Not available.

The correlation coefficients (R^2) for the $q_{max,donor}$ and $K_{S,donor}$ estimates in individual assays involving strain BB1 ranged from 0.89 to 0.91. The R^2 values ranged from 0.90 to 0.92 for strain PCE1. These results are consistent with a previous study that suggested the minimum R^2 value between q_{max} and K_S is approximately 0.9 when Y is estimated independently, due to the inherent correlation of the Monod kinetic parameters (Liu and Zachara 2001). Nevertheless, unique estimates of q_{max} and K_S were identifiable under the intrinsic conditions used in this study as indicated by the collinearity index (γ_K) values calculated for each set of parameter estimates. The γ_K values calculated for the electron donor utilization kinetic parameter estimates for strain BB1 and strain PCE1were 6.53 and 8.61, respectively—well below the maximum value of 10 for identifiable parameter estimates (Brockmann et al. 2008; Brun et al. 2002).

3.3.2 Electron acceptor kinetics

The q_{max} and K_S values for the chlorinated ethenes obtained in this and earlier studies are reported in Table 3.2. As observed under electron donor-limiting conditions, the R^2 calculated in this study ranged from 0.90 to 0.92 for strain BB1 and from 0.88 to 0.90 for strain PCE1, and the γ_K values were less than 10, suggesting that the q_{max} and K_S parameter estimates under electron-acceptor limiting conditions are identifiable.

The q_{max} and K_S values estimated for different organisms vary over more than an order of magnitude. In particular, *Dehalococcoides* strains in pure and mixed cultures tend to exhibit substantially lower q_{max} and K_S values compared to the values reported

for the heterotrophic *Desulfuromonas* and *Desulfitobacterium* strains in this and a recent study (Amos et al. 2007). For example, $q_{max,PCE}$ values of 12.6 and 5.36 μmol/mg VSS/h were obtained in this study for strains BB1 and PCE1, respectively, compared with reported $q_{max,PCE}$ values ranging from 0.3 to 1.8 μmol/mg VSS/h for mixed cultures containing *Dehalococcoides* strains (Table 3.2). The estimates of $K_{S,PCE}$ for strains BB1 and PCE1 in this study are 9.31 and 8.81 μM, respectively, and values reported in earlier studies range from 0.54 to 3.9 μM for mixed cultures containing *Dehalococcoides* strains.

Differences in the fundamental characteristics of the chlorinated ethene reductive dehalogenases in the different dehalorespirers likely influence the values reported in Table 3.2. In addition, the initial conditions of the batch culture assays, including the S_0/X_0 ratios, may also contribute to the reported variability in kinetics of dehalorespiration and other metabolic processes (Grady et al. 1996; Liu and Zachara 2001). For example, previously reported estimates of $q_{max,PCE}$ and $q_{max,TCE}$ for strain BB1 (Table 3.2) are most likely lower than those obtained in the current study because the earlier estimates were obtained in batch culture assays at S_0/X_0=3.6, conditions that did not allow unrestricted growth to occur. However, other experimental differences in the two studies may also have influenced the parameter estimates.

Independent estimates of Y and k_d were also obtained in this study for strains BB1 and PCE1 under electron acceptor-limiting conditions. The Y determined for strain BB1 based on protein measurements (0.0033 mg VSS/μmol Cl^-) is of similar magnitude to that estimated using qPCR (0.0074 mg VSS/μmol Cl^-). In contrast, the

Y reported for strain BB1 in an earlier study was approximately one order of magnitude lower (Sung et al. 2003). Nearly identical estimates of *Y* were obtained in this study for strain PCE1 based on protein analysis (0.0116 mg VSS/µmol Cl^-) and qPCR (0.0118 mg VSS/µmol Cl^-).

Few k_d values have been reported for dehalorespirers in the literature. Fennell and Gossett (1998) estimated that the k_d for dehalorespirers and other anaerobic microorganisms was 0.001 h^{-1}, and this value is often used in modeling studies, e.g., (Becker 2006; Yu et al. 2005). The experimentally determined k_d for a mixed culture containing *Dehalococcoides* sp. strain VS under no-growth conditions (0.00375 h^{-1}; Cupples et al. 2003) was closer to the values obtained in this study for strain BB1 (0.0054 h^{-1}) and strain PCE1 (0.0076 h^{-1}).

3.3.3 Model simulation

To assess the accuracy with which the kinetic parameter estimates predict chlorinated ethene behavior under intrinsic conditions, a new set of batch PCE depletion experiments were conducted at S_0/X_0>20 and compared to model predictions made using the values in Table 3.2. In addition, the model predictions were compared to experimental data collected in batch assays at several lower S_0/X_0 ratios to assess the range of conditions that can be accurately modeled using the intrinsic parameters. The model accurately predicted the experimental data obtained at S_0/X_0>20 for both strains (Figures 3.4A and 3.5A). A good fit between the model simulation results and the experimental data was also observed for $10<S_0/X_0<20$. This is important because previous guidelines recommend that a ratio of S_0/X_0>20 (Grady et al., 1996) be used for estimating intrinsic kinetics. This may be appropriate for

electron donor substrates, but the results of this study showed that, at least for electron acceptor substrates, intrinsic kinetics can be obtained when S_0/X_0 >10. A higher S_0/X_0 ratio may be needed to allow unrestricted growth on electron donor substrates to occur (and estimation of intrinsic kinetic parameters) because a portion of electron donor substrates is consumed in the synthesis of cellular constituents (McCarty 1971). This has the net effect of increasing the requirement for electron donor substrates relative to the demand for electron acceptors, which, in the case of chlorinated ethenes, are consumed only for energy generation. The finding that a lower S_0/X_0 ratio may be used to estimate intrinsic kinetic parameters (at least for electron acceptors) may be very beneficial for environmental contaminants like chlorinated ethenes that can inhibit biodegradation at high concentrations. A high S_0 must be avoided for these substrates when estimating Monod kinetic parameters.

The predicted dechlorination rates were much higher than the experimental observations made at S_0/X_0<10 for strain BB1 (Figures 3.4C, D) and strain PCE1 (Figure 3.5C, D) and the difference between the modeling and experimental results grew larger as the S_0/X_0 ratios became smaller. This reflects the restrictions on growth and the availability of the enzymes that catalyze the biodegradation reactions caused by low substrate concentrations. As noted above, these restrictions on growth appeared to be alleviated at a threshold S_0/X_0 of approximate 10.

The modeling and experimental results shown in Figures 3.4 and 3.5 highlight the need to carefully select the conditions under which kinetic parameters are determined in batch culture assays to ensure that they are relevant to the system that is being modeled. It is also important to note that reports that do not describe the initial

conditions under which the batch culture assays were conducted (S_0 and X_0) are of little value because their relevance to other systems cannot be evaluated by other researchers. In this study, the kinetic parameters were estimated under intrinsic conditions, and thus can be used to predict of PCE dechlorination only when S_0/X_0 is high enough to allow unrestricted growth.

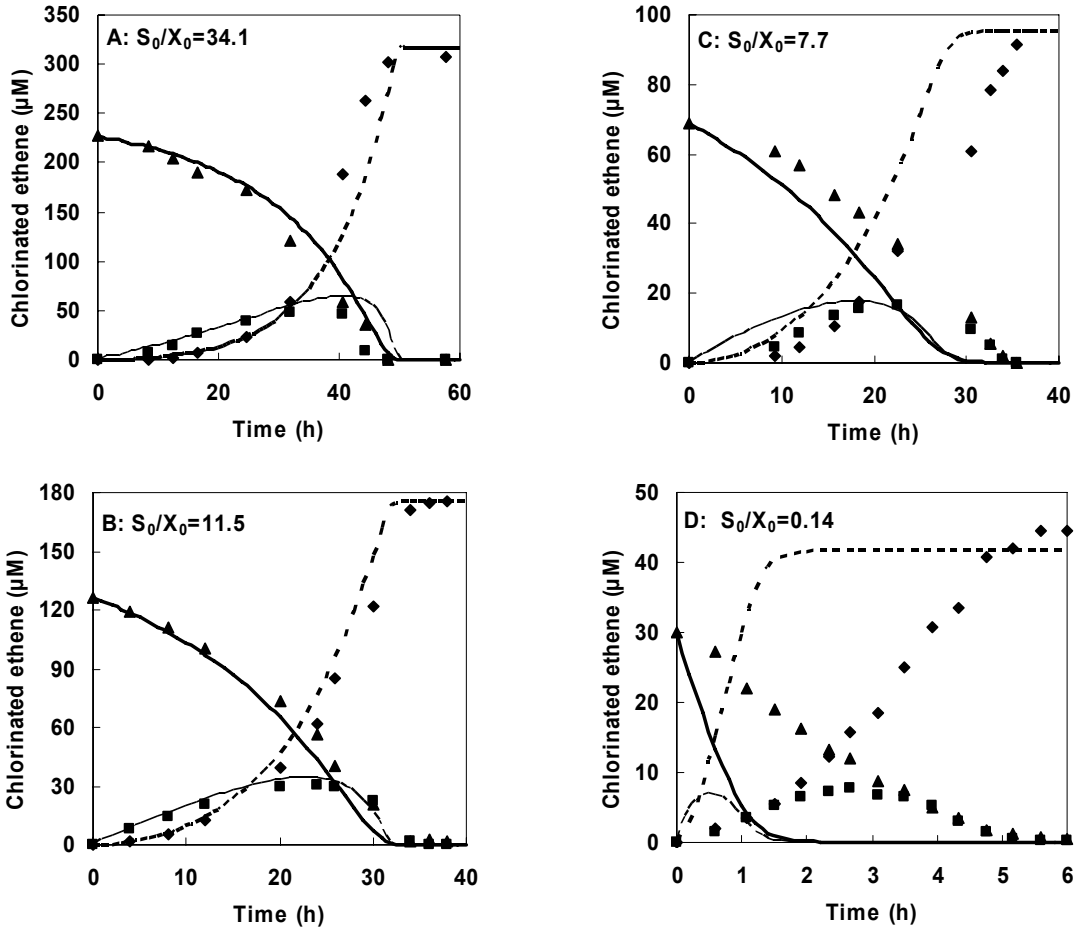

Figure 3.4 PCE dechlorination by *Desulfuromonas michiganensis* strain BB1 under four different sets of initial conditions (S_0/X_0 ratios): (**A**) S_0/X_0 =34.1; (**B**) S_0/X_0 =11.5; (**C**) S_0/X_0 =7.7; (**D**) S_0/X_0 =0.14. S_0/X_0 ratios are given on a COD basis. Data points represent experimental results and lines represent model predictions for PCE (—▲—), TCE (···■···), and DCE (- -◆- -) using the intrinsic kinetic parameter values in Table 3.2.

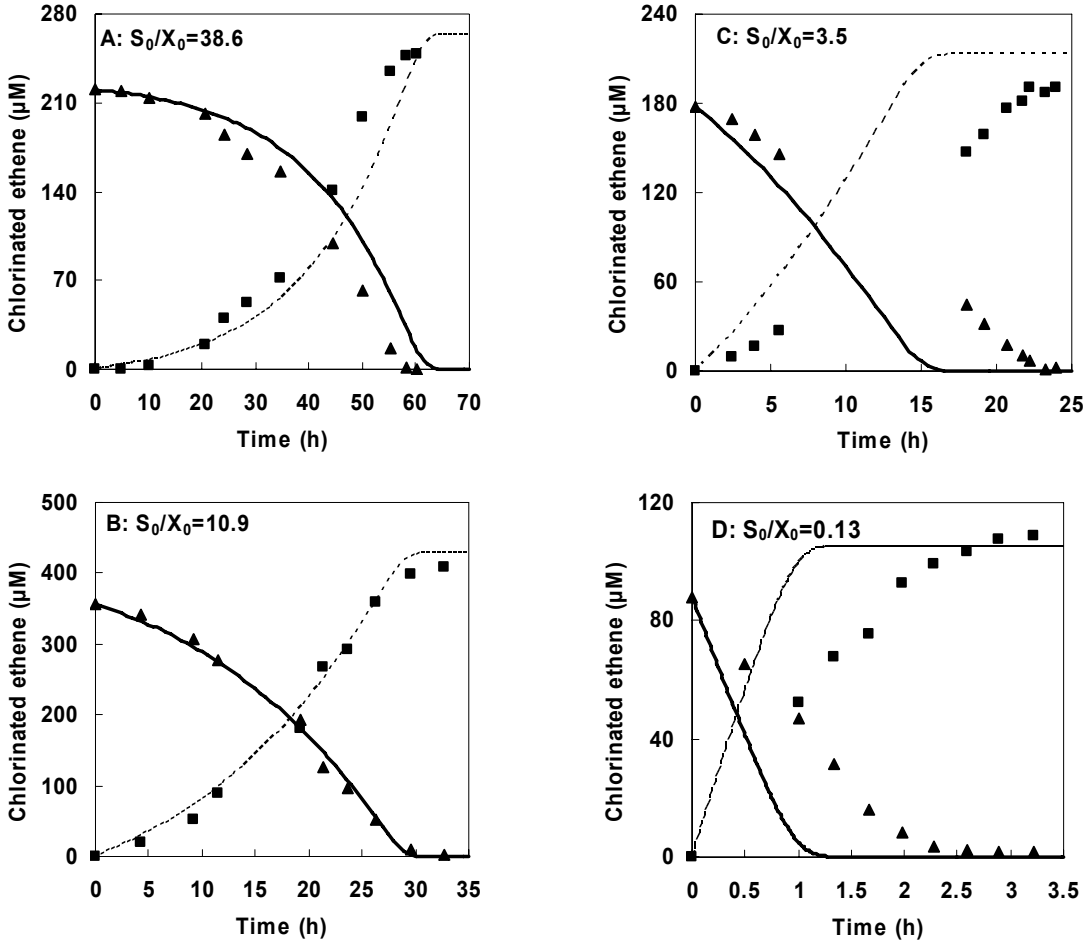

Figure 3.5 PCE dechlorination by *Desulfitobacterium* strain sp. PCE1 under four different sets of initial conditions (S_0/X_0 ratios): (**A**) S_0/X_0 =38.6; (**B**) S_0/X_0 =10.9; (**C**) S_0/X_0 =3.5; (**D**) S_0/X_0 =0.13. Data points represent experimental results and lines represent model predictions for PCE (—▲—) and TCE (···■···) using the intrinsic kinetic parameter values in Table 3.2.

3.4 Implications for bioremediation practice

Prediction of the fate of chlorinated ethenes at sites undergoing in situ bioremediation requires accurate estimates of microbial kinetic parameters. In many regions of the contaminant plume, chlorinated ethene concentrations may be high enough to allow unrestricted microbial growth, particularly when biostimulation, the addition of electron donors, is implemented upgradient to alleviate electron donor

limitation. Therefore, to predict the fates of chlorinated ethenes under these conditions, intrinsic dechlorination kinetic parameters should be used. However, intrinsic kinetic parameters may overestimate removal of chlorinated ethenes in regions of the contaminant plume where growth is restricted by substrate availability.

To ensure that intrinsic kinetic parameter estimates are obtained, batch laboratory assays should be conducted at $S_0/X_0>10$, for electron acceptor substrates. Additional work is needed to determine if intrinsic kinetic parameter estimates can be obtained for electron donor substrates under these initial conditions. Further, estimates of electron donor and acceptor utilization kinetics should be made independently, because as shown in this and an earlier study (Esteve-Núñez et al., 2005), the stoichiometry of electron donor and acceptor utilization for some organisms may not be constant under different conditions.

As discussed by Becker (2006), the apparently high dechlorination capacity of the *Desulfuromonas* and *Desulfitobacterium* strains evaluated in this study could provide them with a competitive advantage with respect to utilization of PCE and TCE that leads to differences in the roles of dehalorespiring populations in contaminant plumes. In particular, the differences in the q_{max} values reported in Table II suggest that the natural niche of *Dehalococcoides* species may be to respire DCE and VC produced by *Desulfuromonas* and *Desulfitobacterium* strains that dechlorinate PCE and TCE at faster rates.

Chapter 4: Extant kinetics studies of two heterotrophic tetrachloroethene-respiring strains and the impact of initial conditions on kinetic parameter determination

4.1 Introduction

It is well-recognized that meaningful kinetic parameter estimates are key to demonstrating the occurrence of natural attenuation and the successful implementation of engineered bioremediation approaches (Becker, 2006; Shen and Sewell, 2005; Yu et al., 2005; Yu and Semprini, 2004; Waul et al., 2008). For example, kinetic parameter estimates are needed to determine the rates at which stimulatory materials should be added to the subsurface, size treatment systems, and estimate clean-up times (Song and Seagren, 2008). However, it is often less clear what constitutes meaningful kinetic parameter estimates.

The present study was motivated by a need for a consistent set of meaningful kinetic parameter that could be input into a model describing the interactions among tetrachloroethene (PCE)-respiring populations in a continuous-flow stirred tank reactor (Becker, 2006). At high concentrations, chlorinated ethenes can become self-inhibitory or inhibit the reductive dechlorination of other chlorinated ethenes. Dehalorespiration of chlorinated ethenes under non-inhibitory concentrations is generally described by a dual Monod model (Equation 4.1) in which both the electron acceptor (chlorinated ethene) and electron donor can limit the dechlorination rate (Bagley, 1998; Fennell and Gossett, 1998; Haston and McCarthy, 1999),

$$\frac{dS_a}{dt} = -q_{\max} X \left(\frac{S_d}{K_{S,d} + S_d} \right) \left(\frac{S_a}{K_{S,a} + S_a} \right) \quad (4.1)$$

where S_a [$M_S \cdot L^{-3}$] and $K_{S,a}$ [$M_S \cdot L^{-3}$] are the aqueous concentration and the half-saturation constant of the electron acceptor, respectively. This chapter focuses on the factors that affect our ability to obtain meaningful estimates of $q_{\max}$ and $K_{S,a}$, assuming Y and $K_{S,d}$ are estimated in separate experiments.

Meaningful estimates of q_{max} and K_S share three key characteristics. First, the estimated values must reflect the relevant cellular and microbial community composition. This is important because the rates of biodegradation and microbial growth are frequently limited by low in situ substrate concentrations. At the community level, limited substrate availability tends to select for slow-growing populations with a high affinity for the contaminant. At the cellular level, low substrate concentrations limit the size of the protein synthesizing systems (PSS), which determines the availability of the enzymes that catalyze the biodegradation. Consequently, the rate of biodegradation is less than optimal for a given organism and is controlled by the culture history. The term "extant" is used to describe kinetic parameter estimates that reflect the biodegradation rates occurring in situ (Grady et al., 1999) at natural attenuation sites or in bioreactors where growth and substrate utilization are controlled by the cell age or solids retention time (SRT).

At the other end of spectrum are "intrinsic" kinetics, which are measured under conditions of abundant substrate availability that result in unrestricted microbial growth. At the community level, high substrate concentrations tend to select for fast-

growing populations with low affinity for the contaminant. At the cellular level, the PSS, and consequently the biodegradation rate, are optimal during unrestricted growth. The substrate utilization kinetics that occurs during unrestricted growth is independent of growth history of a culture, including substrate availability in its source environment. Thus, intrinsic kinetics could potentially be useful for comparing the inherent capabilities of culture that are being evaluated for use in bioaugmentation applications or for predicting contaminant removal rates under conditions of high electron donor availability through implementation of biostimulation (Grady et al., 1999).

The second important characteristic of meaningful kinetic parameter estimates is that they are unique and identifiable. With respect to the Monod kinetic parameters, parameter uniqueness determines whether q_{max} and K_S values can be fit to experimental substrate depletion data independently or only as lumped parameters. Identifiability determines the amount of correlation between q_{max} and K_S. Correlation is undesirable because when two parameters are highly correlated, different combinations of the parameter estimates may fit the experimental data equally well.

Third, meaningful kinetic parameter estimates have relatively low uncertainties (Liu and Zachara, 2001). Uncertainty arises from the inverse nature of the problem of using measurement data to estimate unknown kinetic properties and cannot be eliminated. However, uncertainty increase with parameter correlation, and large standard deviations are observed for highly correlated parameter estimates. The sample number and sampling time points also affect parameter uncertainties.

Biodegradation kinetic parameters are typically estimated in batch culture assays because they are simple to prepare and maintain. However, the kinetic parameter values estimated for a given compound in batch culture assays often vary widely, even for pure cultures. Frequently, the parameter values estimates in batch culture assays also over predict the contaminant removal rates observed in-situ. Clearly, obtaining meaningful estimates of in-situ biodegradation kinetics in batch culture assays can be challenging.

Several steps should be taken to help ensure that kinetic parameter estimates are meaningful. First, to describe biodegradation activity in situ, parameter estimates must be estimated under extant conditions that do not allow growth of the cells removed from the environment of interest so that the PSS cannot change. According to Grady et al. (1996), this criterion can generally be met by providing a small initial substrate concentration relative to the initial biomass concentration, on the order of $S_0/X_0<0.025$ when both concentrations are expressed in terms of chemical oxygen demand (COD). A high S_0/X_0 ratio on the order of $S_0/X_0>20$ on a COD basis is recommended to allow for the unrestricted growth required for the estimation of intrinsic kinetic parameters.

Selection of the initial conditions in batch culture assays also impacts the amount of correlation between the parameter estimates. Grady et al. (1996) suggested that the best independent estimates of q_{max} and K_S can be obtained at $S_0/X_0 \geqslant 20$ on a COD basis; however, these conditions are not compatible with extant parameter determinations. Other studies have focused on the relationship between the S_0/K_S ratio

and parameter correlation by computing the partial derivative of the substrate concentration with respect to μ_{max}, where μ_{max}, the maximum specific growth rate, equals $Y\,q_{max}$, and K_S at various time intervals and visually compared the resulting sensitivity curves obtained under different S_0/K_S ratios. The sensitivity curves for μ_{max} and K_S tracked together at small S_0/K_S ratios ($S_0<K_S$), suggesting that independent estimates of μ_{max} and K_S cannot be obtained under these initial conditions (Ellis et al., 1996; Robinson and Tiedje, 1983). In contrast, visual inspection of the sensitivity curves suggested that there was little correlation between the parameter estimates at S_0/K_S =4 (Robinson and Tiedje, 1983) or S_0/K_S =1 (Ellis et al., 1996). More recently, Liu and Zachara (2001) calculated the correlation coefficients and standard deviations of the Monod kinetic parameter estimates obtained under different initial conditions and for different parameter estimates and observed a high degree of correlation between μ_{max} and K_S at S_0/K_S =1. The correlation was reduced, but not eliminated, at higher S_0/K_S ratios.

In the present study, extant kinetic parameters were estimated in batch assays to describe dehalorespiration activity in an anaerobic CSTR (Becker, 2006). However, analysis of the correlation coefficients for the estimated q_{max} and K_S values indicated that the parameters were nearly perfectly correlated. A series of experimental and simulated batch kinetic assays were then conducted to determine if parameter correlation could be reduced by optimizing the initial conditions in the batch assays. These evaluations showed that S_0/K_S, but not S_0/X_0, strongly influenced parameter correlation.

4.2 Materials and methods

4.2.1 Chemicals

PCE (99%, Spectrum Chemical Mfg. Corp.) were used in kinetic experiments. The PCE (99.99%), TCE (99.99%) and *cis*-DCE (99.99%) used to prepare analytical standards were purchased from Sigma-Aldrich (St. Louis, MO). Sodium acetate (Certified ACS) was obtained from Fisher Scientific Inc. (Pittsburgh, PA) and sodium L-lactate (60% wt. solution) was obtained from Acros Organics (Geel, Belgium). Methanol (Certified ACS) was obtained from Fisher.

4.2.2 Culture maintenance

Desulfuromonas michiganensis strain BB1 (DSM 15941, Sung et al., 2003) and *Desulfitobacterium* sp. strain PCE1 (DSM 10344, Gerritse et al., 1996) were used to evaluate the extant kinetic parameter determination. They were grown in semi-continuous cultures maintained at a 20 d SRT, as described in Chapter 3.

4.2.3 Kinetic assays

Kinetic parameter estimation was performed using batch assays. To obtain the small S_0/X_0 ratio required for extant kinetics, the batch assays must be conducted using a large amount of biomass. A large amount of biomass could be generated by transferring culture from the semi-continuous stocks to fresh medium containing high substrate concentrations to promote growth. However, the biomass grown under these conditions would no longer reflect the constraints of the 20 d SRT on microbial activity in the source culture. Therefore, the extant experiments were conducted using

the undiluted semi-continuous stock cultures of strains BB1 and PCE1, which contain relatively high biomass concentrations due to the addition of large amounts of PCE and electron donor at regular intervals. Replication was obtained by repeating each experiment twice in a given stock culture. It was possible to do this without affecting the long-term performance and physiological state of the semi-continuous cultures because the extant kinetic assays have a short duration and do not result in biomass growth. The batch assays were initiated using the following approach. When approximately 90% of the last dose of PCE added to the semi-continuous cultures was dechlorinated, the cultures were purged of chlorinated ethenes with O_2-free N_2/CO_2 (80%/20%, Airgas East) for 20 min, and a 5-ml sample was aseptically removed so that the biomass concentration could be measured. Fresh media (5-ml) was added to maintain 100-ml of liquid in the semi-continuous culture bottles. To ensure that the chlorinated ethene was limiting, 1mM of acetate and lactate was provided to strain BB1 and strain PCE1, respectively. PCE (6.5 μM) or TCE (13 μM) was added to the cultures from sterile methanol stock solution. The total volume of methanol added to a given semi-continuous culture did not exceed 0.1% of the total culture volume. At this concentration, methanol does not have a significant affect on the partitioning of chlorinated ethenes between the gaseous and aqueous phases (Gossett, 1987). The amount of PCE (or TCE) added to the strain BB1 semi-continuous culture would consume 4 μM of acetate, which is equivalent to 0.2 mg/L on a COD basis. The source culture contained approximate 5.5 mg VSS/L of biomass. Thus, assuming 1.42 g COD/g VSS (Grady et al., 1999), the initial conditions were equivalent to the S_0/X_0=0.025, the criterion for extant kinetics specified by Grady et al.

(1996) for electron donor substrates. The same approach was used to select the initial conditions for strain PCE1. The batch cultures were incubated at 30°C or room temperature (22°C) for 30 min to equilibrate the temperature in the batch cultures.

After shaking by hand for several seconds, the batch cultures were amended with the chlorinated ethene and incubated at either 30°C or room temperature in an orbital shaker at 120 rpm. Headspace samples of 0.5 ml or 0.1 ml were taken from the batch cultures every 6 to 10 min for determination of chlorinated ethene concentrations via a gas chromatograph (GC) equipped with flame ionization detector (FID) or electron capture detector (ECD), respectively. After the PCE or TCE in the batch culture assays was completely removed, the cultures were purged with 80% N_2/20% CO_2 again, so that the experiment could be repeated or routine maintenance of the semi-continuous cultures could continue.

Kinetic parameters were estimated by fitting the experimental data to a simplified form of the integrated Monod equation (Equation 4.2) that is obtained by assuming that the biomass concentration is constant and the electron donor concentration is non-limiting.

$$t = \frac{1}{q_{\max} X_0}\left[K_S \ln\left(\frac{S_0}{S}\right) + \left(S_0 - S\right)\right] \tag{4.2}$$

Two sets of experiments were also conducted to evaluate the impact of the initial conditions (S_0/X_0 and S_0/K_S) in the batch culture assays on the amount of correlation between the Monod kinetic parameter estimates and the uncertainty associated with

these estimates. The experiments were conducted in duplicate using strain BB1. PCE was supplied as the electron acceptor at the S_0/X_0 and S_0/K_S ratios shown in Table 4.1 by transferring different volumes of the semi-continuous culture to batch assays containing varying amounts of PCE following the same general approach used in the intrinsic kinetic parameter assays (Chapter 3). Estimates of q_{max} and K_S were obtained by fitting Equation 4.2 to the PCE depletion data using Y = 0.0033 mg VSS/µmol Cl^-, which was estimated under intrinsic conditions (Chapter 3).

Table 4.1 Summary of the initial conditions used to evaluate the impact of S_0/X_0 and S_0/K_S on Monod parameter correlation and uncertainty in batch kinetic assays involving *Desulfuromonas michiganensis* strain BB1

Initial biomass concentration, X_0		Initial PCE concentrations, S_0		S_0/X_0	S_0/K_S [a]	S_0/K_S [b]
(mg VSS/L)	(mg COD/L)	(µM)	(mg COD/L)			
0.35	0.49	7.13	0.23	0.4	3.0	1
0.65	0.90	11.31	0.37	0.4	1.8	2
1.29	1.83	26.51	0.85	0.4	4.5	4
2.58	3.66	54.35	1.76	0.5	6.0	8
4.55	6.45	90.10	2.92	0.5	13.2	14
5.00	7.09	105.45	3.42	0.5	12.3	17
0.65	0.92	26.05	0.84	0.9	4.0	4
0.13	0.18	27.39	0.89	4.9	4.6	4
0.07	0.09	26.50	0.86	9.5	4.3	4
0.04	0.06	27.68	0.89	14.8	4.0	4

[a] Initially S_0 for each condition was based on K_S parameter values estimates in preliminary experiments. The S_0/K_S values reported here are based on the K_S estimates obtained in individual batch kinetic assays under the corresponding initial conditions. [b] The values of K_S used to calculate the S_0/K_S values is the average of the K_S estimates obtained under the different initial conditions.

4.2.4 Numerical experiments

PCE dechlorination by strain BB1 was numerically simulated so that the effects of initial batch assay conditions on parameter correlation and identifiability could be systematically evaluated for a broad range of conditions ($0.025 \leq S_0/X_0 \leq 50$ and $0.04 \leq S_0/K_S \leq 40$). Because the sample number also influences the identifiability of Monod kinetic parameter estimates (Liu and Zachara, 2001), each synthetic data set contained a uniform number of samples (n=50), which were evenly collected from time 0 to the time when the residue PCE concentrations in the batch culture assays were lower than 0.05 μM. The synthetic PCE dechlorination data were predicted by Equations 3.1-3.3, which were solved using STELLA Version 8.0 (isee systems; Lebanon, NH) and a fourth-order Runge Kutta numerical method. The intrinsic kinetic parameter estimates for PCE dechlorination by strain BB1 (Table 3.2) were used as model inputs in these numerical simulations.

4.2.5 Parameter identifiability analysis

Parameter identifiability was quantitatively evaluated by calculating correlation coefficients for estimates of q_{max} and K_S based on numerical and experimental data following the method described by Liu and Zachara (2001). Sensitivity coefficients were calculated as the first derivatives of S with respect to a Monod kinetic parameters, $\frac{\partial S}{\partial \theta}$, where θ is a Monod parameter. The uncertainties of parameter estimates is evaluated by calculating the relative standard deviation, $\sigma(\theta)/\theta$, where σ is the standard deviation of the replicate parameter estimates (Liu and Zachara,

2001). The linear dependence of the sensitivity functions was quantified by calculating the collinearity index, γ_K according to Equation 4.3 (Brun et al., 2002),

$$\gamma_K = \frac{1}{\sqrt{\min\left(EV(\tilde{S}^T\tilde{S})\right)}} \tag{4.3}$$

where $\tilde{S}$ is the normalized sensitivity coefficient matrix and EV is the eigenvalue of $\tilde{S}^T\tilde{S}$. As the degree of parameter dependence increases, γ_K approaches infinity, whereas for linearly independent parameters, γ_K is equal to one. Brun et al. (2002) suggested that parameter estimates are poorly identifiable for all γ_K>10-15.

4.2.6 Analytical methods

The concentrations of chlorinated ethenes were determined using a Hewlett Packard 5890 II Plus GC equipped with an electron capture detector (ECD) and a DB-624 capillary column (30 m x53 mm (I.D.) x 3 μm film thickness) or a Hewlett Packard 5890 II Plus GC equipped with an flame ion detector (FID) and a stainless-steel column packed with 1% SP-1000 on 60/80 Carbopack-B (2.4 m by 3.2 mm, Supelco Inc.). When the GC-ECD was used, the GC injector and detector temperatures were 250^{o}C and 300^{o}C, respectively. Helium and nitrogen were provided as carrier and make-up gases at flow rates of 6 and 60 mL/min, respectively. The oven temperature was programmed to ramp at 4.0^{o}C/min from an initial temperature of 31ºC to 60ºC, hold at 60ºC for 5 min, followed by a second ramp of 15.0ºC/min to 200°C and a final hold at 200°C for 2 min. When the GC-FID was used,

the method described by Gossett (1987) was employed to determine PCE concentrations.

The initial biomass concentrations in the batch culture assays were quantified using the Bradford reagent kit from Sigma-Aldrich (St. Louis, Mo) following the manufacturer's instructions.

4.3 Results and Discussion

The substrate depletion curves in the initial experiment conducted to estimate extant q_{max} and K_S values were highly reproducible for *Desulfuromonas michiganensis* strain BB1. However, there was a significant amount of uncertainty associated with these parameters estimates, as indicated by the large standard deviations. Further, the q_{max} and K_S estimates were highly correlated. The calculated correlation coefficients (R^2 values) were 0.99.

Previous studies point to the importance of the initial conditions in determining the amount of correlation between parameter estimates (Checchi and Marsili-Libelli, 2005; Ellis et al., 1996; Grady et al., 1996; Kesavan and Law, 2005; Liu and Zacharam, 2001; Robinson and Tiedje, 1983; Seagren et al., 2003). However, these studies are inconsistent with regard to their conclusions about how the initial conditions affect parameter identifiability. Therefore, a preliminary evaluation consisting of ten batch kinetic assays with different combinations of S_0, X_0, and K_S was conducted to ascertain whether the parameter correlation and uncertainty associated with the extant parameter estimates could be reduced by optimizing the initial conditions. The S_0/X_0 ratios ranged from 0.025 to 22 and thus spanned the

range from extant to intrinsic conditions. The S_0/K_S ratios covered a similar range in the preliminary study. As shown in Figure 4.1, the estimated q_{max} values grew larger as S_0/X_0 increased up to approximately 10 because as greater amounts of substrate became available, the restrictions on growth and substrate utilization were lessened, consistent with the observations of Grady et al. (1996) and the references cited therein. The q_{max} value measured at $S_0/X_0 = 10$ represents the intrinsic q_{max} because substantial increases in the parameter estimates were not observed at S_0/X_0 greater than 10. These results are consistent with the findings of Chapter 3, which showed that intrinsic kinetics could be obtained at $S_0/X_0 \geq 10$.

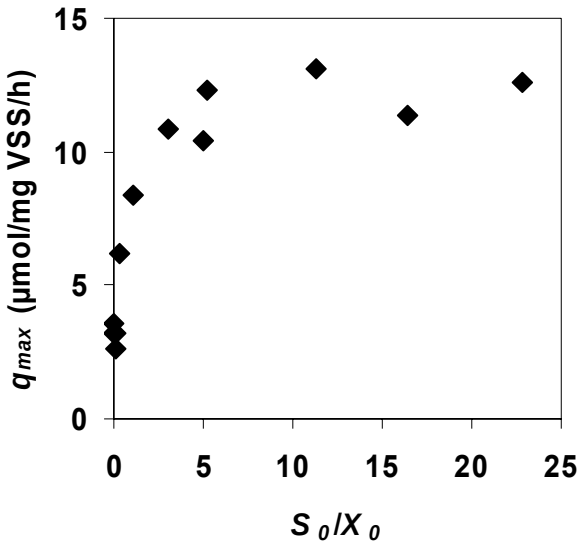

Figure 4.1 Monod kinetic parameters estimates (q_{max}) for PCE dechlorination by *Desulfuromonas michiganensis* strain BB1 in batch cultures with different initial substrate-to-biomass ratios (S_0/X_0).

The amount of correlation between q_{max} and K_S did not appear to be influenced by the S_0/X_0 ratio. As shown in Figure 4.2B, correlation coefficients (R^2) were approximately 0.9 or higher at all of S_0/X_0 ratios tested. This contrasts with guidelines that suggest independent estimates can be obtained at $S_0/X_0 \geq 20$ (Grady et al., 1996). However, the data in Figure 4.2A suggest a trend of decreasing R^2 with increasing S_0/K_S. Because S_0/X_0 was not constant in the kinetic parameter determinations conducted at different S_0/K_S values, the possibility that the S_0/X_0 ratio influenced parameter correlation in these experiments could not be discounted.

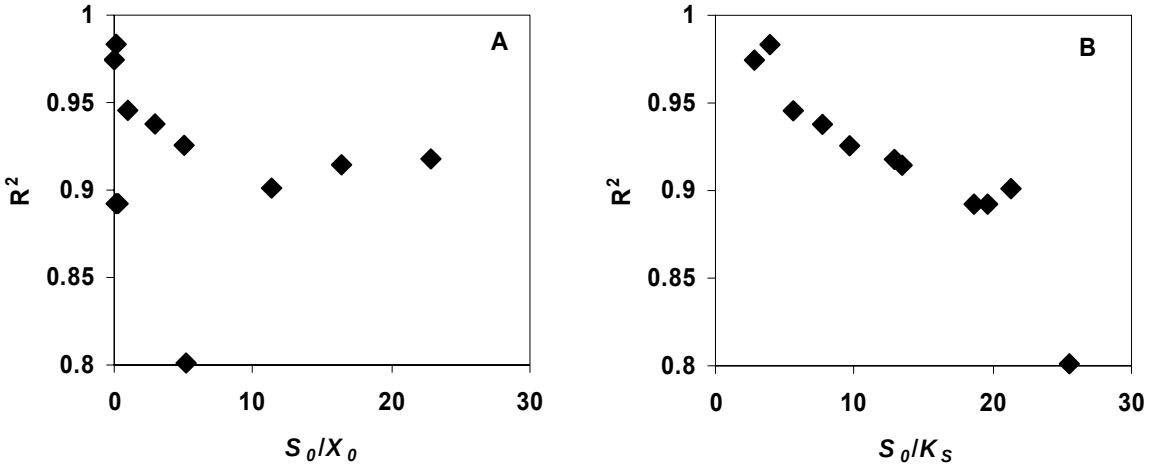

Figure 4.2 Correlation coefficients between the Monod kinetic parameters (q_{max} and K_S) for PCE dechlorination by *Desulfuromonas michiganensis* strain BB1 in batch cultures as a function of (A) initial substrate-to-half saturation constant ratios (S_0/K_S); and (B) initial substrate-to-biomass concentration ratios (S_0/X_0).

Therefore, correlation coefficients (R^2) were computed for numerically simulated batch PCE depletion for strain BB1 under wide range initial conditions ($0.025 \leq S_0/X_0 \leq 50$ and $0.04 \leq S_0/K_S \leq 40$; Table 4.2). This allowed us to unambiguously evaluate

the relationship between S_0/K_S and parameter correlation at constant S_0/X_0, as well as any effects of S_0/X_0 on parameter correlation at constant S_0/K_S. The simulated kinetic parameter determinations are consistent with previous studies that suggest that independent estimates cannot be obtained at $S_0/K_S < 1$ (Ellis et al., 1996; Robinson and Tiedje, 1983). Correlation coefficients (R^2) of approximately 0.99 were calculated for $S_0/K_S = 1$. Based on these results, choosing $S_0=K_S$, as suggested by Ellis et al. (1996), is also not recommended for estimation of independent parameters. While Table 4.2 clearly shows that, for a given S_0/X_0 ratio, the amount of parameter correlation decreases with increasing S_0/K_S, increasing S_0/X_0 does not reduce parameter correlation at a constant S_0/K_S ratio. These data suggest that in theory, it should be possible to obtain independent parameter estimates of q_{max} and K_S at the low S_0/X_0 ratios required for extant kinetics, provided K_S is small enough to result in a large S_0/K_S ratio (10 or higher).

Table 4.2 Correlation coefficients (R^2) for q_{max} and K_S parameter values that were fit to numerically simulated data for a range of initial conditions in the batch culture assays

		S_0/K_S							
		0.04	0.1	0.4	1	4	10	20	40
S_0/X_0	0.025	1	0.9996	0.9958	0.9883	0.9434	0.8984	0.8705	0.8401
	0.1	1	0.9999	0.9971	0.9869	0.9432	0.9065	0.8747	0.8462
	1	1	1	0.9990	0.9925	0.9581	0.9231	0.8968	0.8783
	5	1	1	1	0.9959	0.9738	0.9524	0.9375	0.9280
	10	1	1	1	0.9968	0.9793	0.9633	0.9525	0.9449
	20	1	1	1	0.9970	0.9829	0.9701	0.9620	0.9558
	50	1	1	1	0.9968	0.9853	0.9749	0.9673	0.9601

Liu and Zachara (2001) suggested that due to the inherent correlation between the Monod kinetic parameters, it is difficult to obtain R^2<0.9 for estimates of q_{max} and K_S. The R^2 values calculated in this study using the numerical data were somewhat lower at the highest S_0/K_S ratios. However, it is difficult to determine whether kinetic parameter estimates are identifiable and meaningful based on R^2 values alone. Therefore, the collinearity index, γ_K, which represents the linear dependence of the sensitivity functions of the parameter estimates, was also calculated for each numerical data set evaluated in this study (Table 4.3). Parameter estimates with a γ_K value greater than 10 to 15 are considered poorly identifiable (Brockmann et al., 2008; Brun et al., 2002). Therefore, in this study, synthetic data obtained under initial conditions that resulted in $\gamma_k < 10$ are considered identifiable. Generally, this required a S_0/K_S of 20 or higher, although identifiable parameter estimates could be obtained at lower S_0/K_S ratios provided the S_0/X_0 ratio was also low. R^2 values for parameter estimates with $\gamma_k < 10$ ranged from 0.8401 to 0.9673 (Table 4.2).

Table 4.3 Collinearity index values (γ_K) calculated for q_{max} and K_S parameter values that were fit to numerically simulated data for a range of initial conditions in the batch culture assays

		S_0/K_S							
		0.04	0.1	0.4	1	4	10	20	40
S_0/X_0	0.025	∞	∞	32.12	21.35	9.69	7.51	6.78	6.27
	0.1	∞	∞	58.17	17.86	9.73	7.97	6.92	6.40
	1	∞	∞	38.23	23.19	10.82	8.36	7.32	6.78
	5	∞	∞	∞	24.01	12.54	9.37	8.56	8.06
	10	∞	∞	∞	32.36	13.18	10.18	9.34	8.56
	20	∞	∞	∞	29.43	13.24	11.02	9.8	9.16
	50	∞	∞	∞	28.11	13.24	11.34	9.92	9.30

The results of the numerically simulated batch culture assays made it possible to determine a priori the range of initial conditions that are expected to yield substantial differences in the calculated R^2 values. This information was then used to select the initial conditions for the experimental kinetic assays evaluating the effect of S_0/K_S on parameter correlation at constant S_0/X_0 and the effect of S_0/X_0 on parameter correlation at constant S_0/K_S. For example, based on Table 4.2, an increase in the S_0/K_S ratio from 1 to over 10 was expected to result in a decrease of approximately 0.1 in R^2 value at low S_0/X_0 ratios.

As shown in Figure 4.3A, R^2 decreased from approximately 0.98 at S_0/K_S in the range of 1-2 to approximately 0.88 at S_0/K_S =14 at a constant S_0/X_0 = 0.45. Further, the uncertainty (measured as the dimensionless variance, $\sigma(\theta)/\theta$) associated with the q_{max} and K_S parameter estimates generally decreased with increases in the S_0/K_S ratio (Figure 4.3B). Similarly, Liu and Zachara (2001) found that higher correlation coefficient values resulted in large standard deviations of parameter estimates. In contrast, the S_0/X_0 ratio did not appear to affect the amount of correlation between parameter estimates or their uncertainties (Figure 4.4). Consequently, when designing batch assays for the estimation of kinetic parameters for dehalorespiring populations, choosing a large S_0/X_0 as previously suggested (Grady et al. 1996), appears to be less important than selecting conditions that will result in a high S_0/K_S ratio and thus yield parameter estimates with relatively low correlation and uncertainty.

The results of this study suggest that under both intrinsic and extant conditions, it should be possible to obtain independent Monod kinetic parameter estimates as long

as the S_0/K_S ratio is sufficiently high. However, achieving a high S_0/K_S ratio for PCE and other chlorinated ethenes may be constrained by several factors. First, at high concentrations, PCE is self-inhibitory to some dehalorespiring organisms, including *Desulfuromonas michiganensis* (Amos et al., 2007; This study, Chapter 3). If S_0 is increased to inhibitory levels in an effort to achieve a high S_0/K_S, substrate utilization will not follow Monod kinetics and accurate estimates of q_{max} and K_S cannot be obtained. When estimating extant kinetics, the availability of biomass may also limit how high S_0 can be while still achieving a S_0/X_0 ratio that will prevent biomass growth from occurring. Finally, if K_S is relatively high, it may not be possible to select a S_0 that is below inhibitory conditions but still high enough to yield independent parameter estimates.

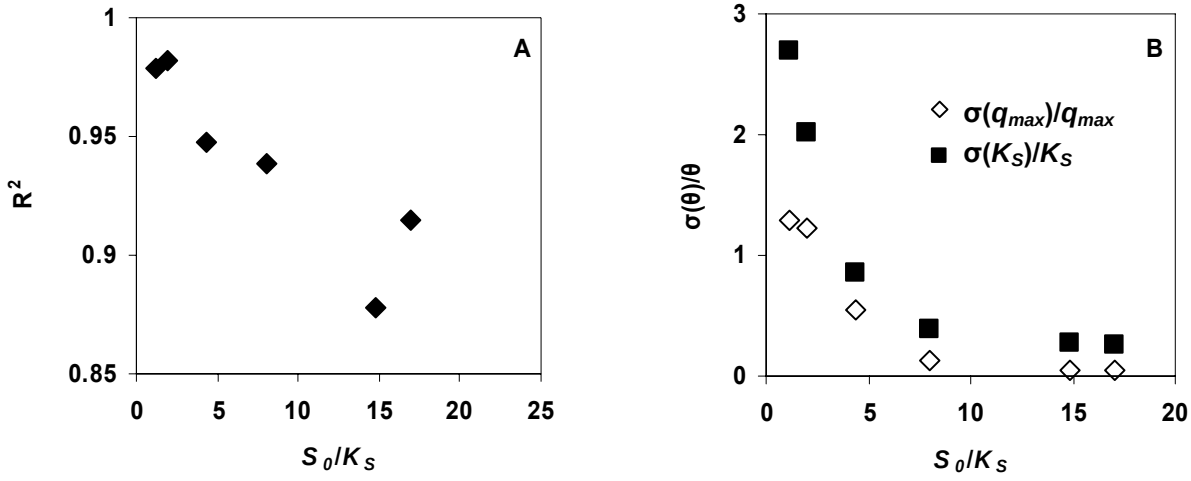

Figure 4.3 (A) Correlation coefficients (R^2); and (B) dimensionless variances ($\sigma(\theta)/\theta$) of the Monod kinetic parameters (q_{max} and K_S) for PCE dechlorination by strain BB1 in batch cultures with different initial substrate-to-half saturation constant ratios (S_0/K_S) and constant $S_0/X_0 = 0.45$.

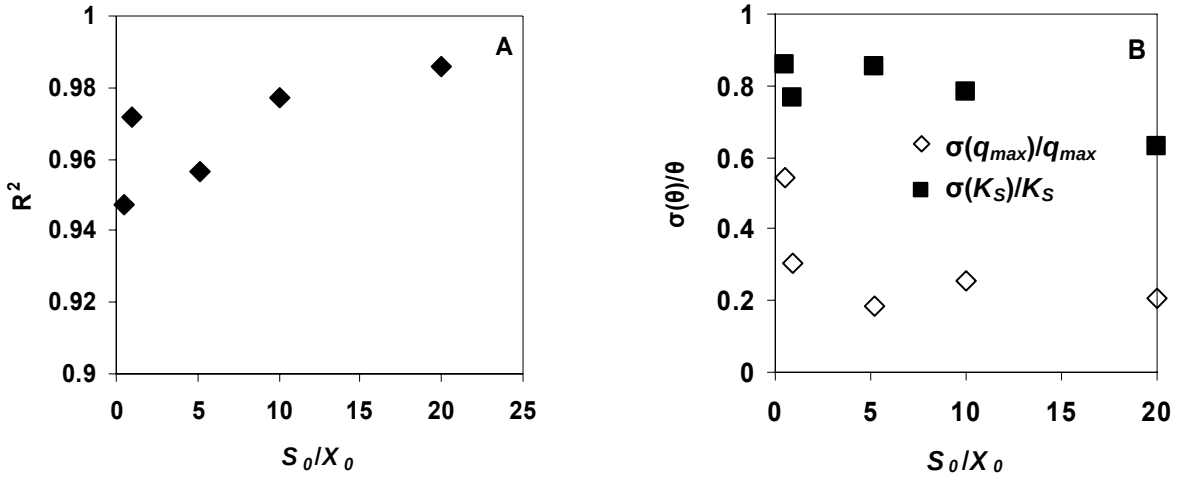

Figure 4.4 (A) Correlation coefficients (R^2); and (B) dimensionless variance ($\sigma(\theta)/\theta$) of the Monod kinetic parameter (q_{max} and K_S) for PCE dechlorination by strain BB1 in batch cultures with different initial substrate-to-biomass concentration ratios (S_0/X_0) and constant $S_0/K_S = 4$.

Because of these constraints, an iterative approach is needed to select the appropriate conditions in batch assays used to determine extant parameters to describe dehalorespiration kinetics. First, preliminary experiments should be conducted to estimate K_S. If K_S is relatively high, then estimation of unique and extant Monod kinetic parameters may not be possible. In this case, estimation of a lumped first-order biodegradation rate coefficient (q_{max}/K_S) may be most appropriate. If K_S is relatively low, then theoretical evaluation of the correlation coefficients (as was done to generate Table 4.3 in this study) should be used to select an S_0 that will minimize the correlation between the estimated q_{max} and K_S values. The X_0 needed to ensure that extant kinetic parameters are estimated should then be determined based on the S_0. Finally, R^2, $\sigma(\theta)/\theta$, and γ_k should be calculated for the experimental parameter

values obtained under these conditions to confirm that they meet all the criteria for identifiable parameter estimates.

To accurately predict the removal of PCE and other chlorinated ethenes in environments in which dehalorespiration activity is limited by substrate availability or the solids retention time, kinetic parameters must be estimated under extant conditions. This can be achieved by controlling the S_0/X_0 ratio in batch kinetic assays. The initial conditions also influence parameter correlation and uncertainty. However, S_0/K_S was shown to be much more important than S_0/X_0 in determining parameter independence and uncertainty. Theoretical and experimental evaluations suggest that an S_0/K_S of at least 4 under extant conditions and 10 under intrinsic conditions should be employed to obtain independent estimates of q_{max} and K_S. Higher S_0/K_S ratios could theoretically lower parameter correlation further, but may not be practically feasible for the determination of dehalorespiration kinetics due to the self-inhibitory effects of PCE in some organisms and the availability of biomass, particularly when working with pure cultures. When initial conditions corresponding to extant conditions (S_0/X_0 = 0.025 on a COD basis) and S_0/K_S>4 were used, independent estimates of q_{max} and K_S were successfully obtained for strains BB1 and PCE1, as summarized in Table 4.4. The corresponding substrate depletion curves are shown in Figures 4.5 and 4.6 for strain BB1 and strain PCE1. Because of the nature of extant kinetic parameters, the values reported in Table 4.2 are applicable to strain BB1 and strain PCE1 maintained at a 20-d SRT. However, the procedure used to obtain these parameter estimates is universally applicable.

Table 4.4 Extant kinetic parameter estimates for chlorinated ethene utilization by strain BB1 and strain PCE1 maintained at a 20-d SRT at room temperature (22°C) or 30°C

	Electron acceptor	Temperature	q_{max} (μmol/mg VSS·h)[a]	K_S (μM)[a]
Desulfuromonas michiganensis strain BB1	PCE	30°C	3.20 (0.43)	1.19 (0.08)
		22°C	2.59 (0.22)	0.84 (0.17)
	TCE	22°C	7.13 (0.89)	2.15 (0.57)
Desulfitobacterium sp. strain PCE1	PCE	30°C	3.12 (0.22)	3.98 (0.57)
		22°C	2.42 (0.30)	2.63 (0.87)

[a] Values in parentheses represent the standard deviation of the estimates from the triplicate batch culture assays.

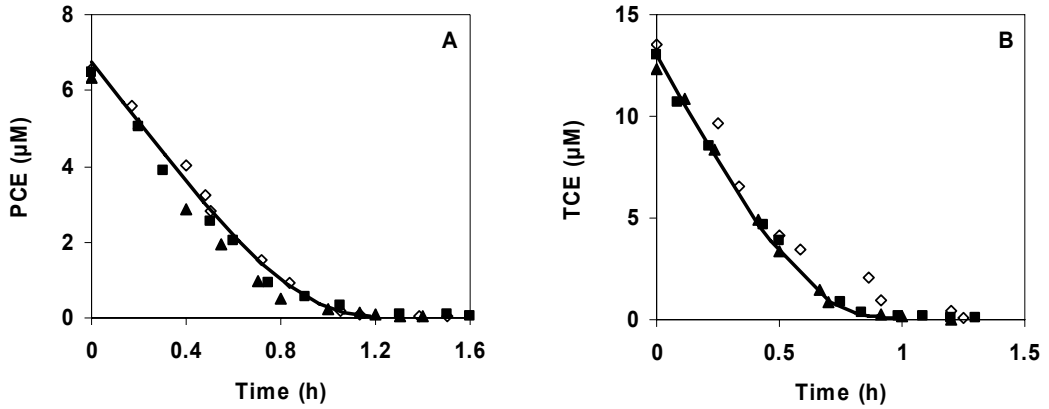

Figure 4.5 Extant kinetic substrate depletion curves in batch culture assays of *Desulfuromonas michiganensis* strain BB1 maintained at a 20-d SRT at room temperature (22°C). (A) PCE and (B) TCE. Data points represent individual data points obtained in replicate experiments. Solid lines represent the best fit of Equation 4.2 to the pooled data.

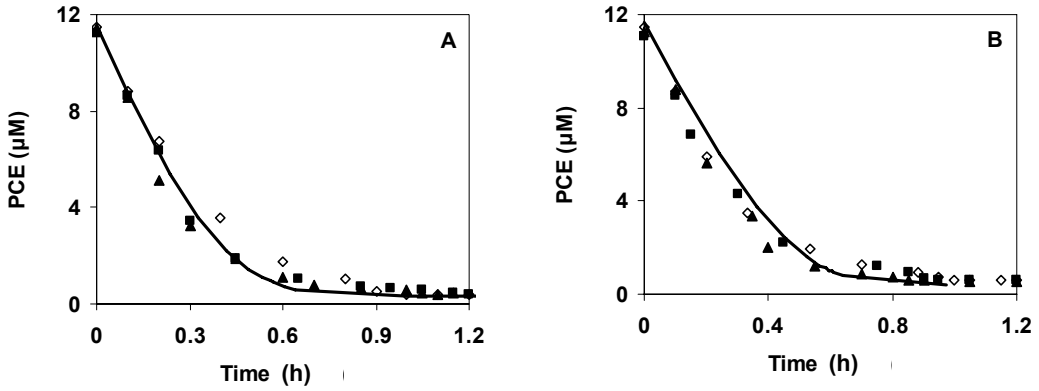

Figure 4.6 Extant kinetic substrate depletion curves in batch culture assays of *Desulfitobacterium* sp. strain PCE1 under extant conditions at (A) 30^{o}C and (B) room temperature (22^{o}C). Data points represent individual data points obtained in replicate experiments. Solid lines represent the best fit of Equation 4.2 to the pooled data.

In chlorinated ethene-contaminated groundwater, the concentrations of PCE and populations of dehalorespirers can vary significantly depending on the distance of the contaminated sites from contaminated source zones and the nature of the bioremediation approach (engineered bioremediation or natural attenuation). Intrinsic kinetic parameter estimates such as those reported in Chapter 3 may be appropriate for describing the activity of dehalorespirers where substrate availability is high, provided chlorinated ethene levels are not toxic. The activity of dehalorespirers elsewhere in the contaminant plume however should be described using extant kinetics obtained following the parameter estimation techniques recommended above. However, the applicability of reported kinetic parameter estimates that do not describe the conditions of the kinetic assays under which they were obtained is not clear. Kinetic estimates with relative high uncertainties are not meaningful either. Becker (2006) used mathematical modeling to demonstrate that the results for

bioremediation of PCE using dehalorespirers can change significantly due to small changes in the K_S values of the dehalorespirers. Therefore, it is clearly important that modeling predictions of PCE bioremediation must be based on identifiable extant kinetic parameter estimates, which can be obtained using the approach outlined in this study.

Chapter 5: Substrate inhibition and dechlorination kinetics at high PCE concentration

5.1 Introduction

Tetrachloroethene (PCE) is widely used as a raw material for the manufacture of hydrofluorocarbon refrigerant and as a dry cleaning solvent and metal cleaning and degreasing agent. Improper handling and disposal of PCE have contributed to the contamination of groundwater with PCE, which is among the most frequently detected chemical contaminants in groundwater at Superfund sites (EPA, 2005). Since complete reductive dechlorination of PCE to ethene was demonstrated by Freedman and Gossett (1989), natural attenuation and engineered bioremediation approaches using microorganisms that couple reductive dechlorination to energy metabolism (dehalorespiration) have increasingly been used in the *in situ* clean up of aqueous-phase PCE and lesser dechlorinated ethenes such as trichloroethene (TCE). However, because of its low aqueous solubility (1.459 mM at 22^{o}C; Knauss et al., 2000) and high hydrophobicity (partitioning coefficient Log K_{OC}=2.2-2.7; ATSDR, 1997), PCE tends to form dense non-aqueous phase liquids (DNAPLs) in subsurface environments when sufficient quantities are present.

Biotransformation of chlorinated ethene DNAPLs previously was not believed possible because of the toxicity of chlorinated ethenes at high concentrations (Bouwer 1994; Robertson and Alexander 1996), but recent investigations suggest that under certain conditions, biodegradation of PCE at aqueous concentration approaching

saturation levels is possible (Adamson et al. 2004; Carr et al. 2000; Cope and Hughes 2001; Dennis et al. 2003; Sleep et al., 2006; Sung et al. 2003; Yang and McCarty 2000, 2002).

To model biodegradation of high aqueous concentrations of PCE and its effect on dissolution of PCE from the NAPL phase (Becker and Seagren, 2009), an accurate kinetic model is needed. The Monod model has been used to describe reductive dechlorination of chlorinated ethenes at relatively low aqueous concentrations (Cupples et al., 2003; Fennell and Gossett, 1998; Haston and McCarty, 1999) according to

$$\frac{dS_a}{dt} = -q_{\max} X \frac{S_a}{K_S + S_a} \tag{5.1}$$

where S_a is the aqueous concentration of the chlorinated ethene electron acceptor [M_S L^{-3}]; q_{max} [M_S M_X^{-1} T^{-1}] is the maximum specific substrate utilization rate for the chlorinated ethene electron acceptor; X is the biomass concentration [M_X L^{-3}]; and K_S [M_S L^{-3}] is the half-saturation constant for the chlorinated ethene electron acceptor. Biomass growth is described according to

$$\frac{dX}{dt} = -Y \frac{dS_a}{dt} - k_d X \tag{5.2}$$

where Y is the true yield coefficient [M_X M_S^{-1}] and k_d [T^{-1}] is the endogenous decay coefficient.

At higher concentrations, chlorinated ethenes may inhibit their own dechlorination through toxicity effects or the dechlorination of other chlorinated ethenes through competition for key enzymes. Thus, in some cases, Andrews kinetics (self-inhibition) and/or kinetic models that include terms describing competitive inhibition between chlorinated ethenes are used instead of Monod kinetics (Cupples et al., 2004; Garant and Lynd, 1998; Tandoi et al., 1994; Yu and Semprini, 2004). The Andrews kinetic model (Andrews 1968) includes a self-inhibition coefficient, K_{AI} [M_S L^{-3}], according to

$$\frac{dS_a}{dt} = -q_{\max} X \frac{S_a}{K_S + S_a + \frac{S_a^2}{K_{AI}}} \tag{5.3}$$

Competitive inhibition between chlorinated ethenes is modeled according to

$$\frac{dS_a}{dt} = -q_{\max} X \frac{S_a}{K_S\left(1 + \frac{S_I}{K_{CI}}\right) + S_a} \tag{5.4}$$

where S_I [M_S L^{-3}] is the aqueous concentration of the chlorinated ethene inhibitor, and K_{CI} [M_S L^{-3}] is the competitive inhibition coefficient. Recently, the Luong model (Luong, 1987)

$$\frac{dS_a}{dt} = -q_{\max} X \frac{S_a}{K_S + S_a}\left(1 - \frac{S_a}{S_{a-\max}}\right)^n \tag{5.5}$$

where $S_{a\text{-}max}$ [M_S L^{-3}] is the maximum concentration of the chlorinated ethene electron acceptor at which dechlorination will occur and n is a power term that determines

how the specific growth rate q [$M_S M_X^{-1} T^{-1}$], responds to changes in S_a as it approaches $S_{a\text{-}max}$, was applied to describe the activity of dehalorespiring strains that stopped dechlorinating at aqueous PCE concentration exceeding a maximum level (540 μM; Amos et al., 2007). Several factors including temperature (Heimann et al., 2007; Friis ea al., 2007), and differences in the source culture characteristics or history (Grady et al., 1996) may explain in part why dechlorination of PCE proceeds at high aqueous concentrations in some systems (Sung et al., 2003, Yang and McCarty, 2000), but not in others (Amos et al., 2007). In addition, the low octanol-water portion coefficient (K_{OW}) of PCE (log K_{OW}=3.40; ATSDR, 1997) suggests that it will preferentially accumulate in and disrupt the cytoplasmic membrane (Ramos et al., 2002). The inactivation of biomass caused by the toxicity of the products of cometabolism of chlorinated solvents and trinitrotoluene has been modeled by incorporating a biomass inactivation term in the biomass growth equation (Alvarez-Cohen and McCarty, 1991; Alvarez-Cohen and Speitel, 2001; Yin et al., 2005). This type of solvent effect results in an irreversible inactivation of biomass, and therefore, the active biomass concentration may decrease due to exposure of PCE with the transformation of substrate (Sikkema et al., 1995).

5.2 Materials and methods

5.2.1 Chemicals

Sodium acetate (Certified ACS) was obtained from Fisher Scientific and sodium L-lactate (60% wt. solution) was obtained from Acros Organics. PCE, TCE, *cis*-dichloroethene (*cis*-DCE), sodium acetate and sodium L-lactate were obtained from

the sources described in Chapter 3. All of the other chemicals used were reagent grade or better, unless otherwise specified.

5.2.2 Culture maintenance and medium

Two pure cultures *Desulfuromonas michiganensis* strain BB1 (DSM 15941) and *Desulfitobacterium* sp. strain PCE1 (DSM 10344) were used in this study. They were maintained in semi-continuous cultures, as described in Chapter 3, to ensure that all cultures used for batch culture experiments had the same culture history and were always at the same physiological state.

5.2.3 Determination of competitive inhibition coefficients

Five sets of experiments were conducted to evaluate the inhibition effects among chlorinated ethenes transformed and produced by strains BB1 and strain PCE1, as summarized in Table 5.1. For each experiment, 10 batch assays were prepared in 160-mL serum bottles containing 95 mL of mineral media and 5 mL of inoculum obtained from a semi-continuous culture. The batch assays contained the same concentrations of biomass, electron donor, and chlorinated ethene electron acceptor, but a different concentration (ranging from 0 μM to aqueous saturation) of a chlorinated ethene inhibitor was added to each assay. The ratio of the initial chlorinated ethene electron acceptor concentration to the initial biomass concentration was the same as that used in Chapter 3 and allowed unrestricted growth to occur. Therefore, the estimates of K_S and q_{max} that were obtained under intrinsic conditions (Chapter 3) could be used to fit the inhibition constants. Electron donor concentrations (2.5 mM) were in stoichiometric excess of the chlorinated ethene electron acceptors. Chlorinated

ethenes were added to the batch assays from sterile stock solutions at least two days before inoculation to ensure equilibration between the gas and aqueous phases. All experiments were conducted at 30°C.

Table 5.1 Initial conditions and Monod kinetic parameter estimates used in determination of inhibition constants for *Desulfuromonas michiganensis* strain BB1 and *Desulfitobacterium* sp. strain PCE1.

Organism	q_{max} [a]	K_S [b]	X_0 [c]	Electron acceptor	Electron acceptor concentration (μM)	Inhibitor	Inhibitor concentration (μM)
Dsm. michiganensis strain BB1	12.6	9.31	0.14	PCE	135	TCE	0–922
						cis-DCE	0–1687
				TCE	270	PCE	0–743
						cis-DCE	0–1289
Dsf. sp. strain PCE	5.36	8.81	0.70	PCE	340	TCE	0–1024

[a] From Chapter 3, units of μmol/mg VSS/h; [b] From Chapter 3, units of μM; [c] Units of mg VSS/L.

The initial dechlorination rate, $\frac{dS_a}{dt}$, was calculated for each assay based on substrate depletion data obtained during the first 5 h of the experiment. Growth was insignificant during this period; therefore, X could be treated as a constant. K_{CI} was fit to a linear form of Equation (5.4),

$$\frac{q_{\max} S_a}{\nu_T} = S_a + K_S + \frac{K_S}{K_{CI}} S_I \qquad (5.6)$$

where $\nu_T = \frac{dS_a}{Xdt}$ is the initial unit dechlorination rate [$M_S\ M_X^{-1}\ T^{-1}$]. The estimates of K_S and q_{max} used to fit K_{CI} are shown in Table 5.1.

5.2.4 Evaluation of dechlorination of high PCE concentrations

As described above, dechlorination by several pure dehalorespiring strains did not proceed above a PCE concentration of around 540 μM in one previous study (Amos et al., 2007), whereas others report dechlorination of higher PCE concentrations (Sung et al., 2003, Yang and McCarty, 2000). As described below in Section 5.3, chlorinated ethene levels above 540 μM were completely depleted by relatively high concentrations of strain BB1 and PCE1 in the current study, but not at lower biomass concentrations. One potential explanation for the different results is that the solvent effects of chlorinated ethenes may inactivate a portion of the dechlorinating biomass, and the fraction of biomass that is inactivated may influence the apparent $S_{a\text{-}max}$ in a batch culture.

Two sets of experiments were conducted in this study to examine whether the initial biomass concentration affects the apparent $S_{a\text{-}max}$ value for dechlorination of chlorinated ethenes. Four or six batch assays with different combinations of initial biomass and PCE concentrations (Table 5.2) were prepared for each strain in 160-mL serum bottles. Neat sterile PCE was added to the serum bottles at least two days before adding enough inoculum from the semi-continuous source cultures (described above) to bring the aqueous volume to 100-mL. Electron donor concentrations (5 mM) were provided in stoichiometric excess of PCE. The concentrations of both electron donors and acceptors were high enough relative to the initial biomass concentrations to allow unrestricted growth to occur (in the absence of toxicity effects) and description of dechlorination kinetics using previous estimates of intrinsic q_{max} and K_S

values (Table 5.1). However, at the highest strain PCE1 biomass concentration tested (X_0=27.2 mg VSS/L), the S_0/X_0 ratio is approximately 1.4 (on a COD basis). The cultures were continuously shaken at 120 rpm at an incubation temperature of 30°C. PCE concentrations were monitored through headspace sampling for 20 d or until PCE was depleted.

Table 5.2 Initial conditions for substrate inhibition experiments for *Desulfuromonas michiganensis* strain BB1 and *Desulfitobacterium* sp. strain PCE1

Organism	Biomass concentration (mg VSS/L)	PCE Concentration (μM)
Desulfuromonas michiganensis strain BB1	0.35	550
	0.35	800
	4.5	1200
	9	1200
Desulfitobacterium sp. strain PCE1	1.3	580
	1.3	650
	1.3	750
	1.3	850
	7.2	680
	27.2	1320

When substantial PCE dechlorination was not observed within 20 d, the batch cultures were purged with 80% N_2/20% CO_2 (Airgas East) for 30 min to completely remove the chlorinated ethenes in the batch cultures. Then 100 μM of PCE was added to the batch cultures to test whether the dechlorinating activities of the cells could recover after high PCE concentrations were removed.

5.2.5 Model development

The substrate utilization kinetic models described above, when coupled with the biomass growth expression in Equation 5.2, cannot capture the PCE dechlorination phenomena observed in this study. Specifically, the Monod (Equation 5.1), Andrews (Equation 5.3), and Monod model with competitive inhibition (Equation 5.4) all predict that dechlorination will continue for all $X > 0$ and thus are not appropriate for describing the experimental systems in which PCE removal stopped completely. The Luong model (Equation 5.5) does incorporate a $S_{a\text{-}max}$ term that prevents dechlorination at PCE concentrations above $S_{a\text{-}max}$. The Wayman and Tseng model is essentially a discontinuous version of the Luong model and also incorporates a $S_{a\text{-}max}$ term according to:

$$\frac{dS_a}{dt} = -q_{\max} X \frac{S_a}{K_S + S_a} + I_T \left(S_a - K_{WI}\right) \qquad \text{for } K_{WI} < S_a < S_{a\text{-}max} \qquad (5.7)$$

where the inhibition term I_T [T^{-1}] represents the rate of increase in the extent of inhibition between K_{WI}, the threshold concentration below which no inhibition is observed, and $S_{a\text{-}max}$. At $S_a < K_{WI}$, the Wayman and Tseng model is identical to the Monod model (Equation 5.1), and $\frac{dS_a}{dt} = 0$ at $S_a > S_{a\text{-}max}$, However, neither the Luong or Wayman and Tseng models account for the fact that $S_{a\text{-}max}$ does not appear to be constant and seems to depend on X_0.

Therefore, a new approach to modeling reductive dechlorination of high chlorinated ethene concentrations was developed by adapting the transformation

capacity (T_C) concept used in modeling cometabolic oxidation of TCE to describe the inactivation of biomass by oxidation products (Alvarez-Cohen and McCarty). In the current model, T_C [M_X M_S^{-1} T^{-1}] is incorporated into the biomass growth term (Equation 5.2) according to

$$\frac{dX}{dt} = -Y\frac{dS_a}{dt} - k_d X - T_C S_a \tag{5.8}$$

According to Equation 5.8, the net rate of biomass growth will be decreased due to the toxicity of the chlorinated ethene. The PCE dechlorination rate, $\frac{dS_a}{dt}$, is described using one of the substrate utilization models given by Equations 5.1, 5.3, 5.5, and 5.7. The substrate utilization equations coupled with Equation 5.8 are referred to the Monod, Andrews, Luong, Wayman and Tseng inactivation models, respectively.

The ability of the four inactivation models to describe the inhibitory effects of high PCE concentrations on reductive dechlorination by strains BB1 and PCE1 was evaluated by fitting them to the pooled experimental data obtained from the batch culture experiments conducted with a given strain under various initial conditions as described above. The equations for biomass growth and PCE dechlorination are coupled nonlinear ordinary differential equations. The equations were solved using the fourth-order Runge-Kutta method by employing Matlab (version 7.0). The values for Y, q_{max}, K_S, and k_d used in the above equations, were obtained from Chapter 3. The remaining parameters in Equations 5.3, 5.5, 5.7, and 5.8 were estimated by coupling the above solutions with the nonlinear least-squares optimization function,

LSQNONLIN, in Matlab (version 7.0) to minimize the sum of squared errors (SSE) between the pooled PCE concentrations measured in the experiments and the concentrations predicted by the model. The goodness of fit for each model was measured by calculating the SSE and F-value (DeVore and Peck, 1996).

5.2.6 Analytical methods

Chlorinated ethenes were analyzed in the headspace using the gas chromatograph method described in Chapter 3. The biomass in the batch cultures was estimated based on protein content. Protein was quantified using the Bradford protein quantification kit (Sigma Inc.) following the instructions provided by the manufacturer. Protein standards were prepared using bovine serum albumin (Lyophilized powder; Acros Organics).

5.3 Results and Discussion

The effects of PCE on TCE dechlorination followed a competitive inhibition pattern. The initial TCE dechlorination rate decreased with increasing PCE concentration, and even low PCE concentrations exhibited an effect as shown in Figure 1. At a PCE concentration of nearly 800 μM, TCE dechlorination was barely detectable. Therefore, only data obtained at PCE concentrations lower than 500 μM were used to fit Equation 5.6 and estimate the inhibition coefficient (K_{CI}) values. K_{CI} was estimated as 8.20±1.82 μM, which is similar in magnitude to the previously estimated K_S for TCE dechlorination by strain BB1 (Chapter 3). This result is consistent with competitive inhibition studies involving a *cis*-DCE dehalorespirer, in which the estimates of K_{CI} (Cupples et al., 2004) and K_S (Haston and McCarty, 1999)

for the dehalorespirer were similar in magnitude. Yu and Semprini (2004) also showed that when a K_{CI} equal to K_S was assumed, model predictions of PCE dechlorination fit the experimental data well.

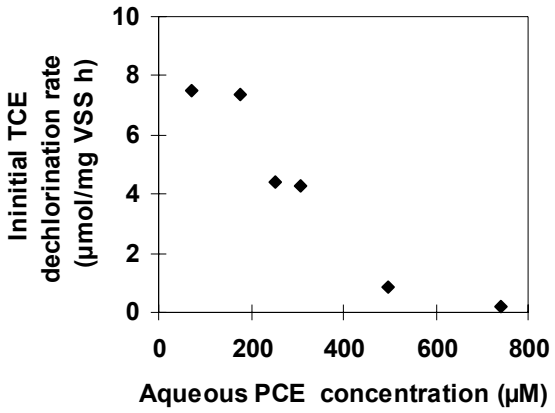

Figure 5.1 Initial TCE dechlorination rate in the presence of different aqueous concentrations of PCE by *Desulfuromonas michiganensis* strain BB1.

In all of the other inhibition scenarios evaluated, a different pattern was observed. For example, the initial PCE dechlorination rate in strain BB1 was not affected by TCE concentrations of less than 700 µM (Figure 5.2A). Under these conditions, the initial PCE dechlorination rate averaged 10.3±0.7 (S.D.) µmol PCE/mg VSS·h. The dechlorination of PCE in all the batch cultures was further monitored after the initial dechlorination rates were obtained. Dechlorination continued until all of the available PCE and TCE was dechlorinated to *cis*-DCE in batch cultures containing less than 700 µM TCE. In batch cultures with more than 700 µM TCE, less than 2% of the total chlorinated ethene was dechlorinated and dechlorination completely ceased after 5 h of inoculation.

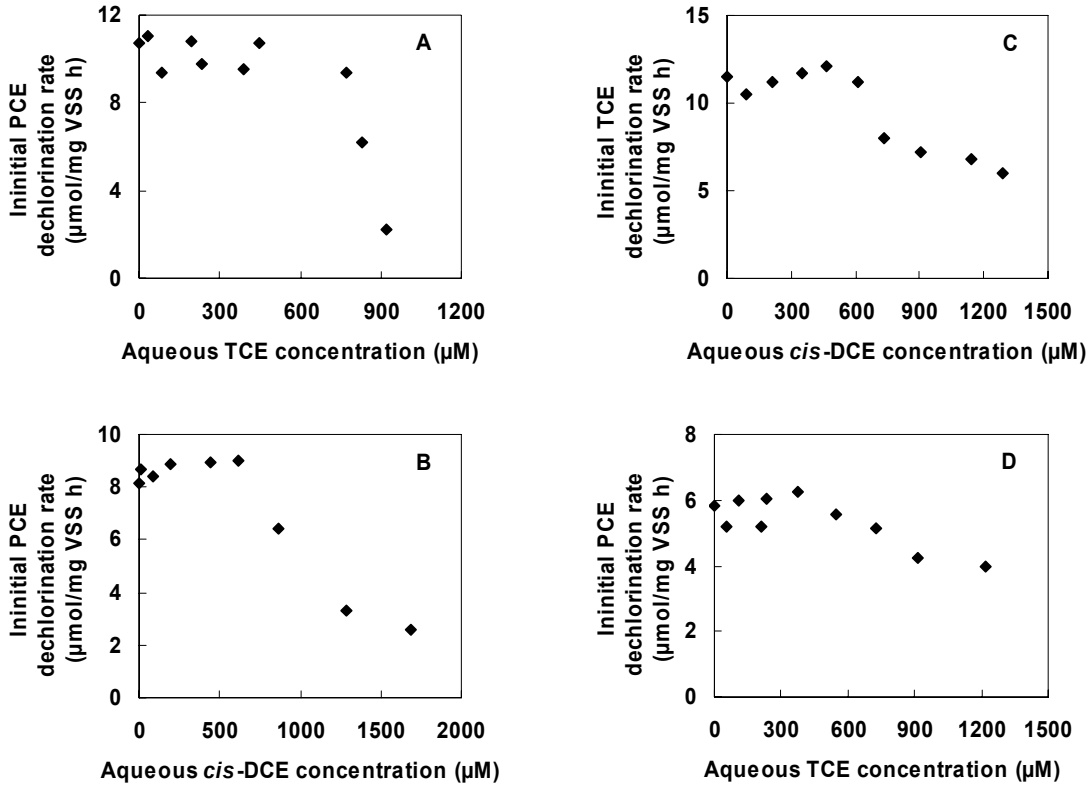

Figure 5.2 Initial dechlorination rates of (A) PCE in the presence of different TCE concentrations; (B) PCE in the presence of different *cis*-DCE concentrations; and (C) TCE in the presence of different *cis*-DCE concentrations by *Desulfuromonas michiganensis* strain BB1; and (D) PCE in the presence of different TCE concentrations by *Desulfitobacterium* sp. strain PCE1.

Similar results were obtained for the inhibition on PCE dechlorination by *cis*-DCE as shown in Figure 5.2B. The initial PCE dechlorination rate was not affected by *cis*-DCE concentrations of less than 700 µM and averaged 8.7±0.3 (S.D.) µmol PCE/mg VSS·h. At *cis*-DCE concentrations ≥ 800 µM, the initial PCE dechlorination rate decreased with increasing *cis*-DCE concentrations. Further, the dechlorination activity in the cultures containing high concentrations of *cis*-DCE completely stopped after 10 h of incubation.

The initial TCE dechlorination rates by strain BB1 in the presence of different aqueous *cis*-DCE concentrations are shown in Figure 5.2C and followed the same pattern observed for PCE dechlorination with varying amounts of TCE and *cis*-DCE. *cis*-DCE has no effect on TCE dechlorination by strain BB1, except at *cis*-DCE concentration ≥ 700 μM. The initial TCE dechlorination rate decreased with increasing *cis*-DCE concentration of greater than 700 μM.

TCE concentrations below 500 μM did not affect the rate of PCE dechlorination by strain PCE1. The average initial PCE dechlorination rate was 5.87±0.63 (S.D.) μmol PCE/mg VSS·h (Figurer 5.2D) consistent with a previous estimate of maximum PCE utilization rate for this strain (Chapter 3).

The results shown in Figure 5.2 suggest that high concentrations of TCE and *cis*-DCE (in excess of 700-800 μM) have an inhibitory or toxic effect on *Desulfuromonas michiganensis* strain BB1. As noted above, a recent evaluation of reductive dechlorination of high concentrations PCE also showed that several pure dehalorespiring cultures (including strain BB1) stopped growing at lower PCE concentrations (≥540 μM) (Amos et al., 2007). One possible explanation for the differences in the apparent $S_{a\text{-}max}$ values in the previous and current studies is that they were the result of differences in the experimental conditions, such as the initial biomass concentrations (X_0). X_0 was 0.35 mg VSS/L for strain BB1 in the kinetic experiments shown in Figures 5.1 and 5.2, but was likely lower (less than 1.8 mg VSS/L) in the study by Amos et al. (2007). Therefore, a series of experiments was undertaken to evaluate the effect of the initial biomass concentration on the self-inhibitory effects of PCE on dechlorination by strains BB1 and PCE1.

The evaluation of *cis*-DCE and TCE inhibition of dechlorination by strain BB1 suggested that at X_0=0.35 mg VSS/L, $S_{a\text{-}max}$ was approximately 700 μM. Therefore, dechlorination of PCE concentration above and below this apparent $S_{a\text{-}max}$ was evaluated for X_0=0.35 mg VSS/L. Dechlorination of PCE at concentrations exceeding 1000 μM was also evaluated with initial biomass concentrations 12.5 (Figure 5.3C) or 25 (Figure 5.3D) times higher than in the batch cultures containing X_0=0.35 mg VSS/L. At the low initial biomass concentration, PCE dechlorination was consistent with the patterns observed in Figure 5.2. PCE was dechlorinated at an initial concentration of approximately 550 μM (Figure 5.3A), but not at 800 μM (Figure 5.3B).

Similar results were obtained with six cultures containing strain PCE1 at X_0 ranging from 1.3 to 27.2 mg VSS/L and PCE concentrations ranging from approximately 600 μM to above the PCE solubility limit. At the lowest initial biomass concentration, PCE dechlorination rates decreased with increasing initial PCE concentrations and stopped completely at an initial PCE concentration of around 860 μM (compare Figures 5.4A-D). As expected, removal of approximately 700 μM PCE occurred more rapidly at a higher X_0 (Figure 5.4E), and at the highest biomass concentration, PCE was completely dechlorinated even at an initial PCE concentration exceeding the aqueous solubility limit (Figure 5.4F).

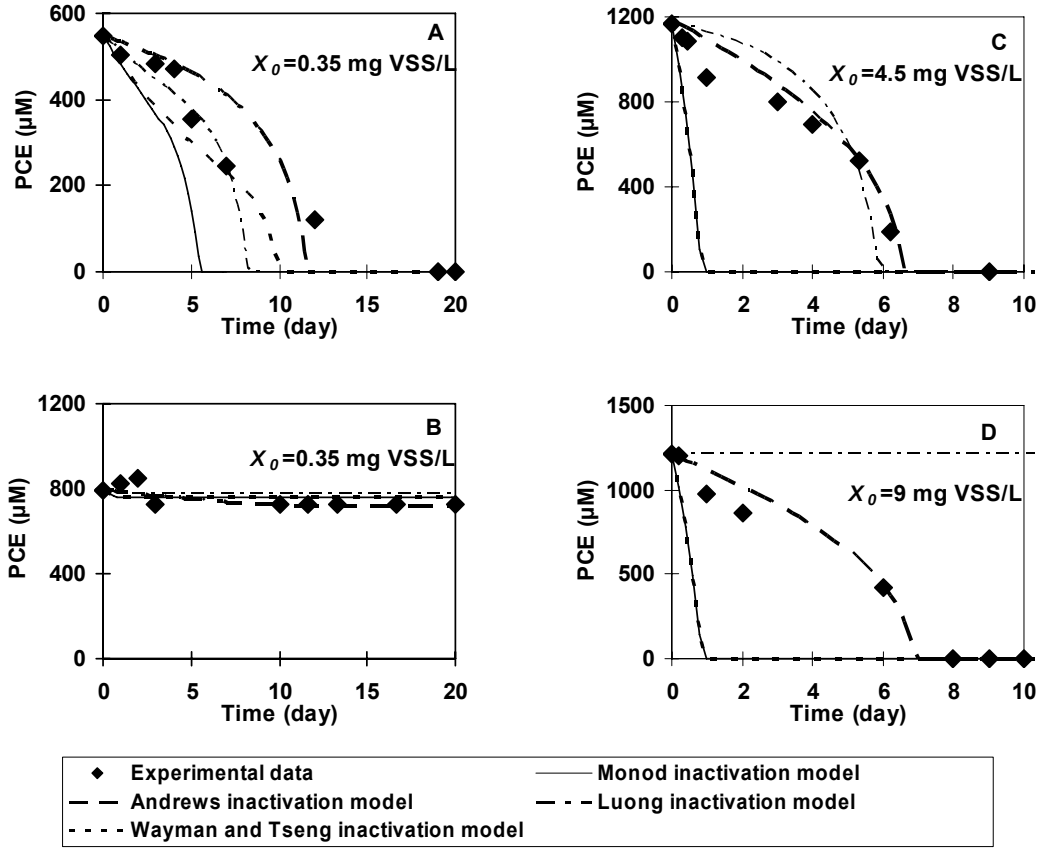

Figure 5.3 Comparison of Monod, Andrews, Luong, and Wayman and Tseng inactivation model predictions and experimental data for dechlorination of high PCE concentrations by different levels of *Desulfuromonas michiganensis* strain BB1. (A) X_0=0.35 mg VSS/L, $S_{PCE,0}$=543 µM; (B) X_0=0.35 mg VSS /L, $S_{PCE,0}$=799 µM; (C) X_0=4.5 mg VSS/L, $S_{PCE,0}$=1183 µM; (D) X_0=9 mg VSS/L, $S_{PCE,0}$=1183 µM. The Wayman and Tseng inactivation model predictions are equivalent to those of the Monod inactivation model in (A), the Andrews inactivation model in (B), and the Luong inactivation model in (C) and (D). The Luong and Andrews inactivation model predictions are equivalent in (B).

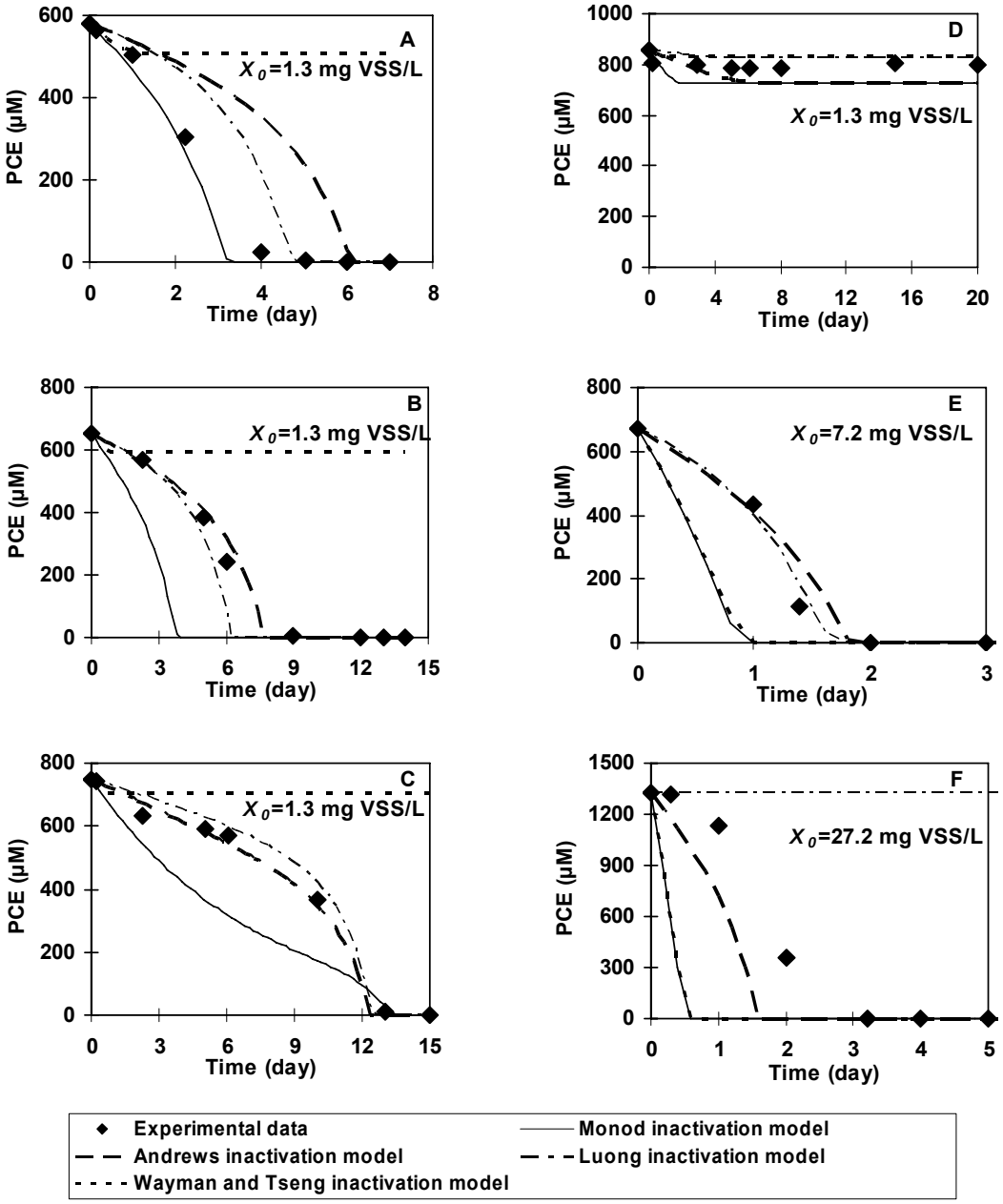

Figure 5.4 Comparison of the Monod, Andrews, Luong, and Wayman and Tseng inactivation model predictions and experimental data for dechlorination of high PCE concentrations by different levels of *Desulfitobacterium* sp. strain PCE1. (A) X_0=1.3 mg VSS/L, $S_{PCE,0}$=581 μM; (B) X_0=1.3 mg VSS /L, $S_{PCE,0}$=645 μM; (C) X_0=1.3 mg VSS /L, $S_{PCE,0}$=774 μM; (D) X_0=1.3 mg VSS /L, $S_{PCE,0}$=839 μM; (E) X_0=7.2 mg VSS/L, $S_{PCE,0}$=671 μM; (F) X_0=27.2 mg VSS/L, $S_{PCE,0}$=1387 μM. The Wayman and Tseng and Monod inactivation model predictions are equivalent in (E) and (F).

The Monod, Andrews, Luong, and Wayman and Tseng inactivation models were fit to the experimental PCE concentration data in Figure 5.3 and 5.4. The parameter estimates for the four models that best fit these data are summarized in Table 5.3. All of the models fit the experimental data showing almost no dechlorination when PCE concentrations of about 800 μM were added to the lowest biomass concentrations (Figure 5.3B and 5.4D).

However, the models differed with respect to their ability to predict the PCE dechlorination curves obtained under the remaining conditions. Qualitative observations suggest the Monod inactivation model fit the PCE dechlorination data well only at the lowest PCE concentration tested with strain PCE1 (Figure 5.4A), in part because the substrate utilization equation does not include an inhibition term. The Luong inactivation model was used by Amos et al. (2007) to describe PCE dechlorination by strain BB1 in the presence of PCE levels that exceeded the solubility limit. In the present study, the Luong inactivation model accurately predicted that PCE dechlorination would not proceed when PCE reaches a critical concentration equal to $S_{a\text{-}max}$ (1210 μM for strain BB1 and 963 μM for strain PCE1), and qualitatively appeared to fit the experimental data when biomass concentrations were relatively low (Figure 5.3A, B, and Figure 5.4A-D). However, the Luong inactivation model predictions did not capture the experimental data obtained at the highest biomass concentrations (Figures 5.3D and 5.4F). In these two cases, the initial PCE concentrations exceeded the apparent $S_{a\text{-}max}$ values for observed strain BB1 and strain PCE1 at low biomass concentrations, but PCE was completely dechlorinated.

Table 5.3 Inhibition coefficient estimates and goodness of fitting measures for the Monod, Andrews, Luong, Wayman and Tseng inactivation models

Model	Inhibition coefficients			SSE (μM^2)		*F*	
		Strain BB1	Strain PCE1	Strain BB1	Strain PCE1	Strain BB1	Strain PCE1
Monod	Tc [a]	3.32×10^{-5}	6.57×10^{-5}	4.60×10^{6}	2.59×10^{6}	2.57	40.5
Andrews	K_{AI} (μM)	135.45	370.72	1.46×10^{5}	5.69×10^{5}	590	264
	T_C [a]	2.14×10^{-6}	1.44×10^{-5}				
Luong	$S_{a\text{-}max}$ (μM)	1210	963.18	6.85×10^{6}	8.09×10^{6}	3.73	2.32
	T_C [a]	1.79×10^{-5}	9.70×10^{-6}				
Wayman and Tseng	K_{WI} (μM)	404.99	697.88	4.48×10^{6}	4.41×10^{6}	3.07	7.75
	T_C [a]	3.34×10^{-5}	1.26×10^{-4}				
	I_T (h^{-1})	8.07×10^{-5}	3.71×10^{-4}				

[a] Units of mg VSS/μmol PCE/h.

At the lowest strain PCE1 biomass concentration, the Wayman and Tseng inactivation model predicted PCE dechlorination would completely cease although the experimental data indicated that up to 774 μM PCE was dechlorinated at this biomass concentration (Figure 5.4A-C). Under the other conditions tested, the Wayman and Tseng and Monod inactivation model predictions were generally similar. This is not surprising because at substrate concentration less than K_{WI}, the two models are identical (Equations 5.1 and 5.7), and the small I_T values (Table 5.3) limit the impact of the inhibition term on PCE dechlorination at substrate concentrations greater than K_{WI} (Equation 5.7). Qualitatively, the Andrews inactivation model fit the overall experimental data better than the other models. In only one case did another

model fit the experimental data better than the Andrews inactivation model (Figure 5.4A).

The calculated SSE and *F* values (summarized in Table 5.3) provided quantitative measures of the goodness of a model fit to the experimental data. Relatively low SSE and high *F* values are indicative of a good model fit. Thus, the quantitative measures are consistent with the qualitative observations in suggesting that the Andrews inactivation model best describes PCE dechlorination at high concentrations.

The treatments showing no significant PCE dechlorination (Figures 5.3B and 5.4D) were treated to remove the high PCE levels, and PCE was re-supplied at a lower concentration to test whether the effects of high PCE concentration on dechlorination were reversible. No TCE or *cis*-DCE was produced within 24 h (data not shown). These results suggest that the dechlorinating activity of the biomass was most likely permanently inactivated by exposure to high chlorinated ethene concentrations.

The PCE tolerance levels reported for several pure cultures vary significantly as shown in Table 5.4. The Gram-positive bacteria *Desulfitobacterium* sp. strain Y51 (Suyama et al., 2001) and *Clostridium bifermentans* DPH-1 (Chang et al., 2000) can grow in media saturated with PCE. The Gram-negative *Desulfuromonas michiganensis* strains BB1 and BRS1 also were initially reported to be able to grow on PCE at saturated levels (Sung et al., 2003). However, PCE was provided in a non-aqueous hexadecane phase and its limited bioavailability under these conditions undoubtedly reduced the toxicity effects. More recently, when PCE was added

directly to the aqueous phase, strain BB1 could not tolerate concentrations above 540 μM (Amos et al., 2007), as described above. Similarly, the Gram-negative *Dehalobacter restrictus* strains PER-K23 and TEA tolerated PCE concentrations of less than 200 μM (Holliger et al., 1993; 1998). PCE concentrations of greater than 100 μM could not be dechlorinated by *Desulfuromonas chloroethenica* TT4B (Krumholz et al., 1996), and *Dehalococcoides ethenogenes* 195 (Maymo-Gatell et al., 1999) cannot tolerate saturated aqueous PCE concentrations.

These findings are somewhat surprising because Gram negative bacteria are generally thought to be more resistant to solvents compared with Gram positive bacteria. The outer membrane, which is present in Gram negative bacteria but lacking in Gram positive bacteria may act as a barrier to solvents, thereby protect the cytoplasmic membrane from damage (Vermuë et al., 1993; Denyer and Maillard, 2002). Although the solvent tolerance of a given culture may be influenced by its physiology and cell structure, such as the possession of a cell wall or S-layer (Ramos et al., 2002), as shown in Table 6.4, the initial experimental conditions (biomass concentrations) also may play a significant role in determining the tolerance level of a given strain. For example, although studies showed that the Gram positive organisms *Clostridium bifermentans* DPH-1 and *Desulfitobacterium* sp. strain PCE1 can tolerate higher level of PCE than some Gram negative strains, it is important to note that the initial biomass concentrations of these two strains were also much higher than in the other reported experiments. Thus, if an organism's inherent tolerance to a solvent relatively weak, it may be possible to compensate by providing higher initial biomass concentrations. The results of this study suggested that the inactivation coefficient, T_C,

is more appropriate than $S_{a\text{-}max}$ for describing the PCE tolerance level of a given strain because T_C includes information about the biomass concentration.

Table 5.4 Reported PCE tolerance levels and cell structure characteristics of several dehalorespirers

Microorganism	PCE tolerance level (μM)	Gram-positive/ negative	Biomass concentration (mg VSS/L)	S-layer	Reference
Desulfitobacterium strain	Sat. [a]	+	156	N/A	Suyama et al., 2001
Clostridium bifermentans	900	+	12.7	N/A	Chang et al., 2000
Desulfuromonas michiganensis [b]	Sat.	-	<2.2	N/A	Sung et al., 2003
Desulfuromonas michiganensis	540	-	<1.8	N/A	Amos et al., 2007
Dehalobacter restrictus	200	-	N/A	Yes	Holliger et al., 1993; 1998
Desulfuromonas chloroethenica	100	-	N/A	N/A	Krumholz et al., 1996
Desulfitobacterium strain	Sat.	+	>7.3	Yes	Gerritse et al., 1996
Dehalococcoides ethenogenes	700	-	7.3	Yes	Maymo-Gatell et al., 1997

[a.] Aqueous saturation with PCE is observed at 1.459 mM for PCE at 22°C, (Knauss et al., 2000). [b.] PCE was added as a separate phase in hexadecane.

When PCE damages the membranes of the dehalorespiring culture cells by impairing the functions or structures, the dehalorespiring activities of the cells will be reduced or terminated (Ramos et al., 2002), and this damage will result in cell lysis and death (Sikkema et al., 1995). This cellular damage is described by the inactivation term in the Andrews inactivation model developed in this research.

The new model and the inactivation coefficient estimates can provide insightful information that will aid in the development of PCE bioremediation strategies near DNAPL source zones. For example, by substituting Equation 5.3 into Equation 5.8, we get

$$\frac{dX}{dt} = q_{\max} YX \frac{S_a}{K_S + S_a + \frac{S_a^2}{K_I}} - k_d X - T_C S_a \tag{5.9}$$

At steady-state $\frac{dX}{dt} = 0$, and equation 5.9 can be solved with respect to X,

$$X = X_{\min} = \frac{T_C S_a}{q_{\max} Y \frac{S_a}{K_S + S_a + \frac{S_a^{\,2}}{K_{AI}}} - k_d} \tag{5.10}$$

where $X_{\min}$ is the minimum biomass concentration required for PCE dechlorination. Equation 5.10 can be used to provide guidelines for the implementation of bioaugmentation of bioremediation at PCE DNAPL source zones. Assuming a PCE concentration of 1200 μM, the intrinsic kinetic parameters for strain BB1 and strain PCE1 in Table 3.2, and the inhibition and inactivation parameters reported in this chapter, the minimum biomass concentrations required for complete removal of PCE by strain BB1 and strain PCE1 are 0.85 and 2.45 mg VSS/L, respectively. The same procedure can be applied to other dehalorespirers, such as *Dehalococcoides* sp., and contamination sites with lower PCE concentrations.

Chapter 6: Experimental evaluation of interactions between *Dehalococcoides ethenogenes* and heterotrophic tetrachloroethene-respiring bacteria

6.1 Introduction

In situ bioremediation of sites contaminated with chlorinated ethenes is generally based on dehalorespiration, a metabolic process in which chlorinated ethenes are used as terminal electron acceptors and undergo reductive dehalogenation in an anaerobic form of respiration. Complete reductive dechlorination of tetrachloroethene (PCE) to ethene was first proven to be feasible under anaerobic conditions 20 years ago (Freedman and Gossett, 1989). However, dechlorination of PCE and trichloroethene (TCE) is often incomplete, which results in accumulation of lesser chlorinated ethenes, such as *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC), at contaminated sites (Major et al. 2002). Because VC is a known human carcinogen and *cis*-DCE is also hazardous to human health, a better understanding of the factors that contribute to the accumulation of these intermediates and approaches for achieving complete dechlorination are needed.

The incomplete dechlorination of PCE and TCE is often thought to be due to the limited availability of electron donors or deficient numbers of dehalorespirers that can dechlorinate *cis*-DCE or VC. Therefore, engineered bioremediation approaches are often implemented to overcome these limitations on the complete dechlorination of PCE and TCE at contaminated sites (Ellis et al., 2000; Harkness et al., 1999; Lendvay et al., 2003; Major et al., 2002).

Dehalococcoides strains are currently the only known microorganisms that can dehalorespire DCE isomers and/or VC and are key to the successful implementation of bioaugmentation (Löffler et al., 2000; Fennell et al., 2001; Hendrickson et al., 2002; Richardson et al., 2002). However, observations of *cis*-DCE or VC accumulation at chlorinated ethene-contaminated sites or in lab/pilot-scale reactors where *Dehalococcoides* species that can respire DCE and/or VC have been detected indicate that the presence of these organisms does not alone guarantee complete dechlorination of PCE (Daprato et al., 2007; Fennell et al., 1997; 2001; Lendvay et al., 2003). A growing number of field and enrichment culture studies have demonstrated that dehalorespirers that cannot dehalorespire beyond *cis*-DCE may be present together with *Dehalococcoides* species (Daprato et al., 2007; Da Silva et al., 2006; Lendvay et al., 2003; Macbeth et al., 2004; Yang et al., 2005; Zheng et al., 2001). These strains differ with respect to the rates at which they respire chlorinated ethenes and the electron donors that can be used to sustain these reactions.

Currently, relatively little is known about how dehalorespiring populations with different physiologies and kinetics interact at contaminated sites undergoing natural attenuation or engineered bioremediation. A better understanding of the microbial ecology of dehalorespiring populations is needed because the interactions could potentially enhance or inhibit efforts to implement bioremediation depending on whether the interactions are complementary or competitive in nature. For example, if two dehalorespiring populations can utilize the same electron donor to sustain dehalorespiration, competition for the electron donor may arise if alternative electron donors are not available at a contaminated site. *Dehalococcoides ethenogenes* strain

195 and *Desulfuromonas michiganensis* strain BB1 are two dehalorespiring organisms that use distinct electron donors. Hydrogen is the only electron donor that can be used by *Dehalococcoides* strains (Maymo-Gatell et al., 1997). Dehalorespiring *Desulfuromonas* strains use organic compounds such as acetate, but not hydrogen, as the electron donor (Sung et al., 2003). However, both strains 195 and BB1 can respire PCE and TCE and competition among the two populations for these electron acceptors could potentially arise at a contaminated site (Becker, 2006). Whereas strain BB1 converts PCE and TCE primarily to *cis*-DCE, an unacceptable bioremediation endpoint, strain 195 has the potential to completely dechlorinate and detoxify higher chlorinated ethenes to ethene. Thus, the outcome of competitive interactions among dehalorespiring populations could have important implications in terms of the environmental fate of PCE. On the other hand, if adequate amounts of hydrogen and organic electron donors are available, it may be possible for strains BB1 and 195 to coexist through complementary interactions if strain 195 can grow by respiring the *cis*-DCE produced by strain BB1.

The objectives of this study were to: (1) experimentally evaluate the influence of interactions between the hydrogenotroph *Dehalococcoides ethenogenes* strain 195 and two heterotrophic PCE-to- *cis*-DCE/TCE dechlorinating cultures (*Desulfuromonas michiganensis* strain BB1 and *Desulfitobacterium* sp. strain PCE1) on the extent of PCE dechlorination in continuous-flow stirred tank reactors (CSTRs), and (2) compare these experimental results to mathematical model predictions using independently measured kinetic parameters. Three experiments were conducted with the defined co-cultures were conducted under two different conditions: (1) strain 195

and strain BB1 under natural attenuation conditions, in which relatively low concentrations of electron donors were provided, and both electron donors and acceptors were limiting; (2) strain 195 and strain BB1 under engineered bioremediation conditions, in which high concentrations of electron donors were provided and electron acceptors were limiting; and (3) strain 195, strain PCE1, and the lactate fermenter, *Acetobacterium woodii* under engineered bioremediation conditions.

6.2 Materials and methods

6.2.1 Chemicals

PCE (99%, Spectrum Chemical Mfg. Corp.; New Brunswick, NJ) was used in the bioreactor experiments. PCE (99.99%), TCE (99.99%), *cis*-DCE (99.99%), VC (2000 µg/mL in methanol, Supelco) and ethene (99.99%) used to prepare analytical standards were purchased from Sigma-Aldrich (St. Louis, MO). Sodium acetate (Certified ACS) and sodium L-lactate (60% by wt. solution) were obtained from Fisher Scientific Inc. (Pittsburgh, PA) and Acros Organics (Geel, Belgium), respectively. [1,2-^{14}C]sodium acetate ([^{14}C]acetate, 98.8%, 110 mCi/mmol) was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA).

6.2.2 Culture and maintenance

Desulfuromonas michiganensis strain BB1 (DSM 15941) and *Desulfitobacterium* sp. strain PCE1 (DSM 10344) were grown on the basal medium as described in Chapter 3. *Acetobacterium woodii* (DSMZ 1030) was grown on the same basal

medium used for *Desulfitobacterium* sp. strain PCE1, as described by Gerritse et al. (1996). *Dehalococcoides ethenogenes* strain 195 was provided by Dr. Steven Zinder (Cornell University), and grown on a previously-described medium (He et al., 2007). These dehalorespiring cultures were maintained in 160-ml serum bottles with thick black butyl rubber septa (Geo-Microbial Technologies Inc.) using a semi-continuous feeding and wasting cycle that resulted in a 20-d solid retention time (SRT) as described in Chapter 3. This ensured that all of the cultures used in the bioreactor experiments had the same culture history and were at the same physiological state (Grady et al., 1996). *Acetobacterium woodii* was fed with 100 μM lactate every 4 d. Strain 195 was fed with 250 μM PCE and excess H_2 every 4 d.

6.2.3 Experimental system

A schematic of the experimental system used to evaluate the interactions among the dehalorespiring strains is shown in Figure 6.1.The entire experimental system is composed of four parts: (1) duplicate bioreactors, (2) feeding system, (3) wasting system, and (4) gassing system. The duplicate bioreactors were constructed from 2-L Pyrex media bottles. The bioreactor inlet was formed by fusing a glass-to-Teflon connector with 1/4″-28 threaded fitting (Supelco, Bellefonte, PA) on the lower side of the bioreactor flask. Teflon tubing (1/8″ I.D., Cole-Parmer Instrument Company) was connected to the reactor inlet with a 1/8″ chlorotrifluoroethylene (CTFE) compression fitting (Valco Instruments Co. Inc. Houston, TX). Glass flanges were fused to the top of the bioreactor flask and to the bottom of a glass cap, and sealed together with a Viton O-ring and a clamp. Two sample ports were installed on the side of the bioreactor flask and the top of the glass top, respectively, which are sealed with black

butyl rubber septum and aluminum cap. The total volume of each bioreactor is 2.3 L. The bioreactor outlet was installed on the top of the glass cap with another glass-to-Teflon connector, which was connected to the wasting reservoir via Teflon tubing. The effluent of the bioreactor was drained to a waste collection vessel, which was sealed with a carbon trap (Supelco) to capture volatile organic compounds. All of the Teflon tubing was covered with 1/4″-Viton tubing which is relatively impermeable to oxygen, to help maintain anaerobic conditions in the reactor system.

The feeding reservoir was made from a 2 L-Pyrex glass bottle, which was sealed with a 3-hole threaded cap (Fisher Scientific). The fresh media in the feeding reservoir was fed to the bioreactors through a syringe pump (Standard pump 22, Harvard Bioscience, Inc. Holliston, MA) equipped with 140 ml sterile syringes (Fisher Scientific). The fresh media feeding rate was 80 μL/min so that the SRT of the bioreactors is 20 d. 80% N_2//20% CO_2 (Air Gas) was supplied to the feeding reservoir via Teflon tubing to maintain a slightly positive pressure in the feeding reservoir while recharging the feeding syringes. In experiments involving strain 195 and strain BB1, hydrogen was provided as electron donor for strain 195 by equilibrating the mineral medium with a gas mixture containing hydrogen and CO_2, as described further below. To recharge the feeding syringe, the 3-way valves on syringes were first switched to shut off flow from the bioreactors and create a flow path from the medium reservoir. Then the feeding pump was turned on “withdraw” mode. After the syringes were full, the 3-way valves were returned to their original position, and the syringe pump was switched to “push” mode.

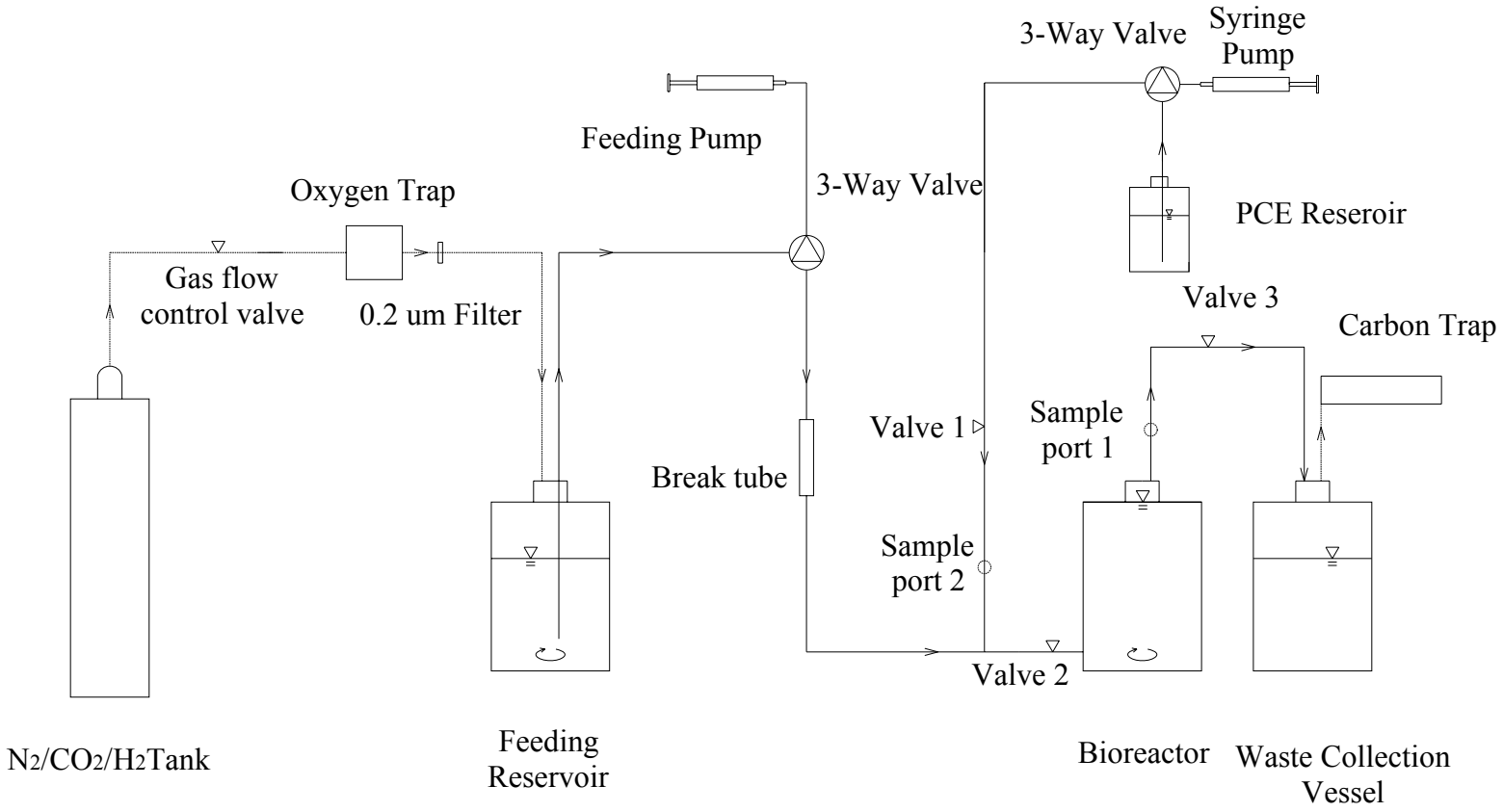

Figure 6.1 Flow diagram of the experimental system used to evaluate interactions among dehalorespiring populations.

The PCE-methanol stock solution (2 mM) was introduced to the influent line using a syringe pump (Sage Instruments, Model 355) equipped with 5-ml glass luer-lock, gas-tight syringes (Hamilton Company, Reno, NV) at a flow rate of 2.4 μL/hour so that the PCE influent concentration was 10 μM. The syringe pump was connected with the bioreactor influent tubing and a PCE-methanol stock solution reservoir via a three-way valve (Swagelok Company). The PCE stock reservoir was made from a 100-ml Pyrex medium bottle sealed with a GL45 1/4"-28 ported Teflon threaded cap (Bio-Chem Valve Inc. Boonton, NJ).

All gases used in media preparation and bioreactor maintenance were scrubbed of O_2 by passing them through reduced copper or O_2 traps and were filter-sterilized (0.2 μM, PTFE, Millipore Corporation). All components of the reactor system were autoclaved for 30 min before being assembled. Fresh medium was prepared in the reactor and feeding reservoir. After being autoclaved for 1.5 h, the liquid medium was cooled to room temperature under 80% N_2/20% CO_2 (Air Gas).

6.2.4 Conceptual substrate interaction models

The known PCE-respiring bacteria are physiologically diverse and include both heterotrophics and mixotrophs and Gram positive and Gram negative species. The conceptual substrate interaction models evaluated in this research (Figure 6.2) reflect this diversity, and, more importantly, capture several important ecological interactions that may arise among multiple dehalorespiring populations at a contaminated site. The specific strains included in the conceptual models and experimental and mathematical evaluations were selected because of the availability

of preliminary biokinetic data at the onset of this study and the availability of the strains in culture collections. In developing the conceptual models, it was assumed that only two dehalorespiring populations compete for chlorinated ethenes, and in some cases, indirectly for electron donors.

The known *Dehalococcoides* strains are limited to the use of H_2 as the electron donor. In contrast, several members of the genus *Desulfuromonas* can respire PCE and TCE using acetate, but not H_2, as the electron donor (Krumholz, 1997; Sung et al., 2003). In scenario A, *Desulfuromonas michiganensis* strain BB1 could potentially compete with *Dehalococcoides ethenogenes* strain 195 for PCE (or TCE, not shown), but the two organisms do not compete for electron donors (Figure 6.2A). In addition, complementary substrate interactions between strain 195 and strain BB1 could occur if strain BB1 grows using PCE and/or TCE as the electron acceptor(s), and strain 195 grows predominantly using *cis*-DCE produced by strain BB1 (Figure 6.2B). Interactions between strain 195 and strain BB1 could conceivably occur in PCE-contaminated sites where H_2 and acetate are produced from the fermentation of organic substrates added to the subsurface as part of a biostimulation approach. In scenario C (Figure 6.2C), an organotrophic dehalorespirer, *Desulfitobacterium* sp. strain PCE1 competes for lactate with a lactate-fermenting organism that also provides H_2 needed by strain 195. In this case, indirect competition for lactate-derived electron donors occurs between strain PCE1 and strain 195. Strain PCE1 may also compete with strain 195 for PCE.

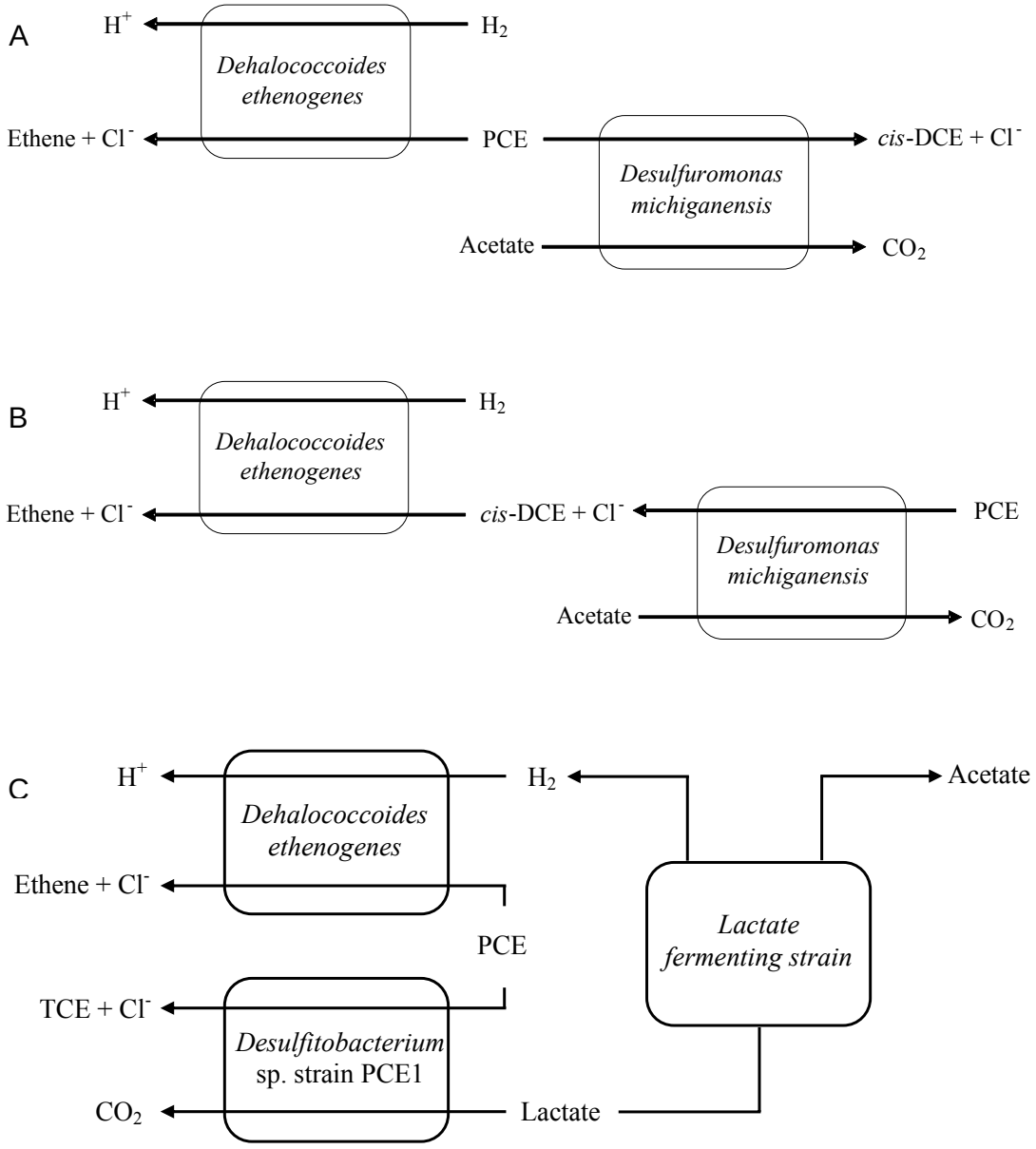

Figure 6.2 Conceptual substrate interaction models involving representative PCE-respiring bacteria. (A) Competition for electron acceptors (TCE not shown) between *Dehalococcoides ethenogenes* strain 195 and *Desulfuromonas michiganensis* strain BB1; (B) Complementary electron acceptor utilization by *Dehalococcoides ethenogenes* strain 195 and *Desulfuromonas michiganensis* strain BB1; and (C) Competition for PCE and lactate-derived electron donors between *Dehalococcoides ethenogenes* strain 195 and *Desulfitobacterium* sp. strain PCE1.

6.2.5 Pure culture control experiments with CSTRs

A series of control experiments were conducted to ensure that each dehalorespiring strain could be maintained in the CSTR and would not be washed out under the conditions used in the defined co-culture experiments. The control experiments were conducted using a single dehalorespiring strain under the conditions shown in Table 6.1. All of the control experiments were conducted for more than one SRT, during which PCE did not accumulate in any case (data not shown). The results indicated that each of the dehalorespiring cultures could be maintained in the bioreactors at a 20-d SRT under conditions similar to those used in the co-culture experiments.

Table 6.1 Experimental conditions used in the substrate interaction and control experiments

		PCE influent concentration (μM)	Electron donor influent concentrations (μM)		
			H_2	Acetate	Lactate
Control experiments	Strain BB1	25	0	100	0
	Strain PCE1	25	0	0	250
	Strain 195	25	250	0	0
Scenario A	Engineered bioremediation	11.5	200	100	0
	Natural attenuation	10	20	10	0
Scenario B	Engineered bioremediation	10	0	0	100

6.2.6 Substrate interaction experiments with CSTRs

All substrate interaction experiments were conducted with duplicate bioreactors using the influent substrate concentrations summarized in Table 6.1. A PCE

concentration of 10 μM was chosen because it is within the concentration range detected at some contaminated sites (Lendvay et al., 2003). However, because the PCE-methanol stock solution for scenario A under engineered bioremediation condition was prepared with a slightly higher concentration, the PCE influent concentration in this case was also slightly higher (11.5 μM) than in the other experiments. Under natural attenuation conditions, the concentrations of electron donors used were somewhat higher than the minimum values recommended for successful natural attenuation of chlorinated ethenes (Wiedemeier et al., 1999) to facilitate their quantification. H_2 and acetate were provided in a 2:1 (mol:mol) ratio in scenario A because lactate is often added to the subsurface as a fermentable source of reducing equivalents according to Equation 6.1.

$$CH_3CHOHCOO^- + 2H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 2H_2 \quad (6.1)$$

Under engineered bioremediation conditions, the electron donor concentrations were 10 times greater than the concentration used under natural attenuation conditions so that the electron donors were not limiting, consistent with biostimulation practices used in the field. The initial substrate concentrations in the reactors were the same as the influent concentrations in all cases. Acetate and lactate were added to the reactors and fresh media in the feeding reservoirs from sterile stock solutions. In scenario A, H_2 was added into the reactors and feeding reservoirs by purging the fresh media with 6% H_2/10% CO_2/84% N_2 under natural attenuation and 80% H_2/20% CO_2 under engineered bioremediation conditions, respectively. Neat PCE was initially added to the bioreactors and allowed to completely dissolve before

initiating an experiment. The influent PCE was provided as a methanol stock solution as describe above. Inoculation with the co-cultures was performed one day after PCE addition to the bioreactors. Immediately after the inoculation, the feeding pumps were started to initiate the experiments at room temperature. Liquid samples were taken at regular intervals from the bioreactors through the sample ports for analysis of chlorinated ethenes, organic acids, hydrogen, and biomass.

6.2.7 Analytical methods

The chlorinated ethene concentrations were determined using headspace gas chromatography and two Hewlett Packard 5890 II Plus GC equipped with either an electron capture detector (ECD) or flame ion detector (FID). The GC-ECD method was used for sample with less than 1 μM chlorinated ethenes, and the GC-FID was used for chlorinated ethane concentration higher than 1 μM. Aqueous samples (1-ml) were obtained from the reactors through the sampling ports using 1-ml disposable sterile syringes (Becton-Dickinson) equipped with sterile needles and transferred to 11.84-ml autosampler vials sealed with Teflon-lined caps. Samples were incubated at 30°C for at least 30 min, and 0.5 ml of the headspace was injected onto GC. Separate experiments were conducted to verify that the dechlorination activity in the autosampler vials ceases immediately after the aqueous samples were transferred to the vials. Therefore, continued biodegradation of the chlorinated ethenes during the incubation period is not a concern. The GC temperature programs were described in Chapter 3. The aqueous concentrations of chlorinated ethenes in continuous stirred

tank reactors (S_a) were determined based on the concentration measured in the vials according to:

$$S_a = \frac{V_g H_C + V_{aq}}{V_{aq}} S_{aq} \tag{6.2}$$

where S_{aq} [M_S L^{-3}]is the chlorinated ethene concentration in the sample vial, H_C is the dimensionless Henrys constant at 30°C (0.917 for PCE, 0.491 for TCE, 0.190 for *cis*-DCE and 1.264 for VC, Gossett, 1987), V_g is the headspace volume in the autosampler vial (10.84-ml), and $V_{aq.}$ is the aqueous sample volume in the autosampler vial (1-ml).

Hydrogen was quantified in the same autosampler vial using a Peak Performer I GC equipped with a reducing compound photometer (RCP) detector and two columns. A 31" UNI 1S guard column filters out the chlorinated ethenes, and a 31" Molecular Sieve 13X analytical column employs clay as the adsorbent (Peak Laboratories, Mountain View, CA). The column and detector were operated at 105°C and 265°C, respectively. Ultra pure carrier grade N_2 (AirGas East) was used as the carrier gas at a flow rate of 20 ml/min. The temperature program was isothermal at 265°C. After the sample was injected, the instrument was programmed to run for 210 sec. The retention time of hydrogen is 48 ± 4 sec. Hydrogen calibration curves were prepared with an 18.7 ppm hydrogen standard (balanced with N_2, AirGas East). The aqueous concentrations of hydrogen were calculated by using Equation 6.3 (Löffler et. al., 1999),

$$C_{L,H_2} = \frac{LP}{RT} \tag{6.3}$$

where C_{L,H_2} is the aqueous concentration of hydrogen (M), L is the Ostwald coefficient for hydrogen solubility, which is 0.01895 at 30°C (Wilhelm et al., 1977), P is the partial pressure of hydrogen (atm), R is universal gas constant (0.0821 $L \cdot atm \cdot K^{-1} \cdot mol^{-1}$), and T is the absolute temperature (K).

The concentrations of acetate in liquid samples from the reactors under natural attenuation conditions were determined using ^{14}C-labeled acetate, as described in Chapter 3.

6.2.8 DNA extraction and real-time PCR analysis

The abundance of each individual strain in the co-culture experiments was quantified by enumerating 16S rRNA gene copies numbers using real-time PCR analysis described in Chapter 3. The primers for strain BB1 and strain PCE1 were the same as those used in Chapter 3. The primers for 195 and *A. woodii* are listed in Table 6.2.

Table 6.2 16S rRNA gene primers used for quantification of *Dehaloccocoides ethenogenes* strain 195 and *Acetobacterium woodii*

Strain	Primer name[a]	Primer sequence	Reference
Dehaloccocoides ethenogenes strain 195	Dhc730 F	5'- GCGGTTTTCTAGGTTGTC -3	Ritalahti et al. (2006)
	Dhc1350 R	5'- CACCTTGCTGATATGCGG -3	
Acetobacterium woodii	Aceto 572F	5’-GGCTCAACCGGTGACATGCA-3’	Duhamel and Edwards (2006)
	Aceto 784R	5’-ACTGAGTCTCCCCAACACCT-3’	

[a] Numbers refer to position in *Escherichia coli* 16S rRNA gene sequence.

The measurements made using real-time PCR were converted to biomass concentration (mg VSS/L) based on the assumptions listed in Chapter 3. The genome size of strain 195 is 1.5 Mbp (NCBI, 2005) and that of *A. woodii* is 4.2 Mbp (Duhamel and Edwards, 2006).

6.2.9 Model simulation

Substrate utilization and growth by co-cultures of dehalorespiring strains in the CSTRs were simulated using a mathematical model developed by Fennell and Gossett (1998) and modified by Becker (2006). The dechlorination of chlorinated ethenes and the growth of dehalorespiring bacteria were described using Equations 6.4-6.9. Competitive inhibition among the chlorinated ethenes has been demonstrated in this study for strain 195 and strain BB1 (Chapter 5). However, because this effect is negligible under the experimental conditions used in this study, it is not incorporated into this model.

$$\frac{dS_{PCE}}{dt} = -\left(\frac{q_{\max,PCE} X S_{PCE}}{K_{S,PCE} + S_{PCE}}\right)\left(\frac{S_d - S_{d,threshold}}{K_{S,d} + S_d - S_{d,threshold}}\right) \tag{6.4}$$

$$\frac{dS_{TCE}}{dt} = \left(\frac{q_{\max,PCE} X S_{PCE}}{K_{S,PCE} + S_{PCE}} - \frac{q_{\max,TCE} X S_{TCE}}{K_{S,TCE} + S_{TCE}}\right)\left(\frac{S_d - S_{d,threshold}}{K_{S,d} + S_d - S_{d,threshold}}\right) \tag{6.5}$$

$$\frac{dS_{DCE}}{dt} = \left(\frac{q_{\max,TCE} X S_{TCE}}{K_{S,TCE} + S_{TCE}} - \frac{q_{\max,DCE} X S_{DCE}}{K_{S,DCE} + S_{DCE}}\right)\left(\frac{S_d - S_{d,threshold}}{K_{S,d} + S_d - S_{d,threshold}}\right) \tag{6.6}$$

$$\frac{dS_{VC}}{dt} = \left(\frac{q_{\max,DCE} X S_{DCE}}{K_{S,DCE} + S_{DCE}} - \frac{q_{\max,VC} X S_{VC}}{K_{S,VC} + S_{VC}} \right) \left(\frac{S_d - S_{d,threshold}}{K_{S,d} + S_d - S_{d,threshold}} \right) \tag{6.7}$$

$$\frac{dS_{ETH}}{dt} = \frac{q_{\max,VC} X S_{VC}}{K_{S,VC} + S_{VC}} \left(\frac{S_d - S_{d,threshold}}{K_{S,d} + S_d - S_{d,threshold}} \right) \tag{6.8}$$

$$\frac{dX}{dt} = \sum \frac{q_{\max,CAHs} X Y S_{CAHs}}{K_{S,CAHs} + S_{CAHs}} \left(\frac{S_d - S_{d,threshold}}{K_{S,d} + S_d - S_{d,threshold}} \right) - k_d X \tag{6.9}$$

where S_{PCE}, S_{TCE}, S_{DCE}, S_{VC} and S_{ETH} [$M_S L^{-3}$] are the concentrations of PCE, TCE, *cis*-DCE, VC and ethene in a bioreactor, respectively; X [$M_X L^{-3}$] is the biomass concentration of a given dehalorespirer in a bioreactor; $q_{max,PCE}$, $q_{max,TCE}$, $q_{max,DCE}$, and $q_{max,VC}$ [$M_S M_X^{-1} T^{-1}$] are the maximum specific substrate utilization rates for PCE, TCE, *cis*-DCE and VC respectively; $K_{S,PCE}$, $K_{S,TCE}$, $K_{S,DCE}$, $K_{S,VC}$ and $K_{S,d}$ [$M_S L^{-3}$] are the half-saturation constants for PCE, TCE, *cis*-DCE, VC and electron donors respectively; Y [$M_X M_S^{-1}$] is the yield coefficient, CAHs refers to chlorinated aliphatic hydrocarbon (PCE, TCE, *cis*-DCE, or VC) and k_d [T^{-1}] is the decay coefficient. Several of these parameters have been defined in previous Chapters. Strain BB1 does not grow on *cis*-DCE or VC. Therefore, for this strain, Equation 6.9 includes only terms for PCE, TCE and decay, and Equations 6.6-6.8 are not applicable. Similarly, for strain PCE1, which grows only on PCE, Equation 6.9 includes only PCE utilization and decay terms and Equations 6.5-6.8 are not applicable. Because there is no headspace in the bioreactors, the chlorinated ethene is present in the aqueous phase only.

Electron donor utilization is simulated based on electron acceptor utilization using Equation 6.10,

$$\frac{dS_{donor}}{dt} = -\frac{1}{f_e}\sum \frac{q_{\max,CAHs} X S_{CAHs}}{K_{S,CAHs} + S_{CAHs}} \left(\frac{S_d - S_{d,threshold}}{K_{S,donor} + S_d - S_{d,threshold}} \right) \tag{6.10}$$

where f_e is the fraction of electron donor equivalents that are used in energy production. When *A. woodii* is included as the lactate fermenter, lactate utilization by, and growth of, *A. woodii* is described using Equations 6.11 and 6.12 respectively,

$$\frac{dS_{Lactate}}{dt} = -\frac{q_{\max,fermenter} X_{fermenter} \left(S_{Lactate} - S_{threshold,fermenter} \right)}{K_{S,Lactate,fermenter} + S_{Lactate}} \tag{6.11}$$

$$\frac{dX_{fermenter}}{dt} = Y_{fermenter} \frac{dS_{Lactate}}{dt} - k_d X_{fermenter} \tag{6.12}$$

where $q_{\max,fermenter}$, $K_{S,fermenter}$, $Y_{fermenter}$ and $S_{threshold,fermenter}$ are maximum specific substrate utilization rate, half-saturation constant, yield coefficient, and threshold of *A. woodii* growing on lactate. The *extant* and intrinsic kinetic parameters used in the model simulations are shown in Table 6.3. The model was implemented in STELLA 8.0 (isee systems, Lebanon, NH) using a fourth-order Runge Kutta integration method and a calculation time step of 0.125 h. Further decreases in the time step resulted in a relative difference of less than 10^{-3}.

Table 6.3 Monod kinetic parameter estimates used in the model simulations

		Dsm. michiganensis strain BB1 [a]	*Dsf.* sp. strain PCE1 [a]	*Dhc. ethenogenes* strain 195 [b]	*A. woodii*
Intrinsic	q_{max} (μmol/mg VSS h)				
	PCE	12.6	5.36	6.76	2.5 [c]
	TCE	17.0		7.93	
	DCE			13.2	
	VC			2.00	
	K_S (μM)[a]				
	PCE	9.31	8.81	21.5	
	TCE	2.83		29.0	
	DCE			33.6	
	VC			637	
	Acetate	5.77			
	Lactate		9.40		2.52 [d]
	H_2			0.005	
Extant	q_{max} (μmol/mg VSS h)				
	PCE	2.59	2.42	3.41	
	TCE	7.13		3.44	
	DCE			6.59	
	VC			2.00	
	K_S (μM)				
	PCE	0.84	2.63	0.97	
	TCE	2.15		2.25	
	DCE			4.61	
	VC			637	
$S_{d,threshold}$ (μM)	Acetate	0.41			
	Lactate		4.33		5 [c]
	H_2			0.0015	
Y (mg VSS/μmol Cl^-)		0.0033	0.0116	0.0047	0.0035 [c]
k_d (h^{-1})		0.0054	0.0076	0.0044	0.001

[a] Values were obtained from Chapters 3 and 4; [b] Values were obtained from previous study of our group; [c] Values were calculated from Peters et al, 1998; [d] Values were obtained from Fennell and Gossett, 1998.

6.3 Results and Discussion

6.3.1 Substrate interactions between strains 195 and strain BB1 under natural attenuation conditions

6.3.1.1 Chlorinated ethene transformation by the co-culture

As shown in Figure 6.3, the duplicate bioreactors performed similarly and reached steady-state with respect to chlorinated ethene concentrations within 10 d. The steady-state concentrations of PCE, TCE and *cis*-DCE were approximately 0.8, 0.6, and 1.0 μM, respectively. VC was the dominant product of PCE during the steady-state period. Ethene was detected in both reactors but the concentrations never exceeded 0.01 μM in either reactor and are not shown in Figure 6.3. The accumulation of VC is likely due to the co-metabolic transformation of VC by strain 195 (Maymó-Gatell et al., 1999; 2001). Not only are the kinetics of the VC transformation slow (Table 6.3), but it is possible that the low concentrations of higher chlorinated ethenes in the bioreactors did not induce VC cometabolism at the same rate typically obtained in batch cultures. The availability of electron donors did not contribute to the accumulation of chlorinated ethenes in the bioreactors because the steady-state concentrations of electron donors were much higher (Figure 6.5) compared to the measured threshold concentrations (Table 6.3).

The steady-state experimental chlorinated ethene data are predicted reasonably well by the model using the extant kinetic parameter estimates (Figure 6.4A). However, the model predicted that the time needed to reach steady-state would be longer than was actually observed. The discrepancy between the model predictions

and experimental data can be attributed to the differences in the physiological state of the microbial cells during the dynamic, start-up phase and steady-state period in the experiments. Use of the extant kinetic parameter estimates to describe dechlorination is appropriate during the steady-state period when microbial activity is limited by the low substrate-to-biomass concentration ratios. However, at the beginning of the experiments, a small inoculum was provided with a large amount of substrate and, in fact, S_0/X_0 exceed 20 (on a COD basis), as recommended by Grady et al. (1996) to allow unrestricted growth and for determination of intrinsic kinetics. Accordingly, rapid microbial growth was observed during the dynamic bioreactor start-up phase (days 0-5), as discussed below.

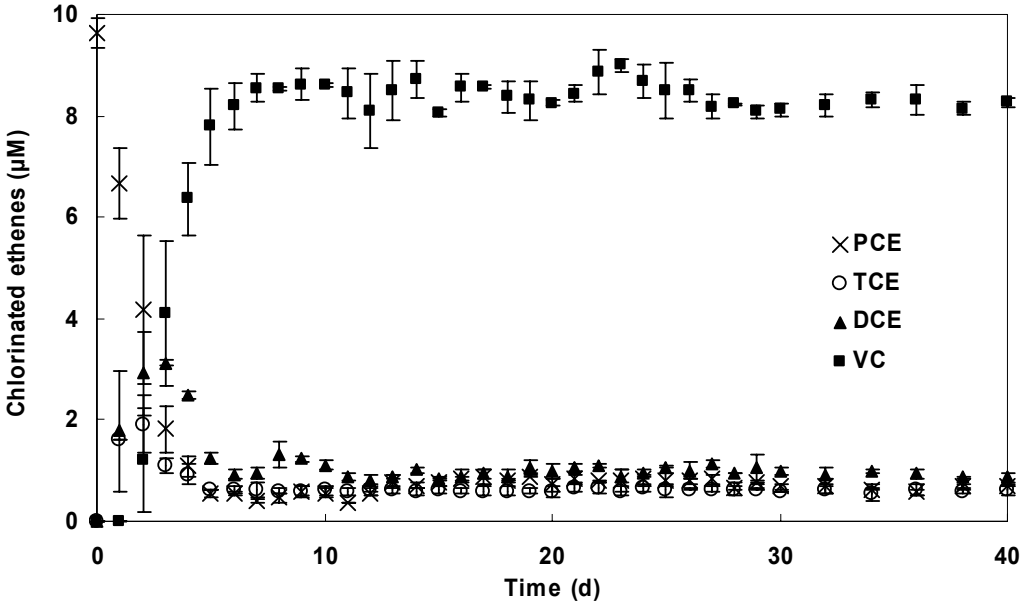

Figure 6.3 Chlorinated ethene concentrations under natural attenuation conditions in the presence of *Dehalococcoides ethenogenes* strain 195 and *Desulfuromonas michiganensis* strain BB1. The error bars represent the standard deviation of the samples from the duplicate reactors.

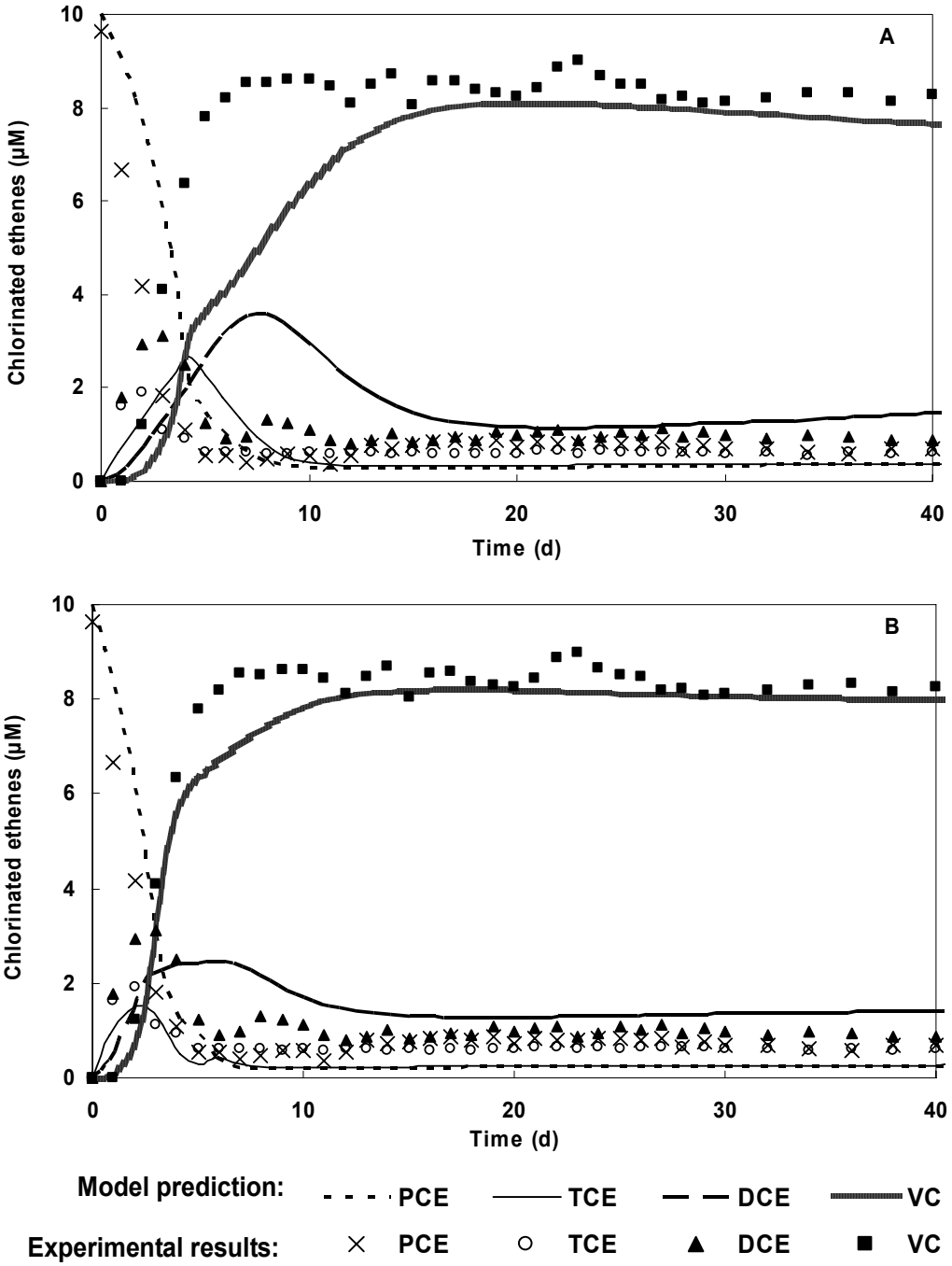

Figure 6.4 Comparison of model predictions (lines) and experimental results (points) of chlorinated ethene concentrations in the co-culture of *Dehalococcoides ethenogenes* strain 195 and *Desulfuromonas michiganensis* strain BB1 under natural attenuation conditions using (A) extant kinetic parameter inputs only; and (B) a combination of intrinsic kinetic parameter inputs for days 0-5 and extant kinetic parameters inputs for >5 d.

When the simulation was performed using a combination of intrinsic kinetic parameter inputs for the dynamic start-up phase (days 0-5) and extant kinetic parameter inputs for the steady-state period (>5 d; Figure 6.4B), the model predicted a more rapid approach to steady-state and did a better job of predicting the full suite of experimental data. The use of intrinsic kinetic parameter inputs to describe the activity of the co-culture during the entire experimental period underestimated the steady-state concentrations of PCE, TCE and *cis*-DCE in the bioreactors (data not shown). This was expected due to the high q_{max} values under intrinsic conditions (Table 6.3), according to the expression for the effluent concentrations of the limiting substrate in a CSTR,

$$S_{SS} = \frac{K_S\left(1+\theta_x k_d\right)}{\theta_x\left(Yq_{\max}-k_d\right)-1} \tag{6.13}$$

where S_{SS} is the effluent substrate concentration at steady-state and θ_x is solid retention time.

Overall, the results demonstrated that to accurately predict microbial activity in a CSTR when a small inoculum is used relative to S_0, it is necessary to utilize a combination of intrinsic kinetic inputs to describe the start-up phase, and extant kinetic inputs to predict the steady-state period.

6.3.1.2 Electron donor consumption by the co-culture

The rapid consumption of electron donors in the reactors operated under natural attenuation conditions (Figure 6.5) coincided with the rapid decrease in the PCE

concentration (Figure 6.3). Similarly, the concentrations of acetate and hydrogen reached a steady-state in the reactors within 10 d at about 5.5 and 6.0 μM respectively. For both acetate and hydrogen, the concentrations at steady-state were higher than the respective $S_{d,threshold}$ values (Table 6.3), which means dehalorespiration was not limited by electron donor availability, as previously mentioned. It is important to note however, that the amounts of the electron acceptors utilized by strain 195 (PCE, TCE, *cis*-DCE) supplied to or produced in the bioreactor exceeded the stoichiometric amount of H_2 supplied to strain 195 in the reactor feed.

The predicted acetate concentration at steady-state (6 μM), is only slightly higher than the measured acetate concentration during this time (Figure 6.6). However, according to the model simulation, hydrogen is rapidly depleted by strain 195 to its $S_{d,threshold}$ (Table 6.3). Thus, strain 195 did not utilize as much hydrogen as predicted by the model.

A mass balance analysis of the consumption of electron donors and acceptors by strain 195 provides insight into the nature of its interactions with strain BB1. According to the experimental results, 14 μM of H_2 was consumed by strain 195 at the steady-state condition (Figure 6.5). The accumulation of 9 μM VC indicates that most of this hydrogen was used by strain 195 to dechlorinate *cis*-DCE to VC with a f_e of 0.95. This suggests that strain 195 used only 4.5 μM of hydrogen in the respiration of PCE or TCE. Consequently, PCE was primarily dechlorinated to *cis*-DCE by strain BB1, consistent with the acetate consumption of 4.5 μM by strain BB1.

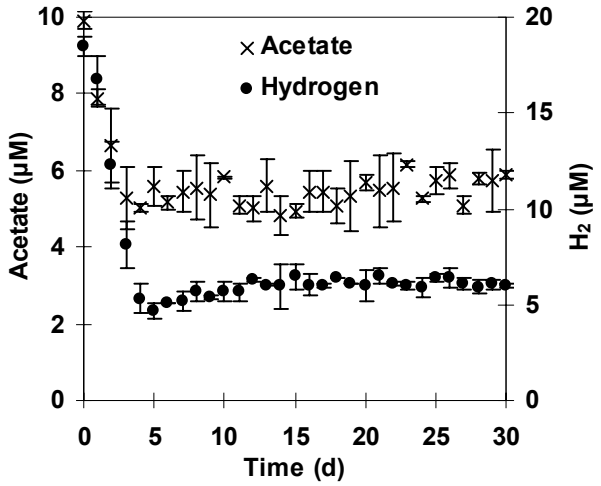

Figure 6.5 Acetate and hydrogen concentration in the duplicate bioreactors in the presence of co-cultures of *Dehalococcoides ethenogenes* strain 195 and *Desulfuromonas michiganensis* strain BB1 under natural attenuation conditions. The error bars represent the standard deviation of the concentrations measured in the duplicate reactors.

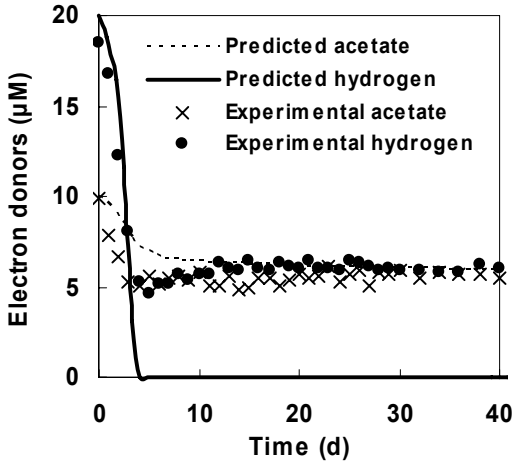

Figure 6.6 Comparison of predicted and measured acetate and hydrogen concentration in the bioreactors in the presence of co-cultures of *Dehalococcoides ethenogenes* strain 195 and *Desulfuromonas michiganensis* strain BB1 under natural attenuation conditions. A combination of intrinsic (days 0-5) and extant (>5 d) kinetic parameter inputs were used.

The discrepancy between the predicted and measured hydrogen concentrations indicates that a phenomenon or interaction between strains 195 and BB1 that limits the ability of strain 195 to compete with strain BB1 for PCE and/or TCE is not yet captured by the mathematical model. There are four likely explanations for the reduced competitiveness of strain 195.

First, to meet strain 195's high requirement for vitamin B_{12}, it was provided to the bioreactor at ten times the concentration used in the batch assays used to estimate the strain BB1 kinetic parameters. Other than supplying the bioreactors with hydrogen, which is not used by strain BB1, all other substrates and growth factors were present at the same concentrations in the bioreactors and batch assays. Thus, it is possible that the PCE and TCE utilization kinetics of strain BB1 in the bioreactors were faster compared to the rates measured in the batch assays. Faster substrate utilization kinetics would improve strain BB1's ability to compete with strain 195 for PCE and TCE.

Second, it is possible that strain BB1 produces a compound that is inhibitory to strain 195 or strain 195 produces a metabolite that enhances PCE/TCE utilization by strain BB1. These types of interactions have been previously observed in other contaminant-degrading bacterial co-cultures. For example, growth of *Pseudomonas putida* F1 on toluene was reduced by *Burkholderia* sp. JS150 in a defined binary culture when toluene was provided as the sole carbon and energy source (Rogers et al., 2000). The reduction in the growth of the *Pseudomonas* strain (relative to pure culture conditions) could be attributed to the production of a compound inhibitory to the *Pseudomonas* strain by the *Burkholderia* strain. When the binary culture was

grown on phenol as the sole substrate, the *Burkholderia* strain produced a compound that could be metabolized by the pseudomonad and enhanced its growth relative to pure culture conditions. Either of these types of scenarios could conceivably have a negative impact on strain 195's ability to respire PCE or TCE and explain why the predictions made using a "pure-and-simple" competition model did not accurately predict the experimental electron donor results.

Third, it is possible that strain BB1 depleted a substrate or growth factor used by strain 195. For example, acetate is utilized as an electron donor and carbon source by strain BB1 and as a carbon source by strain 195. Strain 195 is routinely provided 5 mM acetate in the semi-continuous culture and batch cultures used to estimate electron acceptor utilization kinetics. The acetate concentration provided in the continuous-flow experiments was much lower (10 μM) and was further reduced through uptake by strain BB1. It is possible that the acetate concentration in the bioreactor was below a threshold concentration for acetate utilization by strain 195.

Fourth, it is possible that electron donor utilization for biomass synthesis and energy generation in the two dehalorespiring strains is not constant. In fact, evidence has been obtained in this (Chapter 4) and previous studies (Sung et al., 2003) that reductive dechlorination of chlorinated ethenes by strains BB1 and 195 may become uncoupled from biomass synthesis under certain conditions. This is reflected in the estimated f_e values. In terms of model predictions, electron donor utilization based on coupled dechlorination and growth processes may overestimate the amount of electron donor utilized if dechlorination becomes uncoupled from growth. A series of mathematical and experimental evaluations are currently being conducted to

systemically evaluate why strain 195 is not able to compete for PCE and TCE as well as predicted by the mathematical model. Ultimately, it is hoped that this information will lead to a better conceptual model of how strains 195 and BB1 are interacting and refinement of the mathematical model and kinetic inputs based on an improved understanding of the microbial ecology of co-cultures of dehalorespiring strains.

Until an improved conceptual model can be formulated, the mathematical parameter inputs can be empirically adjusted to reflect the limited amount of PCE and TCE dechlorination` activity in strain 195 indicated by the mass balance on electron donor and acceptor utilization in the bioreactors. As shown in Figure 6.7, when $q_{max,PCE}$ for strain 195 was set to zero and the $q_{max,TCE}$ utilization rates shown for strain 195 in Table 6.3 were reduced by one-half, the model did a better job of predicting the steady-state hydrogen concentration, as expected.

While it is not yet clear why strain 195 specializes in respiring *cis*-DCE in the presence of strain BB1, the fact that the two strains interact primarily in a complementary fashion (Figure 6.2B) has important practical implications. The implementation of biostimulation as an engineered bioremediation approach typically focuses on increasing hydrogen concentrations within contaminant plumes to promote the activity of *Dehalococcoides* strains that can dechlorinate PCE past *cis*-DCE through the addition of a fermentable organic substrate such as lactate. In addition to hydrogen, the fermentation reactions also generate organic by-products such as acetate. If strain 195 outcompeted strain BB1 for the available PCE and TCE via a competitive interaction like that depicted in Figure 6.2A, then the acetate and/or other organic electron donors released to the contaminant plume would be consumed in

anaerobic processes other than dehalorespiration. Ultimately, the amount and extent of PCE transformation that could be achieved by *Dehalococcoides* strains alone is expected to be lower compared to the effects of a heterotrophic populations like strain BB1 working in concert with hydrogenotrophic *Dehalococcoides* strains (Becker and Seagren, 2009). Thus, engineered bioremediation approaches should focus on sustaining multiple, complementary dehalorespiring populations that can maximize utilization of the available electron donors.

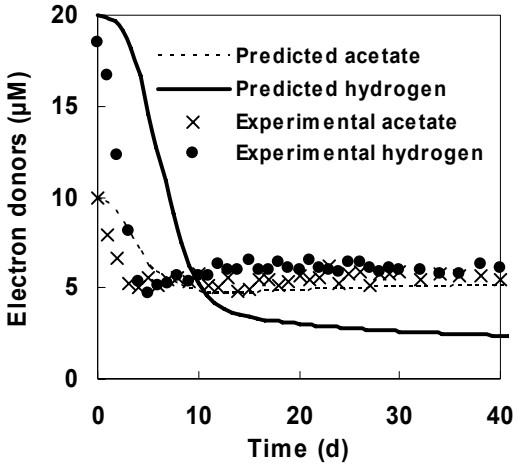

Figure 6.7 Comparison of predicted and measured acetate and hydrogen concentration in the bioreactors in the presence of co-cultures of *Dehalococcoides ethenogenes* strain 195 and *Desulfuromonas michiganensis* strain BB1 under natural attenuation conditions. The predicted concentrations were obtained by manually adjusting the model so that strain 195 does not utilize any PCE and utilizes TCE at one-half the rates given in Table 6.3. A combination of intrinsic (days 0-5) and extant (>5 d) kinetic parameter inputs were used.

6.3.1.3 Biomass growth in the co-culture

The abundance of the two populations based on 16S rRNA gene copy numbers and converted to biomass concentrations were similar throughout the entire experiment (Figure 6.8). Both populations grew rapidly from day 0-5 supporting the

use of intrinsic kinetic parameters to describe their activity during this period. If the model is adjusted to restrict PCE and TCE dechlorination by strain 195 as described above, the biomass concentrations based on the real-time PCR analyses are predicted reasonably well (Figure 6.9A). Thus, the biomass data support the idea that strain 195 and strain BB1 interact primarily in complementary rather than competitive manner. In contrast, the model simulation using the kinetic constants in Table 6.3 without modification predicted that the biomass concentration of strain 195 would be about 1.5 times greater than that of strain BB1 at steady-state (Figure 6.9B).

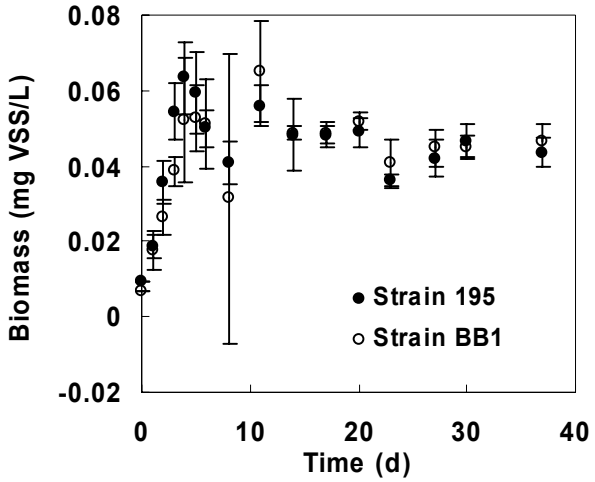

Figure 6.8 Biomass concentrations of *Dehalococcoides ethenogenes* strain 195 and *Desulfuromonas michiganensis* strain BB1 under natural attenuation conditions. Biomass concentrations were calculated based on gene copy numbers quantified with real-time PCR, assuming one 16S rRNA gene copy/cell and a cell weight for 1.6×10^{-14} g VSS.

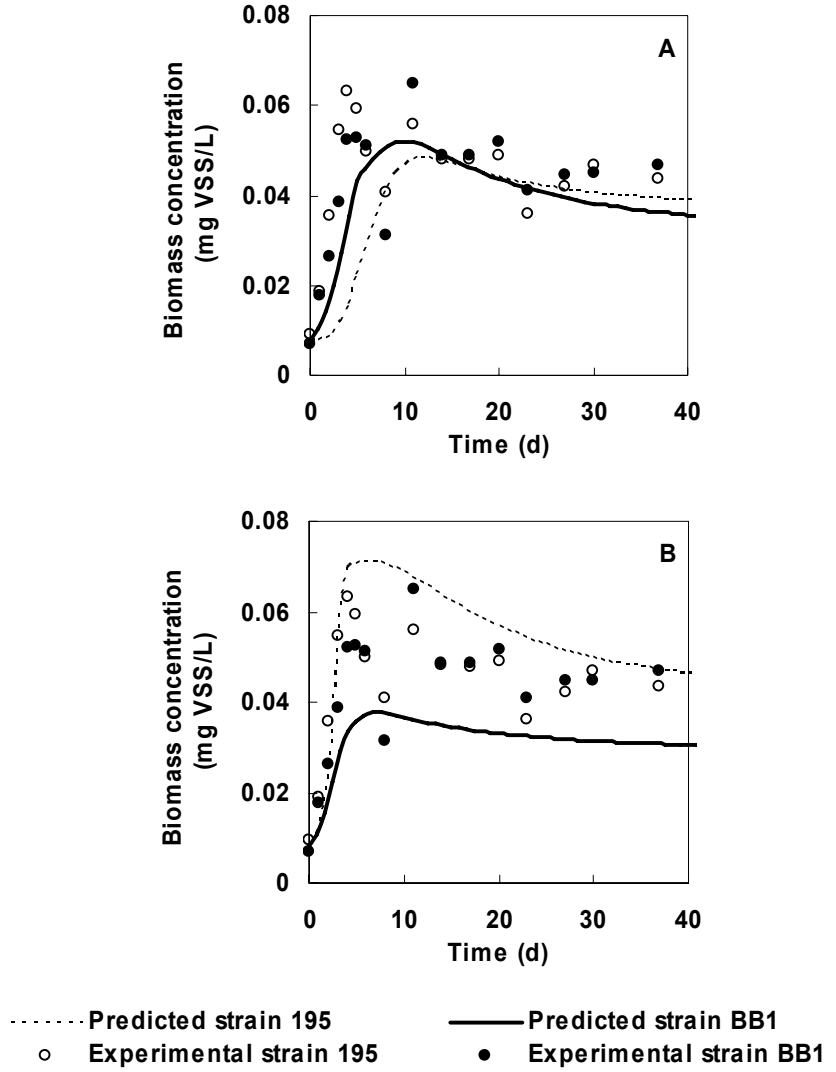

Figure 6.9 Comparison of predicted and measured biomass concentration in the bioreactors in the presence of co-cultures of *Dehalococcoides ethenogenes* strain 195 and *Desulfuromonas michiganensis* strain BB1 under natural attenuation conditions. (A) The predicted concentrations were obtained by manually adjusting the model so that strain 195 does not utilize any PCE and utilizes TCE at one-half the rates given in Table 6.3. (B) The predicted concentrations were obtained using the kinetic parameters in Table 6.3 without modification. A combination of intrinsic (days 0-5) and extant (>5 d) kinetic parameter inputs were used.

6.3.2 Substrate interactions between *Dehalococcoides ethenogenes* and heterotrophic dehalorespirers under engineered bioremediation conditions

Under engineered bioremediation conditions, substrate interactions between strain 195 and either strain BB1 or strain PCE1 plus *A. woodii* in CSTRs were evaluated experimentally and mathematically. The results of these two sets of evaluations are discussed together in the following sections.

6.3.2.1 Chlorinated ethene transformation by the co-cultures

Each co-culture experiment was conducted under engineered bioremediation conditions for more than 2 SRTs (40 d). Steady-state performance was established in all of the reactors within this period. The concentrations of PCE, TCE, and cis-DCE were lower than in the experiment conducted under natural attenuation conditions. With the co-culture of strain 195 and strain BB1 (Figure 6.10A), the PCE concentration dropped below 0.3 μM after 10 days, and the *cis*-DCE concentration was maintained between 1 and 2 μM after a steady-state condition was established. TCE was detected in the duplicate reactors only on day one. VC was the predominant product of PCE dechlorination during the steady-state period. With the co-culture of strain 195, strain PCE1 and *A. woodii*, there was no significant accumulation of either TCE or *cis*-DCE in the reactors during the steady-state period (Figure 6.11A). VC accounted for about 99% percent of the PCE added to the reactors. No significant ethene was detected in either scenario under engineered bioremediation conditions, and thus ethene was not included in Figures 6.10A and 6.11A.

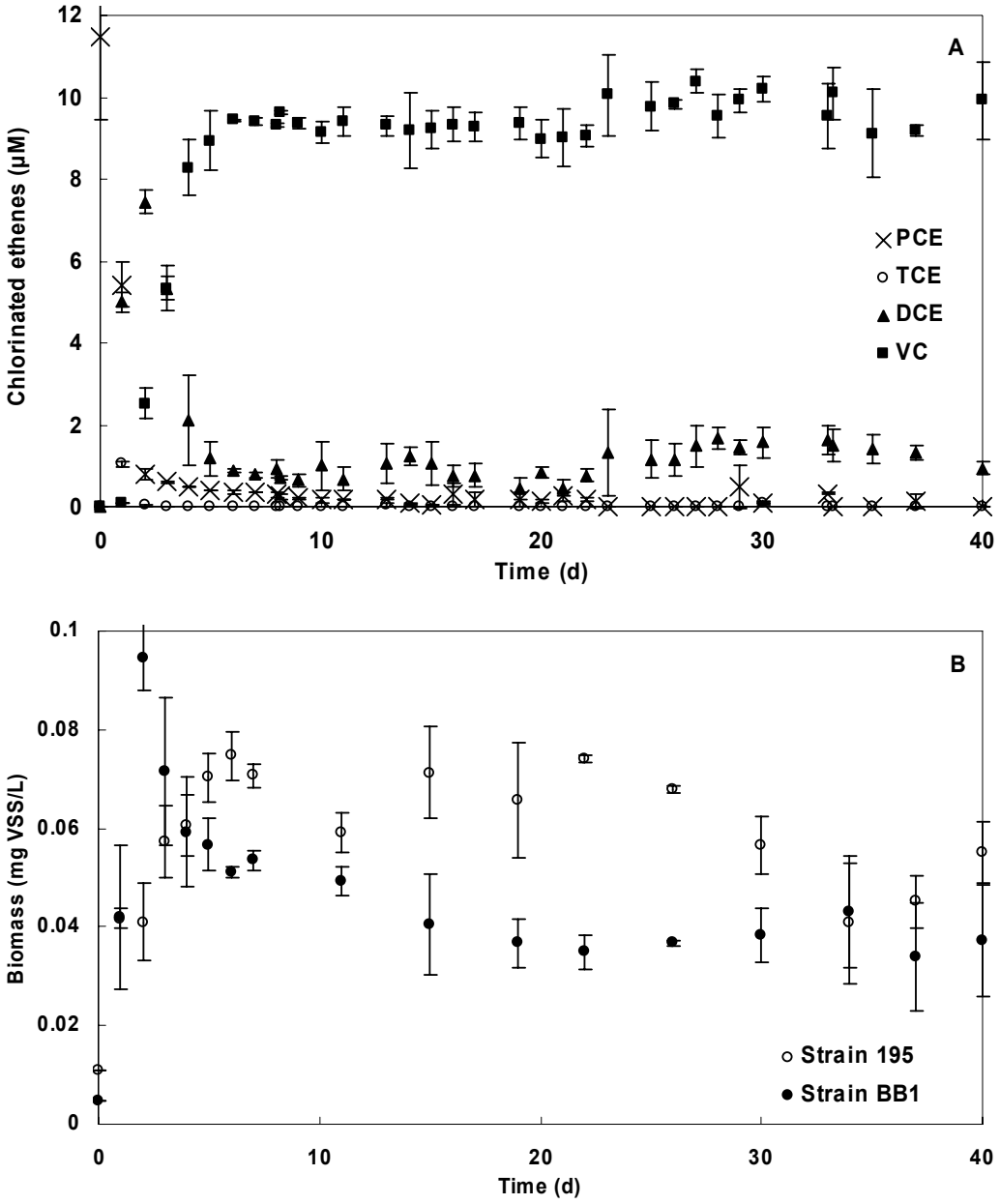

Figure 6.10 (A) Chlorinated ethene and (B) biomass concentrations in the presence of *Dehalococcoides ethenogenes* strain 195 and *Desulfuromonas michiganensis* strain BB1 under engineered bioremediation conditions. The error bars represent the standard deviation of the samples from the duplicate reactors.

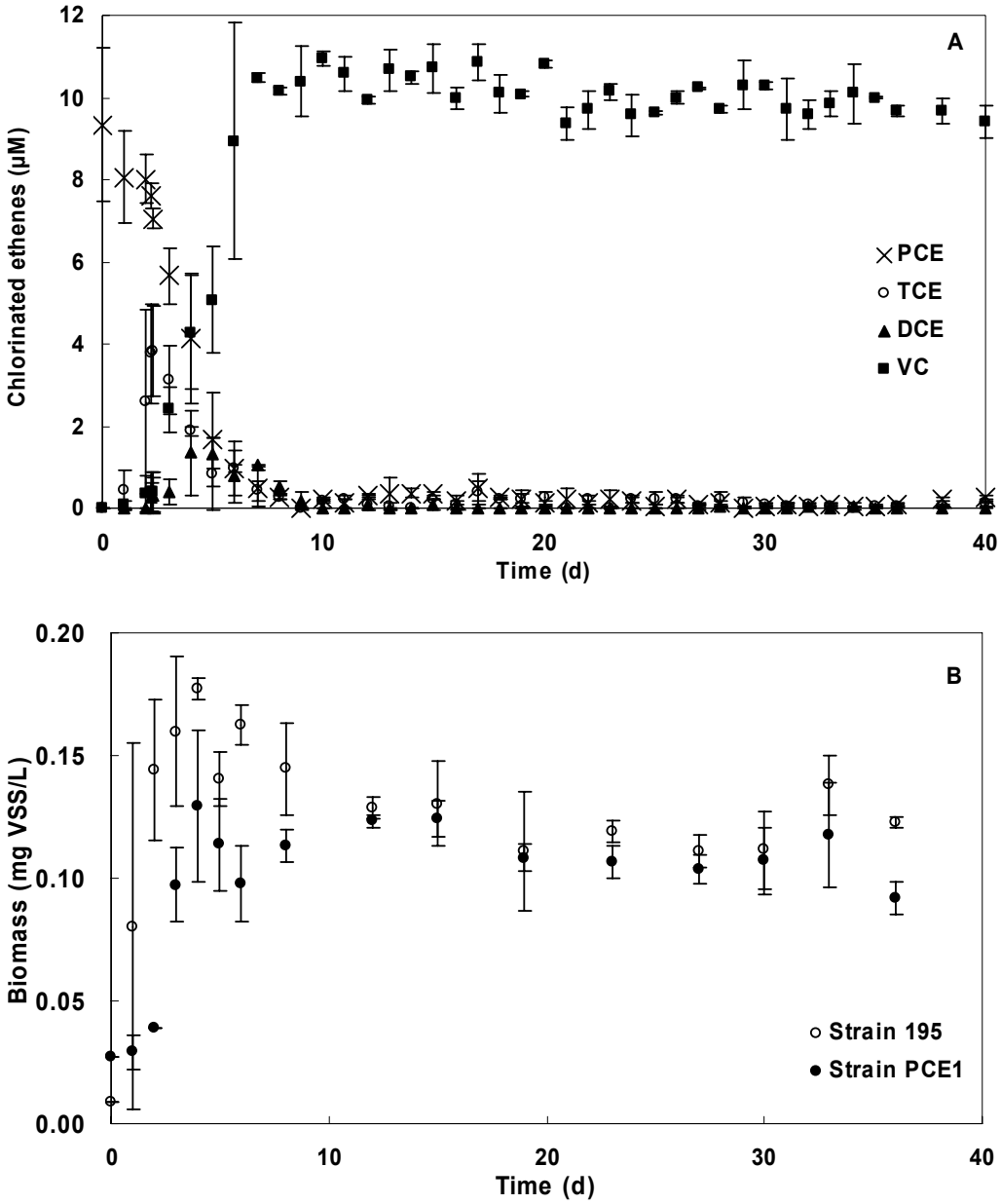

Figure 6.11 (A) Chlorinated ethene and (B) biomass concentrations in the presence of *Dehalococcoides ethenogenes* strain 195, *Desulfitobacterium* sp. strain PCE1, and *Acetobacterium woodii* under engineered bioremediation conditions. The error bars represent the standard deviation of the samples from the duplicate reactors.

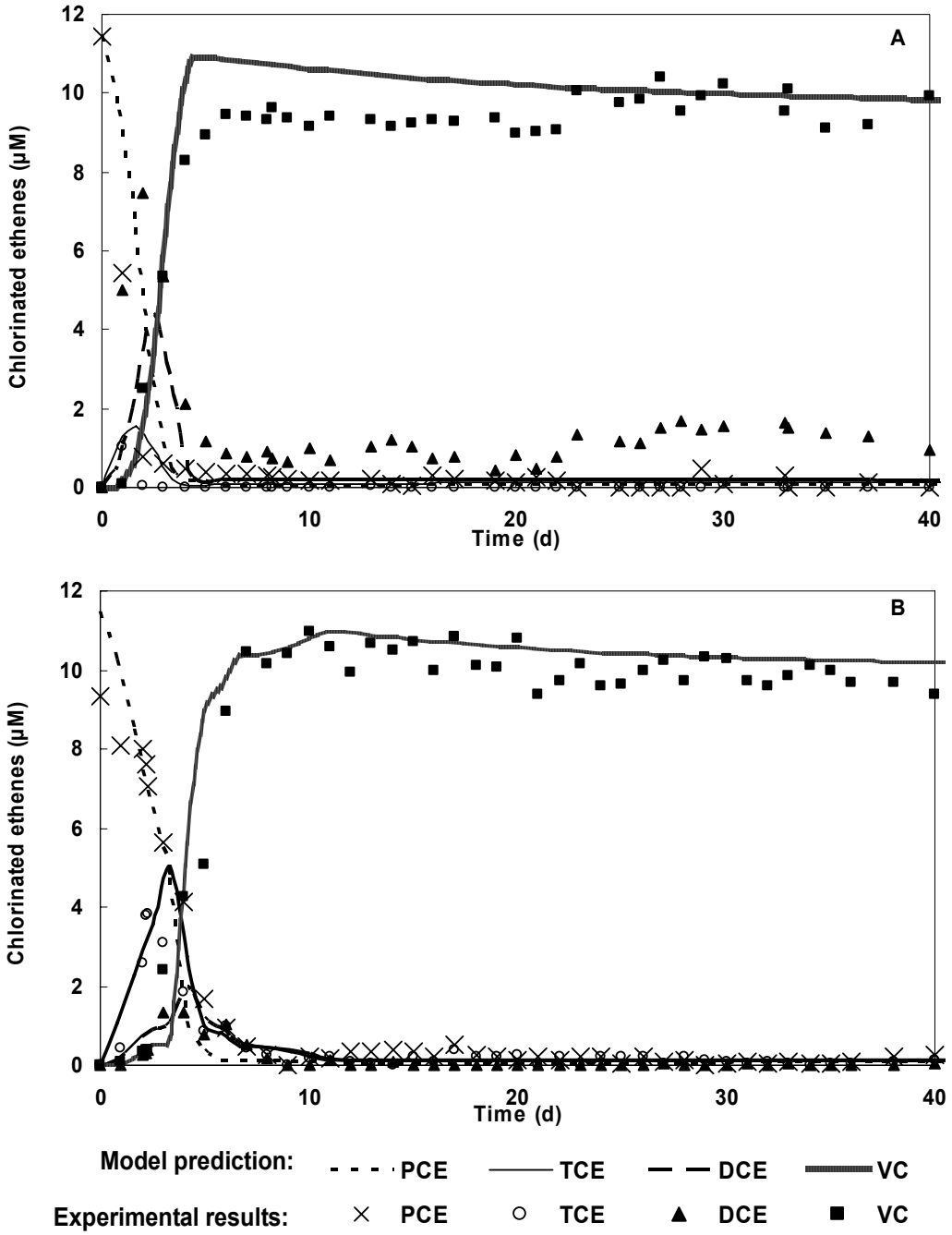

Figure 6.12 Comparison of model predictions (lines) and experimental results (points) of chlorinated ethene concentrations in the co-culture of (A) *Dehalococcoides ethenogenes* strain 195 and *Desulfuromonas michiganensis* strain BB1; and (B) *Dehalococcoides ethenogenes* strain 195, *Desulfitobacterium* sp. strain PCE1, and *Acetobacterium woodii* under engineered bioremediation conditions. A combination of intrinsic kinetic parameter inputs for days 0-5 and extant kinetic parameters inputs for >5 d were used.

The model simulations of the activities of the co-cultures were performed using a combination of intrinsic kinetic parameters (days 0-5) and extant kinetic parameters (> 5d) under engineered bioremediation conditions (Figure 6.12). For both scenarios, the model simulation results matched the experimental results pretty well. However, the predicted steady-state *cis*-DCE concentration was lower than the experimentally measured values for the co-culture of strain 195 and strain BB1 (Figure 6.12A). Again, this implies that strain 195 did not perform as well as expected based on pure culture assays when strain BB1 was absent. In any case, the consistency between the model simulations and experimental results confirms the effectiveness of using a combination of intrinsic and extant kinetics parameter inputs to simulate the entire range of experimental conditions.

Consistent with the experimental results, VC was the most dominant product of PCE dechlorination and no ethene was produced according to the model simulation for both scenarios. The accumulation of VC under engineered bioremediation is undesirable due to the know carcinogenicity of this compound. Initially, the accumulation of VC was surprising given that there was abundant electron donor available. However, electron donors were not limiting under natural attenuation conditions, so it makes sense that the increased availability of electron donors under engineered bioremediation conditions did not alleviate the problem of VC accumulation. The relative slow kinetics of VC transformation by strain 195 undoubtedly contributed to the VC accumulation. Therefore, at the conclusion of the engineered bioremediation experiment conducted with strain PCE1, the bioreactor flow rate was decreased to achieve a SRT of 80 d in an attempt to achieve greater

conversion of VC to ethene. However, the ethene concentration increased less than 1 μM as a result of these measures (data not shown). The inhibition of chlorination of lesser chlorinated ethenes by PCE has been reported (Lai and Becker, unpublished; Yu and Semprini, 2004; Yu et al., 2005). However, the PCE concentration was exceedingly low under engineered bioremediation conditions. Therefore, it is not surprising that when an inhibition term was incorporated into the model to model the PCE inhibition effect, the simulation results were not much different from the results without inhibition term (data not shown). As mentioned above (Chapter 5), the low levels of higher chlorinated ethenes present in the bioreactors may not have been adequate to induce expression of the dehalogenase responsible for VC dechlorination. Currently, another PhD student, Yenjung Lai, is evaluating whether the inclusion of a *Dehalococcoides* strain that respires VC would significantly increase VC conversion to ethene.

6.3.2.2 Population growth of the co-cultures

In both engineered bioremediation scenarios, the co-cultures experienced rapid growth during days 0-5 (Figures 6.10B and 6.11B). This growth accompanied rapid dechlorination of PCE dechlorination in the initial 5 d period. In the co-culture of strain 195 and strain BB1 (Figure 6.10B), strain BB1 initially grew faster than strain 195, which resulted in the accumulation of *cis*-DCE during the first five days (Figure 6.10A). The strain 195 population increased steadily during the period when *cis*-DCE concentrations were decreasing (Figure 6.10B), indicating that strain 195 was growing, at least in part, through dehalorespiration of *cis*-DCE. Presumably, once a stable strain 195 population had been built up, it also began transforming greater

amounts of PCE and TCE. This could explain the decrease in the strain BB1 population after day 2. Interestingly, while the chlorinated ethene concentrations reached a steady-state within about 5 d, it took 10 d for strain BB1 to become relatively constant, and strain 195 biomass levels continued to fluctuate throughout the experiment. This supports the idea that shifts in the chlorinated ethenes utilized by strain 195 were occurring. Overall, strains BB1 and 195 were present at similar levels under natural attenuation and engineered bioremediation conditions (compare Figure 6.8 and Figure 6.10B), despite the fact that the electron donor concentrations in the engineered bioremediation conditions were 10 times higher than under natural attenuation conditions. However, PCE concentrations were similar in the two experiments, indicating that, overall, growth of the two strains was limited by electron acceptor availability. This is consistent with the observation that electron donors were not depleted under natural attenuation conditions. However, the concentration of strain 195 under engineered bioremediation conditions nearly doubled when it was grown with strain PCE1 and *A. woodii* (Figure 6.10B) compared to its growth in the presence of strain BB1 (Figure 6.11B). Presumably, this can be attributed to greater electron acceptor availability for strain 195 in the presence of strain PCE1. Whereas strain BB1 may be able to compete with strain 195 for both PCE and TCE, strain PCE1 can dechlorinate PCE only to TCE, which strain 195 can utilize, along *cis*-DCE, to build up large amounts of biomass.

The model simulations of the engineered bioremediation experiments using the kinetic parameters in Table 6.3 without modification predicted that strain BB1 and strain PCE1 would be washed out (Figure 6.13A, C), due to utilization of increasing

amounts of PCE and/or TCE by strain 195. When the modified kinetic inputs used for strain 195 under natural attenuation conditions were applied under engineered bioremediation conditions, the simulation results did a better job of describing the general trends of the biomass concentrations as shown in Figure 6.13B and D. In particular, the model predicted steady-state biomass concentrations under engineered bioremediation conditions were 0.05 and 0.04 mg VSS/L for strain 195 and strain BB1, respectively in experiment 2 and 0.08 and 0.09 mg VSS/L for strain 195 and strain PCE1, respectively in experiment 3. These predictions compared well to the experimentally measured values (Figure 6.13B, D).

The abundance of the lactate fermenter *A. woodii* in the presence of strain 195 and strain PCE1 under engineered bioremediation conditions was also quantified using real-time PCR technique. *A. woodii* experienced a rapid growth during the first 5 d of the experiment and the maximum value was sustained during the steady-state period (Figure 6.14). The experimental results closely matched the model-predicted *A. woodii* concentration and, consistent with the other data, suggest that the dechlorination activity of strain 195 was not limited by the electron donor supply in this scenario.

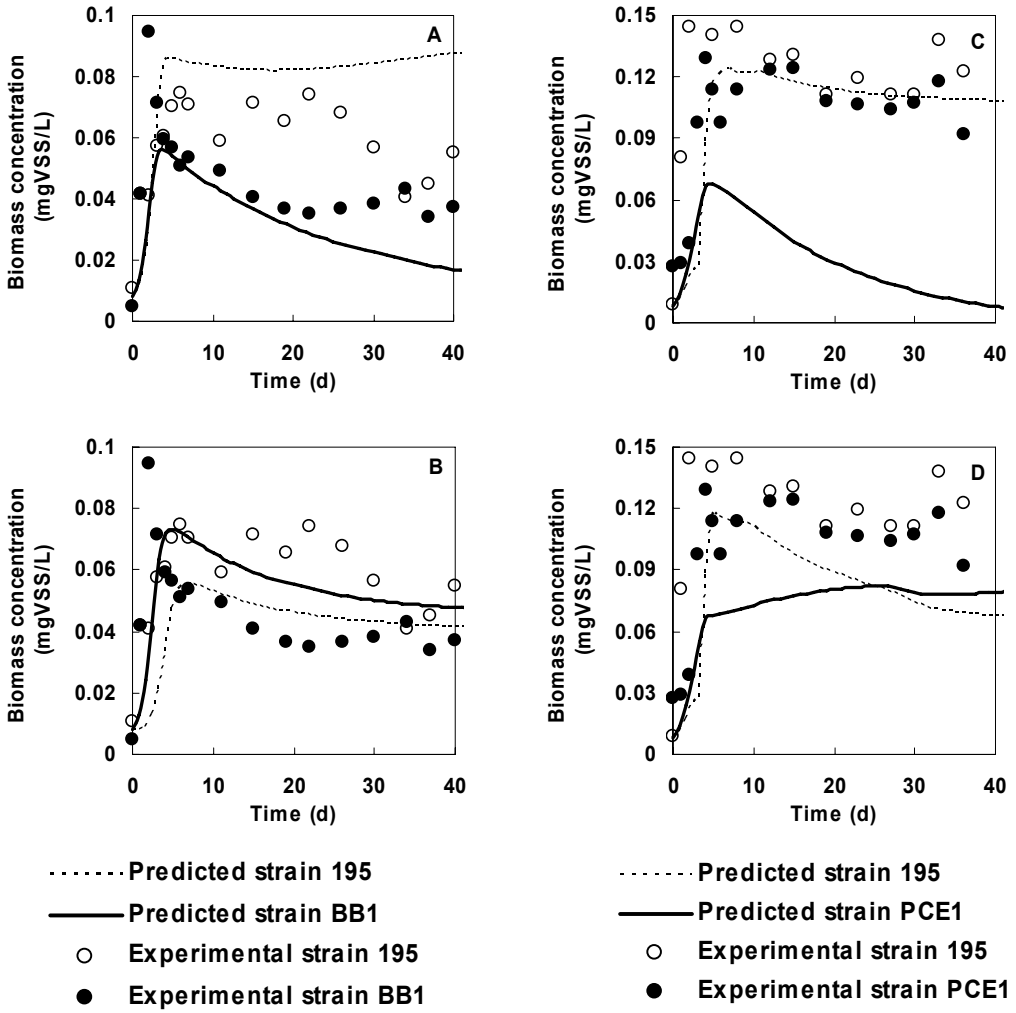

Figure 6.13 Comparison of predicted and measured biomass concentration in the bioreactors in the presence of co-cultures of (A) and (B) *Dehalococcoides ethenogenes* strain 195 and *Desulfuromonas michiganensis* strain BB1 under engineered bioremediation conditions; (C) and (D) *Dehalococcoides ethenogenes* strain 195, *Desulfitobacterium* sp. strain PCE1, and *Acetobacterium woodii* under engineered bioremediation conditions. The predicted concentrations in (A) and (C) were obtained using the kinetic parameters in Table 6.3 without modification. The predicted concentrations in (B) were obtained by manually adjusting the model so that strain 195 does not utilize any PCE and utilizes TCE at one-half the rates given in Table 6.3. The predicted concentrations in (D) were obtained by manually adjusting the model so that strain 195 utilizes no PCE. A combination of intrinsic (days 0-5) and extant (>5 d) kinetic parameter inputs were used.

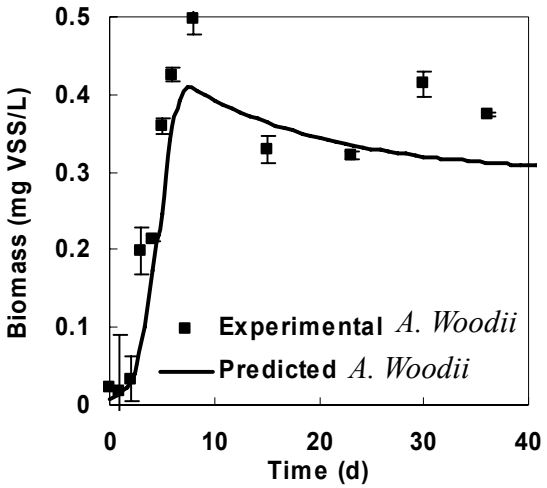

Figure 6.14 Comparison of predicted and measured biomass concentrations of *A. Woodii* under engineered bioremediation in the presence of co-cultures of *Dehalococcoides ethenogenes* strain 195, *Desulfitobacterium* sp. strain PCE1, and *Acetobacterium woodii*.

6.4 Modeling PCE dechlorination by the co-cultures of Dehalococcoides species and PCE-to-TCE/cis-DCE dehalorespirers

The model described in Section 6.2.8 can successfully describe chlorinated ethene utilization by co-cultures of strain 195 and a PCE-to-*cis*-DCE (or TCE) dehalorespirer when a combination of intrinsic and extant kinetic parameters is used. However, the comparison of the experimental and modeling data clearly demonstrated that the activity of dechlorination by strain 195 can be impacted by the presence of a PCE-to-*cis*-DCE (or TCE) dehalorespirer. This phenomenon or interaction between the *Dehalococcoides* species and the PCE-to-*cis*-DCE (or TCE) dehalorespirers results in less consumption of electron donor by strain 195 and less biomass growth of strain 195 than is predicted using the current model.

As discussed above, the additional phenomena or interactions between *Dehalococcoides* species and PCE-to-TCE/*cis*-DCE dehalorespirers should be incorporated into the current model to accurately describe growth and electron donor utilization by multiple dehalorespiring strains. However, the experimental and theoretical demonstration that *Dehalococcoides* strains can coexist with PCE-to-*cis*-DCE (or TCE) dehalorespirers in a continuous-flow systems is a significant finding of this study that has important potential implications. In the presence of a PCE-to-*cis*-DCE (or TCE) dehalorespirer, strain 195 appears to function primarily as a *cis*-DCE respiring organism. This approach may result in the most efficient utilization of organic and inorganic electron donors available in the contaminant plume and ultimately the greatest amount of contaminant removal and detoxification. However, the accumulation of VC in this study suggests that other *Dehalococcoides* strains that can dehalorespire VC, such as *Dehalococcoides* strain BAV1 (He et al., 2003), may be needed to drive PCE dechlorination to non-toxic ethene.

Chapter 7: Conclusions and recommendations

PCE dechlorination kinetics for two heterotrophic dehalorespirers, *Desulfuromonas michiganensis* strain BB1 and *Desulfitobacterium* sp. strain PCE1, were investigated using batch culture assays under both electron donor- and acceptor-limiting conditions. Acetate and lactate utilization kinetics including the thresholds of the electron donors, have not previously been reported for dehalorespirers. The initial conditions in the batch assays were found to have a significant impact on both the kinetic parameter estimates themselves and their identifiability. This study showed that it is possible to obtain identifiable intrinsic and extant kinetic parameters for PCE dechlorination by controlling the initial conditions in the batch assays. Kinetic parameters estimated under different initial conditions in batch culture assays describe microbial populations in different physiological states and thus have different applications. Therefore, reports of kinetic parameter estimates that do not describe the conditions under which they were obtained are of limited.

For some microorganisms that grow on toxic substrates, there may be a tolerance level above which the microorganisms cease to grow and utilize the toxic substrates. However, for some dehalorespirers, such as strain BB1 and strain PCE1, the level of PCE tolerated is not constant. Instead, the level of PCE tolerated and transformed by these dehalorespirers is influenced by by the biomass concentration. None of the existing models of inhibitory substrate utilization described this phenomenon successfully. Therefore, a new model of PCE dechlorination at high concentrations was developed in this study by incorporating a biomass inactivation term into the

biomass growth equation. When coupled with the Andrews substrate utilization model for self-inhibitory substrates, it provided a good description of the dechlorination of high concentrations of PCE by varying amounts of strain BB1 and strain PCE1 biomass.

The interactions between a *Dehalococcoides* strain and PCE-to-TCE/ PCE-to-*cis*-DCE dehalorespirers was also investigated. Both experimental and theoretical results suggested that co-existence of *Dehalococcoides* and heterotrophic dehalorespiring strains is possible due to the ability of *Dehalococcoides* strains to respire lesser chlorinated ethenes produced by the heterotrophic populations. However, competition for carbon source or some other interaction with the heterotrophic dehalorespirers appears to limit the *Dehalococcoides* strain's ability to compete for the higher chlorinated ethenes. Nevertheless, maintenance of dehalorespirers that utilize both organic and inorganic electron donors is advantageous and may optimize the bioremediation of chlorinated ethenes in continuous-flow systems.

Based on the results of this research, the following specific conclusions can be drawn and several recommendations can be made:

(1) Identifiable intrinsic electron donor and acceptor utilization kinetic parameters, as well as extant electron acceptor utilization kinetics for PCE dechlorination by strain BB1 and strain PCE1 can be obtained using batch culture assays. The relatively low K_S and threshold values for electron donor utilization suggest that it is unlikely that strain BB1 and strain PCE1 will be restricted by the availability of electron donors under engineered bioremediation conditions. The

intrinsic kinetic parameter estimates for PCE dechlorination can successfully predict PCE dechlorination in batch cultures with initial substrate-to-biomass ratios (S_0/X_0) higher than 10, but not in batch cultures with $S_0/X_0<5$.

(2) The initial conditions of the batch culture assays have a significant impact on Monod kinetic parameter determination. q_{max} estimates are highly correlated with the initial substrate-to-biomass concentration ratios (S_0/X_0), which represent the relative substrate availability in the batch culture assays. Generally, higher S_0/X_0 ratios result in higher q_{max} estimates. However, when $S_0/X_0>10$, the q_{max} estimates reach a maximum value that equals the intrinsic q_{max} estimate.

(3) The initial substrate-to-half-saturation constant ratio (S_0/K_S) in the batch culture assays largely determines the identifiability of the Monod kinetic parameter estimates obtained in batch assays. Generally, better identifiability or lower correlation of the parameter estimates can be obtained in batch assays with higher S_0/K_S ratios. Although lower S_0/X_0 ratios can also improve parameter identifiability, this influence is much less significant than S_0/K_S. Therefore, when a batch kinetic assay is designed, a S_0/K_S ratio higher than 10 is generally needed to obtain identifiable parameter estimates, although somewhat lower S_0/K_S ratios may be adequate when the extant S_0/X_0 criterion is met. An iterative procedure that can be used to select the initial conditions needed to ensure that parameter estimates are identifiable was outlined in Chapter 4.

(4) Strain BB1 and strain PCE1 can be inhibited by high concentrations of chlorinated ethenes. However, at sufficiently high biomass concentrations, these

populations may be able to transform PCE at concentrations up to the aqueous solubility limit. This discovery suggests that these organisms may be suitable for in situ bioremediation of PCE DNAPL source zones in contaminated groundwater.

(5) Dechlorination of high concentrations of PCE by strain BB1 and strain PCE1 can be described by a new model that includes a biomass inactivation term in the biomass growth equation and describes PCE utilization with the Andrews kinetic model. The intrinsic kinetic parameter estimates and inhibition, and biomass inactivation coefficient estimates obtained in this study can be used along with the model developed in this study to predict the fate of PCE and dehalorespirer populations during the bioremediation of PCE DNAPL source zones. In addition, this information may be useful for determining the amount of dehalorespiring culture that must be added in order to achieve successful bioaugmentation of PCE DNAPLs.

(6) The co-existence of a *Dehalococcoides* strain and PCE-to-TCE/*cis*-DCE dehalorespirers was shown to be possible in continuous-flow systems. However, strain 195, the *Dehalococcoides* strain used in this study dechlorinates VC via cometabolism. As a result, no significant ethene was produced in the co-culture experiments. Therefore, PCE dechlorination by co-cultures that include VC-respiring strains, such as *Dehalococcoides* strain BAV1 (He et al., 2003), as well as PCE-to-*cis*-DCE dehalorespirers should be studied in the future.

One of the most important findings in this study is that the utilization of PCE and TCE by *Dehalococcoides ethenogenes* strain 195 may be negatively impacted by the presence of a PCE-to- *cis*-DCE/TCE dehalorespirer. As a result, it functioned primarily as a *cis*-DCE-to-ethene dehalorespirer in the co-cultures. However, additional investigations are needed to determine why the activity of strain 195 to compete for PCE and TCE appears to be reduced in the presence of another dehalorespirer.

Bibliography

1. Adamson DT, Lyon DY, Hughes JB. 2004. Flux and product distribution during biological treatment of tetrachloroethene dense non-aqueous-phase liquid. *Environ Sci Technol*. 38: 2021–2028.
2. Agency for Toxic Substances and Disease Registry (ATSDR). 1997. Toxicological profile for Tetrachloroethylene (PERC). Atlanta, GA: U.S. Department of Health and Human Services.
3. Alagappan G, Cowan RM. 2001. Biokinetic models for representing the complete inhibition of microbial activity at high substrate concentrations. *Biotechnol Bioeng*. 75:393–405.
4. Alagappan, G, Cowan RM. 2003. Substrate inhibition kinetics for toluene and benzene degrading pure cultures and a method for collection and analysis of respirometric data for strongly inhibited cultures. *Biotechnol. Bioeng*. 83: 798–810.
5. Alvarez-Cohen L, Speitel Jr. GE. 2001. Kinetics of aerobic cometabolism of chlorinated solvents. *Biodegradation*. 12:105–126.
6. Alvarez-Cohenm L, McCarty PL. 1991. A cometabolic biotransformation model for halogenated aliphatic compounds exhibiting product toxicity. *Environ Sci Technol*. 25:1381–1387.
7. Amos BK, Christ JA, Abriola LM, Pennell KD, Loffler FE. 2007. Experimental evaluation and mathematical modeling of microbially enhanced tetrachloroethene (PCE) dissolution. *Environ Sci Technol*. 41: 963 – 970.
8. Andrews JF. 1968. A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates. *Biotechnol Bioeng*. 10:707-723.
9. Atlas RM. 1997. Handbook of microbiological media. CRC Press, New York, N.Y.
10. Aulenta F, Bianchi A, Majone M, Petrangeli PM, Potalivo M, Tandoi V. 2005. Assessment of natural or enhanced in situ bioremediation at a chlorinated solvent-contaminated aquifer in Italy: a microcosm study. *Environ Int*. 31:185-190.
11. Aulenta F, Rossetti S, Majone M, Tandoi V. 2004. Detection and quantitative estimation of Dehalococcoides spp. in a dechlorinating bioreactor by a combination of fluorescent in situ hybridisation (FISH) and kinetic analysis. *Appl Microbiol Biotechnol*. 64: 206-212.
12. Bagley DM. 1998. Systematic Approach for modeling tetrachloroethene biodegradation. *J Environ Eng*. 124: 1076-1086.
13. Ballapragada BS, Stensel HD, Puhakka JA, Ferguson JF. 1997. Effect of hydrogen on reductive dechlorination of chlorinated ethenes. *Environ Sci*

Technol. 31:1728–1734.

14. Baptista, IIR, Zhou NY, Emanuelsson EAC, Peeva LG, Leak DJ, Mantalaris A, Livingston AG. 2008. Evidence of species succession during chlorobenzene biodegradation. *Biotechnol Bioeng*. 99: 68-74.
15. Beck JV, Arnold KJ. Parameter Estimation in Engineering and Science; John Wiley & Sons: New York, 1977.
16. Becker JG. 2006. A Modeling study and implications of competition between *Dehalococcoides ethenogenes* and other tetrachloroethene- respiring bacteria. *Environ Sci Techno.* 40: 4473-4480.
17. Becker JG, Seagren EA. 2009. Modeling the effects of microbial competition and hydrodynamics on the dissolution and detoxification of dense nonaqueous phase liquid contaminants. *Environ Sci Technol*. 43: 870-877.
18. Bielefeldt AR, Stensel HD. 1999. Biodegradation of aromatic compounds and TCE by a filamentous bacteria-dominated consortium. *Biodegradation.* 10: 1–13.
19. Bouwer EJ. 1994. Bioremediation of chlorinated solvents using alternate electron acceptors. In: In Situ Bioremediation (Rittmann BE, Seagren E, Wrenn BA, Valocchi AJ, Ray C, Raskin L, eds). 2nd ed. Park Ridge, NJ: Noyes Publishers, 149–168.
20. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 72: 248-254.
21. Bradley PM. 2003. History and ecology of chloroethene biodegradation: A review. *Bioremediation Journal*. 7: 81-109.
22. Bradley PM, Chapelle FH. 1996. Anaerobic mineralization of vinyl chloride in Fe(III)-reducing aquifer sediments. *Environ Sci Technol.* 30:2084–2086.
23. Bradley PM, Landmeyer JE, Dinicola RS. 1998. Anaerobic oxidation of [1,2-^{14}C]dichloroethene under MN(IV)-reducing conditions. *Appl Environ Microbiol*. 64:1560–1562.
24. Brennan RA, Sanford RA, Werth CJ. 2006a. Biodegradation of Tetrachloroethene by Chitin Fermentation Products in a Continuous Flow Column System. *J Environ Eng*. 132: 664-673.
25. Brennan RA, Sanford RA, Werth CJ. 2006b. Chitin and corncobs as electron donor sources for the reductive dechlorination of tetrachloroethene. *Water Research.* 40: 2125 – 2134.
26. Breznak JA. 1994. Acetogenesis from carbon dioxide in termite guts, p. 303-330. In H. L. Drake (ed.), Acetogenesis. Chapman & Hall, Inc., New York, N.Y.
27. Brockmann D, Rosenwinkel KH, Morgenroth E. 2008. Practical identifiability of biokinetic parameters of a model describing two-step nitrification in biofilms. *Biotechnol Bioeng*. 101: 497-514.

28. Brun R, Kühni M, Siegrist H, Gujer W, Reicher P. 2002. Practical identifiability of ASM2d parameters-systematic selection and tuning of parameter subsets. *Wat Res*. 36: 4113-4127.

29. Bunge M, Kleikemper J, Miniaci C, Duc L, Muusse MG, Hause G, Zeyer J. 2007. Benzoate-driven dehalogenation of chlorinated ethenes in microbial cultures from a contaminated aquifer. *Appl Microbiol Biotechnol*. 76: 1447-1456.

30. Carr CS, Garg S, Hughes JB. 2000. Effect of dechlorinating bacteria on the longevity and composition of PCE-containing nonaqueous phase liquids under equilibrium dissolution conditions. *Environ Sci Technol*. 34:1088–1094.

31. Carrera J, Jubany I, Carvallo L, Chamy R, Lafuente J. 2004. Kinetic models for nitrification inhibition by ammonium and nitrite in a suspended and an immobilised biomass systems. *Process Biochem*. 39: 1159-1165.

32. Chang YC, Hatsu M, Jung K, Yoo YS, Takamizawa K. 2000. Isolation and characterization of a tetrachloroethylene dechlorinating bacterium, *Clostridium bifermentans* DPH-1. *J Biosci Bioeng*. 89:489–491.

33. Checchi N, Marsili-Libelli S. 2005. Reliability of parameter estimation in respirometric models. *Wat Res*. 39:3686-3696.

34. Chiu SY, Fan LT, Erickson LE. 1972. Kinetic behavior of mixed populations of activated sludge. *Biotechnol Bioengng.* 14: 179-199.

35. Christ JA, Ramsburg CA, Abriola LM, Pennell KD, Löffler FE. 2005. Coupling aggressive mass removal with microbial reductive dechlorination for remediation of DNAPL source zones: a review and assessment. *Environ Health Perspect*. 113:465–477.

36. Christiansen N, Christensen SR, Arvin E, Ahring BK. 1997. Transformation of tetrachloroethene in an upflow anaerobic sludge blanket reactor. *Appl Microbiol Biotechnol*. 47: 91-94.

37. Chudoba P, Capdeville B, Chudoba J. 1992. Explanation of biological meaning of the S0/X0 ratio in batch culture. *Water Sci Technol.* 26:743–751.

38. Cole JR, Fathepure BZ, Tiedje JM. 1995. Tetrachloroethene and 3-chlorobenzoate activities are co-induced in *Desulfomonile tiedjei* DCB-1. *Biodegradation*. 6: 167–172.

39. Coleman NV, Mattes TE, Gossett JM, Spain JC. 2002a.Biodegradation of *cis*-dichloroethene as the sole carbon source by a β-proteobacterium. *Appl Environ Microbiol*. 68: 2726–2730.

40. Coleman NV, Mattes TE, Gossett JM. Spain JC. 2002b. Phylogenetic and kinetic diversity of aerobic vinyl chloride-assimilating bacteria from contaminated sites. *Appl Environ Microbiol*. 68: 6162–6171.

41. Conrad R. 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). *Microbiol Rev*. 60: 609-640.

42. Cope N, Hughes JB. 2001. Biologically-enhanced removal of PCE from NAPL source zones. *Environ Sci Technol*. 35:2014–2021.

43. Cord-Ruwisch R, Seitz, HJ, Conrad R. 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. *Arch Microbiol*. 149:350-357.

44. Cupples AM, Spormann AM, Mccarty PL. 2003. Growth of a *Dehalococcoides*-like microorganism on vinyl chloride and cis-dichloroethene as electron acceptors as determined by competitive PCR. *Appl Environ Microbiol*. 69: 953-959.

45. Cupples AM, Spormann AM, McCarty PL. 2004. Vinyl chloride and *cis*-dichloroethene dechlorination kinetics and microorganism growth under substrate limiting conditions. *Environ Sci Technol*. 38:1102– 1107.

46. Da Silva, M. L. B.; Daprato, R. C.; Gomez, D. E.; Hughes, J. B.; Ward, C. H.; Alvarez, P. J. J. 2006. Comparison of bioaugmentation and biostimulation for the enhancement of dense nonaqueous phase liquid source zone bioremediation. *Water Environment Research*. 78: 2456-2465.

47. Damborsky J. 1999. Tetrachloroehene-dehalogenating bacteria. *Folia Microbiol*. 44: 247-262.

48. Daprato RC, Loffler FE, Hughes JB. 2007. Comparative analysis of three tetrachloroethene to ethene halorespiring consortia suggests functional redundancy. *Environ Sci Technol*. 41: 2261 – 2269.

49. Dennis PC, Sleep BE, Fulthorpe RR, Liss SN. 2003. Phylogenetic analysis of bacterial populations in an anaerobic microbial consortium capable of degrading saturation concentrations of tetrachloroethylene. *Can J Microbiol*. 49:15–27.

50. Denyer SP, Maillard JY. 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. *J Appl Microbiol*. 92 (Suppl. 1): 35s-45s.

51. Devore JL, and Peck R. 1996. Statistics: The exploration and analysis of data. 3rd Edition. Publisher Duxbury Press.

52. DeWeerd, K. A.; Mandelco, L.; Tanner, R. S.; Woese, C. T.; Suflita, J. M. 1990. *Desulfomonile tiedjei*, new genus new species, a novel anaerobic, dehalogenating, sulfate-reducing bacterium. *Arch Microbiol*. 154:23–30

53. Dijkhuizen DE, Hartle DL. 1983. Selection in chemostats. *Microbiol Rev*. 47: 150-168.

54. Distefano TD. 1999. The effect of tetrachloroethene on biological dechlorination of vinyl chloride: Potential implication for natural bioattenuation. *Water Res*. 33: 1688-1694.

55. DiStefano TD, Gossett JM, Zinder SH. 1991. Reductive dechlorination of high concentration of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogensis. *Appl Environ Microbiol*. 57: 2287-2292.

56. Distefano TD, Gossett JM, Zinder SH. 1992. Hydrogen as an electron donor for dechlorination of tetrachloroethene by anaerobic mixed culture. *Appl Environ Microbiol*. 58:3622 – 3629.

57. Dolfing J. 2000. Energetics of anaerobic degradation pathways of chlorinated aliphatic compounds. *Microbiol Ecol*.40:2–7.

58. Dolfing J. 2003. Thermodynamic considerations for dehalogenation. in *Microbial Processes and Environmental Applications*. Boston/Dordrecht/London: Kluwer: 89-114.

59. Dolfing J, Harrison BK. 1992. Gibbs free energy of formation of halogenated aromatic compounds and their potential as electron acceptors in anaerobic environments. *Environ Sci Technol*. 26:2213–2218.

60. Dolfing J, Janssen DB. 1994. Estimation of Gibbs free energies of formation of chlorinated aliphatic compounds. *Biodegradation.* 5:21–28.

61. Dolfing J, Tiedje JM. 1987. Growth yield increase linked to reductive dechlorination in a defined 3-chlorobenzoate degrading methanogenic coculture. *Archives of Microbiology*. 149:102-105.

62. Duhamel M, Edwards EA. 2006. Microbial composition of chlorinated ethene-degrading cultures dominated by *Dehalococcoides*. *FEMS Microbiol Ecol.* 58:538-549.

63. Egli T. 1995. The ecological and physiological significance of the growth of heterotrophic microorganisms with mixtures of substrates. *Adv Microbiol Ecol*. 14: 305-386.

64. Ehrlich GG, Goerlitz DF, Bourell JH, Eisen GV, Godsy EM. 1981. Liquid chromatographic procedure for fermentation product analysis in the identification of anaerobic bacteria. *Appl Environ Microbiol*. 42:878-885.

65. El Fantroussi S, Naveau H, Agathos SN. 1998. Anaerobic dechlorinating bacteria. *Biotechnol Prog*. 14:167-88.

66. Ellis DE, Lutz EJ, Odom JM, Buchanan RJ. Jr, Bartlett CL, Lee MD, Harkness MR, Deweerd KA. 2000. Bioaugmentation for accelerated in situ anaerobic bioremediation. *Environ Sci Technol*. 34: 2254-2260.

67. Ellis TG, Anselm CV. 1999. Effect of batch discharges on extant biodegradation kinetics in activated-sludge systems. *Water Environ Res*. 71, 290–298.

68. Ellis TG, Barbeau DS, Smets BF, Grady CPL Jr. 1996a. Respirometric technique for determination of extant kinetic parameters describing biodegradation. *Water Environ Res*. 68: 917-926.

69. Ellis TG, Barbeau DS, Smets BF, Grady CPL Jr. 1996a. Respirometric technique for determination of extant kinetic parameters describing biodegradation. *Water Environ Res*. 68: 917-926.

70. Ellis TG, Smets BF, Magbanua BS, Grady CPL Jr. 1996b. Changes in measured

biodegradation kinetics during the longterm operation of completely mixed activated sludge (CMAS) bioreactors. *Water Sci Technol*. 34, 35–42.

71. Esteve-Núñez A, Rothermich M, Sharma M, Lovley D. 2005. Growth of *Geobacter sulfurreducens* under nutrient-limiting conditions in continuous culture. *Environmental Microbiology*. 7: 641–648.

72. Fathepure BZ, Boyd SA. 1988a. Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by *Methanosarcina* sp strain DCM. *Appl Environ Microbiol*. 54:2976–2980.

73. Fathepure BZ, Boyd SA. 1988b Reductive dechlorination of perchloroethylene and the role of methanogens. *FEMS Microbiol Lett*. 49:149–156.

74. Fennell DE, Carroll AB, Gossett JM, Zinder SH. 2001. Assessment of indigenous reductive dechlorinating potential at a TCE-contaminated site using microcosms, polymerase chain reaction Analysis, and site data. *Environ Sci Technol*. 32: 1830-1839.

75. Fennell DE, Gossett JM. 1998. Modeling the production of and competition for hydrogen in a dechlorinating culture. *Environ Sci Technol*. 32: 2450-2460.

76. Fennell DE, Gossett JM, Zinder SH. 1997. Comparison of butyric acid, ethanol, lactic ccid, and propionic acid as hydrogen donors for the reductive dechlorination of tetrachloroethene. *Environ Sci Technol*. 31: 918-926.

77. Freedman DL, Gossett JM. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. *Appl Environ Microbiol*. 55: 2144-2151.

78. Friis AK, Heimann AC, Jakobsen R, Albrechtsen HJ, Cox E, Bjerg P. L. 2007. Temperature dependence of anaerobic TCE-dechlorination in a highly enriched *Dehalococcoides*-containing culture. *Water Res.* 41: 355-364.

79. Furukawa K, Suyama A, Tsuboi Y, Futagami T, Goto M. 2005. Biochemical and molecular characterization of a tetrachloroethene dechlorinating *Desulfitobacterium* sp. strain Y51: a review. *J Ind Microbiol Biotechnol*. 32: 534-41.

80. Futagami T, Tsuboi Y, Suyama A, Goto M, Furukawa K. 2006. Emergence of two types of nondechlorinating variants in the tetrachloroethene-halorespiring *Desulfitobacterium* sp. strain Y51. *Appl Microbiol Biotechnol*. 70**:**720-728.

81. Gantzer CJ, Wackett LP. 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. *Environ SciTechnol*. 25: 715-722.

82. Garant H, Lynd L. 1998. Applicability of competitive and noncompetitive kinetics to the reductive dechlorination of chlorinated ethenes. *Biotechnol Bioeng*. 57: 751-755.

83. Gerritse J, Drzyzga O, Kloetstra G, Keijmel M, Wiersum LP, Hutson R, Collins MD, Gottschal JC. 1999. Influence of different electron donors and acceptors on dehalorespiration of tetrachloroethene by *Desulfitobacterium frappieri* TCE1. *Appl Environ Microbiol*. 65:5212-5221.

84. Gerritse J, Renard V, Pedro Gomes TM, Lawson PA, Collins, MD, Gottschal JC. 1996. *Desulfitobacterium* sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or ortho-chlorinated phenols. *Arch Microbiol*. 165:132-140.

85. Gosset JM. 1987. Measurement of Henry's Law Constant for C1 and C2 chlorinated hydrocarbons. *Environ Sci Technol*. 21: 202-208.

86. Grady CPL Jr, Daigger GT, Lim HC. 1999. Biological wastewater treatment, Sec. ed. Marcel Dekker, Inc.

87. Grady CPL Jr, Smets BF, Barbeau DS. 1996. Variability in kinetic parameter estimates: A review of possible causes and a proposed terminology. *Water Res*. 30: 742-748.

88. Griffin BM, Tiedje JM, Löffler FE. 2004. Anaerobic microbial reductive dechlorination of tetrachloroethene to predominantly *trans*-1,2-dichloroethene. *Environ Sci Technol.* 38: 4300-4303.

89. Grostern A, Edwards EA. 2006. Growth of *Dehalobacter* and *Dehalococcoides* spp. during Degradation of Chlorinated Ethanes. *Appl Environ Microbiol.* 72: 428-436.

90. Gu AZ, Hedlund BP, Staley JT, Strand SE, Stensel HD. 2004. Analysis and comparison of the microbial community structures of two enrichment cultures capable of reductively dechlorinating TCE and *cis*-DCE. *Environ Microbiol.* 6: 45–54.

91. Han K, Levenspiel O. 1988. Extended Monod kinetics for substrate, product, and cell inhibition. *Biotechnol Bioeng*. 32: 430-437.

92. Harder W, Dijkhuizen L. 1982. Strategies of mixed substrate utilization in microorganisms. *Philos Trans R Soc*. London B 297: 459-480.

93. Harkness MR, Bracco AA, Brennan MJ Jr., Deweerd KA, Spivack JL. 1999. Use of bioaugmentation to stimulate complete reductive dechlorination of trichloroethene in Dover soil columns. *Environ Sci Technol*. 33: 1100-1109.

94. Hartmans S, deBont JA. 1992. Aerobic vinyl chloride metabolism in *Mycobacterium aurum* L1. *Appl Environ Microbiol.* 58:1220–1226.

95. Haston ZC, McCarty PL. 1999. Chlorinated ethene half-velocity coefficients (Ks) for reductive dehalogenation. *Environ Sci Technol*. 33: 223-226.

96. He J, Holmes VF, Lee PK, Alvarez-Cohen L. 2007. Influence of vitamin B12 and cocultures on the growth of *Dehalococcoides* isolates in defined medium. *Appl Environ Microbiol.* 73: 2847-2853.

97. He J, Ritalahti KM, Aiello MR, Löffler FE. 2003a. Complete detoxification of vinyl chloride by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* species. *Appl Environ Microbiol*. 69: 996-1003.

98. He J, Ritalahti KM, Yang Y, Koenigsberg SS, Löffler FE. 2003b. Detoxification

of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. *Nature*. 424: 62–65.

99. He J, Sung Y, Dollhopf ME, Fathepure BZ, Tiedje JM, Löffler FE. 2002. Acetate versus hydrogen as direct electron donors to stimulate the microbial reductive dechlorination process at chloroethene-contaminated sites. *Environ Sci Technol.* 36:3945–3952.

100. He J, Sung Y, Krajmalnlk-Brown R, Ritalahti KM, Löffler FE. 2005. Isolation and characterization of *Dehalococcoides* sp. strain FL2, a trichloroethene (TCE) and 1,2-dichloroethene-respiring anaerobe. *Environmental Microbiology*. 7: 1442-1450.

101. He Q, Sanford RA. 2003. Characterization of Fe(III) reduction by chlororespiring *Anaeromyxobacter dehalogenans*. *Appl Environ Microbiol*. 69:2712–2718.

102. He Q, Sanford RA. 2004. Acetate threshold Concentrations suggest varying energy requirements during anaerobic respiration by *Anaeromyxobacter dehalogenans*. *Appl Environ Microbiol*. 70:6940-6943.

103. Heimann AC, Friis AK, Scheutz C, Jakobsen R. 2007. Dynamics of reductive TCE dechlorination in two distinct H(2) supply scenarios and at various temperatures. *Biodegradation*. 18:167-179.

104. Hendrickson ER, Payne JA, Young RM, Starr MG, Perry MP, Fahnestock S, Ellis DE, Ebersole RC. 2002. Molecular analysis of *Dehalococcoides* 16S ribosomal DNA from chloroethene-contaminated sites throughout North America and Europe. *Appl Environ Microbiol*. 68:485-495.

105. Holliger C, Hahn D, Harmsen H, Ludwig W, Schumacher W, Tindall B, Vazquesz F, Weiss N, Zehnder AJB. 1998. *Dehalobacter restrictus* gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra- and trichloroethene in an anaerobic respiration. *Arch Microbiol*. 169: 313-321.

106. Holliger C, Schraa G, Stams AJ, Zehnder AJ. 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. *Appl Environ Microbiol*. 59:2991–2997.

107. Holliger C, Wohlfarth G, Diekert G. 1999. Reductive dechlorination in the energy metabolism of anaerobic bacteria. *FEMS Microbiology Reviews*. 22: 383-398.

108. Holmberg A. 1982. On the practical identifiability of microbial growth models incorporating Michaelis-Menten type non-linearities. *Math Biosci*. 62: 23-43.

109. Holmes VF, He J, Lee PKH, Alvarez-Cohen L. 2006. Discrimination of functionally distinct *Dehalococcoides* strains in a trichloroethene enrichment by quantification of their functional reductases. *Appl Environ Microbiol.* 72:5877-5883.

110. Hopkins BT, McInerney MJ, Warikoo V. 1995. Evidence for an anaerobic syntrophic benzoate degradation threshold and isolation of the syntrophic

benzoate degrader. *Appl Environ Microbiol*. 61**:**526-530.

111. Hu Z, Ferraina RA, Ericson JF, Smets BF. 2005. Effect of long-term exposure, biogenic substrate presence, and electron acceptor conditions on the biodegradation of multiple substituted benzoates and phenolates. *Water Res*. 39:3501-3510.

112. Interstate Technology Regulatory Council. 2005. Overview of in situ bioremediation of chlorinated ethene DNAPL source zones. http://www.itrcweb.org/documents/BioDNAPL-1.pdf

113. Janssen DB, Oppentocht JE, Poelarends GJ. 2001. Microbial dehalogenation. *Curr Opin Biotechnol*. 12:254–258.

114. Jetten MSM, Stams AJM, Zehnder AJB. 1990. Acetate threshold values and acetate activating enzymes in methanogenic bacteria. *FEMS Microbiol Ecol.* 73:339–344.

115. Jones LJ, Haugland RP, Singer VL. 2003. Development and characterization of the NanoOrange protein quantitation assay: a fluorescence-based assay of proteins in solution. *Biotechniques*. 34:850-4, 856, 858 passim.

116. Kan E, Deshusses MA. 2006. Cometabolic degradation of TCE vapors in a foamed emulsion bioreactor. *Environ Sci Technol*. 40:1022-1028.

117. Kesavan P, Law VJ. 2005. Practical identifiability of parameters in Monod kinetics and statistical analysis of residuals. *Biochemical Engineering Journal*. 24: 95-104.

118. Kim DJ, Choi JW, Choi NC, Mahendran B, Lee CE. 2005. Modeling of growth kinetics for *Pseudomonas spp*. during benzene degradation. *Appl Microbiol Biotechnol*. 69: 456-462.

119. Knauss KG, Dibley MJ, Leif RN, Mew DA, Aines RD. 2000. The aqueous solubility of trichloroethene (TCE) and tetrachloroethene (PCE) as a function of temperature. *Applied Geochemistry*. 15: 501-512.

120. Kovárová-Kovar K, Egli T. 1998. Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. *Microbiol Mol Biol Rev.* 62: 646-666.

121. Krumholz LR. 1997. *Desulfuromonas chloroethenica* sp. nov. uses tetrachloroethylene and trichloroethylene as electron acceptors. *Int. J. Syst. Bacteriol*. 47:1262–1263.

122. Krumholz LR, Sharp R, Fishbain S. 1996. A freshwater anaerobe coupling acetate oxidation to tetrachloroethene dehalogenation. *Appl Environ Microbiol* .62:4108–4113.

123. Kuo MCT, Liang KF, Han YL, Fan KC. 2004. Pilot studies for in-situ aerobic cometabolism of trichloroethylene using toluene-vapor as the primary substrate. *Water Res.* 38: 4125–4134.

124. Lanthier M, Villemur R, Lepine F, Bisaillon JG, Beaudet R. 2001. Geographic

distribution of *Desulfitobacterium frappieri* PCP-1 and *Desulfitobacterium* spp. in soils from the province of Quebec, Canada. *FEMS Microbiology Ecology*. 36: 185-191.

125. Lee PK, Johnson DR, Holmesm VF, He J, Alvarez-Cohen L. 2006. Reductive dehalogenase gene expression as a biomarker for physiological activity of *Dehalococcoides* spp. *Appl Environ Microbiol*. 72: 6161-6168.

126. Lendvay JM, Löffler FE, Dollhopf M, Aiello MR, Daniels G, Fathepure BZ, Gebhard M, Heine R, Helton R, Shi J, Krajmalnik-Brown R, Major CL Jr., Barcelona M J, Petrovskis E, Hickey R, Tiedje JM, Adriaens P. 2003. Bioreactive barriers: a comparison of bioaugmentation and biostimulation for chlorinated solvent remediation. *Environ Sci Technol*. 37: 1422-1431.

127. Liu C, Zachara JM. 2001. Uncertainties of Monod kinetics parameters nonlinearly estimated from batch experiments. *Environ Sci Technol*. 35: 133-141.

128. Liu Y, Liu Q, Tay J. 2005. Initial conditions-dependent growth kinetics in microbial batch culture. *Process Biochemistry*. 40: 155-160.

129. Löffler FE, Li J, Urbance JW, Tiedje JM. 1998. Characterization of strain BB1, a tetrachloroethene (PCE)-dechlorinating anaerobe. Presented at the 98th General Meeting of the American Society for Microbiology, Atlanta, GA, May 17-21, Abstract Q-177.

130. Löffler FE, Sun Q, Li J, Tiedje JM. 2000. 16S rRNA gene-based detection of tetrachloroethene (PCE)-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. *Appl Environ Microbiol*. 66:1369-1374.

131. Löffler FE, Tiedje JM, Sanford RA. 1999. Fraction of electrons consumed in electron acceptor reduction and hydrogen thresholds as indicators of halorespiratory physiology. *Appl Environ Microbiol*. 65: 4049-4056.

132. Lovley DR. 1985. Minimum threshold for hydrogen metabolism in methanogenic bacteria. *Appl Environ Microbiol*. 49:1530-1531

133. Lovley DR, Chapelle FH, Woodward JC. 1994. Use of dissolved H_2 concentrations to determine distribution of microbially catalyzed redox reactions in anaerobic groundwater. *Environ Sci Technol*. 28:1205-1210.

134. Lovley DR, Goodwin S. 1988. Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. *Geochim Cosmochim Acta*. 52:2993-3003.

135. Lu XX, Wilson JT, Kampbell DH. 2006. Relationship between *Dehalococcoides* DNA and dechlorination rates at field scale. *Water Res.* 40: 3131-3140.

136. Luijten ML, de Weert J, Smidt H, Boschker HT, De Vos WM, Schraa G, Stams AJ. 2003. Description of *Sulfurospirillum halorespirans* sp.nov., an anaerobic, tetrachloroethene-respiring bacterium, and transfer of *Dehalospirillum multivorans* to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans* comb.

nov. *Int J Syst Evol Microbiol.* 53: 787-793.

137. Luijten MLGC, Roelofsen W, Langenhoff AAM, Schraa G, Stams AJM. 2004. Hydrogen threshold concentrations in pure cultures of halorespiring bacteria and at a site polluted with chlorinated ethenes. *Environmental Microbiology*. 6: 646-650.

138. Luong JHT. 1987. Generalization of Monod kinetics for analysis of growth data with substrate inhibition. *Biotechnol Bioeng*. 29:242 – 248.

139. Macbeth TW, Cummings DE, Spring S, Petzke LM, Sorenson KS. 2004. Molecular characterization of a dechlorinating community resulting from in situ biostimulation in a trichloroethene-contaminated deep, fractured basalt aquifer and comparison to a derivative laboratory culture. *Appl Environ Microbiol*. 70:7329-7341.

140. Magbanua BS Jr, Smets BF, Bowyer RL, Rodieck AG, Sanders RW II, Sowers WW, Stolze SB, Grady CPL Jr. 2003. Relative efficacy of intrinsic and extant parameters for modeling biodegradation of synthetic organic compounds in activated sludge: steady-state systems. *Water Environ Res*. 75:126-137.

141. Magnuson JK, Romine MF, Burris DR, Kingsley MT. 2000. Trichloroethene reductive dehalogenase from *Dehalococcoides ethenogenes*: sequence of tceA and substrate range characterization. *Appl Environ Microbiol*. 66:5141-5147.

142. Magnuson JK, Stern RV, Gossett JM, Zinder SH, Burris DR. 1998. Reductive dechlorination of tetrachloroethene to ethene by a two-component enzyme pathway. *Appl Environ Microbiol.* 64: 1270-1275.

143. Mahadevan V, Stenroos L. 1967. Quantitative analysis of volatile fatty acids in aqueous solution by gas chromatography. *Analytical Chemistry*. 39:1652-1654.

144. Major DM, McMaster ML, Cox EE, Edwards EA, Dworatzek SM, Hendrickson ER, Starr MG, Payne JA, Buonamici LW. 2002. Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. *Environ Sci Technol.* 36:5106 – 5116.

145. Maymo-Gatell X, Anguish T, Zinder SH. 1999. Reductive dechlorination of chlorinated ethenes and 1,2-dichloroethane by '*Dehalococcoides ethenogenes*' 195. *Appl Environ Microbiol*. 65:3108–3113.

146. Maymó-Gatell X, Chien YT, Gossett JM, Zinder SH. 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science*. 276:1568-1571.

147. Maymó-Gatell X, Nijenhuis I, Zinder SH. 2001. Reductive dechlorination of cis-1,2-dichloroethene and vinyl chloride by "*Dehalococcoides ethenogenes*". *Environ. Sci. Technol*. 35: 516-521

148. Mazur CS, Jones WJ. 2001. Hydrogen concentrations in sulfate-reducing estuarine sediments during PCE dehalogenation. *Environ Sci Technol*. 35: 4783-4788.

149. McCarty PL. 1971. "Energetics and bacterial growth" In Organic Compounds in

Aquatic Environments (Editor Faust SD, Hunter JV), Marcel Dekker, Inc. New York, pp: 495-531.

150. McCarty PL. 1997. Breathing with Chlorinated Solvents. *Science*. 276, 1521-1522.

151. McCarty PL, Goltz MN, Hopkins GD, Dolan ME, Allan JP, Kawakami BT, Carrothers TJ. 1998. Full-scale evaluation of in situ cometabolic degradation of trichloroethylene in groundwater through toluene injection. *Environ Sci Technol*. 32: 88–100.

152. Middeldorp PJM, Luijten MLGC, Van de Pas BA, Van Eekert MHA, Kengen SWM, Schraa G, Stams AJM. 1999. Anaerobic microbial reductive dehalogenation of chlorinated ethenes. *Bioremediation J*. 3: 151–169.

153. Miller E, Wohlfarth G, Diekert G. 1997. Comparative studies on tetrachloroethene reductive dechlorination mediated by *Desulfitobacterium* sp. strain PCE-S. . *Arch Microbiol*. 168: 513-519.

154. Miller E, Wohlfarth G, Diekert G. 1998. Purification and characterization of the tetrachloroethene reductive dehalogenase of strain PCE-S. *Arch Microbiol*. 169:497-502.

155. Miller GS, Milliken CE, Sowers KR, May HD. 2005. Reductive dechlorination of tetrachloroethene to trans-dichloroetheneand *cis*-dichloroethene by PCB-dechlorinating bacterium DF-1. *Environ Sci Technol*. 39: 2631-2635.

156. Min H, Zinder SH. 1989. Kinetics of acetate utilization by two thermophilic acetotrophic methanogens: *Methanosarcina* sp. strain CALS-1 and *Methanothrix* sp. Strain CALS-1. *Appl Environ Microbiol*. 55:488–491.

157. Mohn WW, Tiedje JM. 1992. Microbial reductive dehalogenation. *Microbial Reviews*. 56: 482-507.

158. Molchanov S, Gendel Y, Ioslvich I, Lahav O. 2007. Improved experimental and computational methodology for determining the kinetic equation and the extant kinetic constants of Fe(II) oxidation by *Acidithiobacillus ferrooxidans*. *Appl Environ Microbiol*. 73: 1742-1752.

159. Monod J. 1949. The growth of bacterial cultures. *Ann Rev Microbiol*. 3: 371-394.

160. Moran MJ, Zogorski JS, Squillace PJ. 2007. Chlorinated solvents in groundwater of the United States. *Environ Sci Technol*. 41: 74 -81.

161. Müller JA, Rosner BM, von Abendroth G, Meshulam-Simon G, McCarty PL, Spormann AM. 2004. Molecular identification of the catabolic vinyl chloride reductase from *Dehalococcoides* sp. strain VS and its environmental distribution. *Appl Environ Microbiol*. 70:4880-4888.

162. National Research Council. 2000. Natural attenuation for groundwater remediation. Washington, D.C. National Academy Press.

163. NCBI. 2005. Genome database for *Desulfuromonas acetoxidans* DSM 684.

164. Neidhardt FC, Ingraham JI, Schaechter M. 1990. Physiology of the bacterial cell: A molecular Approach. Sinauer, Sunderland, Mass.

165. Nemati M, Webb C. 1997. A kinetic model for biological oxidation of ferrous iron by *Thiobacillus ferrooxidans*. *Biotechnol Bioeng*. 53:478–486.

166. Neuman A, Scholtz-Muramatsu H, Diekert G. 1994. Tetrachloroethene metabolism of *Dehalospirillum multivorans*. *Arch Microbiol*. 162, 295-301.

167. Neumann A, Wohlfarth G, Diekert G. 1995. Properties of the tetrachloroethene and trichloroethene dehalogenase of *Dehalospirillum multivorans*. *Arch Microbiol*. 163: 276-281.

168. Neumann A, Wohlfarth G, Diekert G. 1996. Purification and characterization of tetrachloroethene reductive dehalogenase from *Dehalospirillum multivorans*. *J Biol Chem.* 271:16515-16519.

169. Newman LM, Wackett LP. 1997. Trichloroethylene oxidation by purified toluene 2-monooxygenase: products, kinetics, and turnover-dependent inactivation. *J Bacteriol.* 179: 90-96.

170. Nijenhuis I, Nikolausz M, Köth A, Felföldi T, Weiss H, Drangmeister J, Großmann J, Kästner M, Richnow HH. 2007. Assessment of the natural attenuation of chlorinated ethenes in an anaerobic contaminated aquifer in the Bitterfeld/Wolfen area using stable isotope techniques, microcosm studies and molecular biomarkers. *Chemosphere*. 67: 300-311.

171. Nonaka H, Keresztes G, Shinoda Y, Ikenaga Y, Abe M, Naito K, Inatomi K, Furukawa K, Inui M, Yukawa H. 2006. Complete genome sequence of the dehalorespiring bacterium *Desulfitobacterium hafniense* Y51 and comparison with *Dehalococcoides ethenogenes* 195. *J Bacteriol*. 188: 2262–2274.

172. Noutsopoulos C, Mamais D, Andreadakis A. 2006. Effect of solids retention time on *Microthrix parvicella* growth. *Water SA*. 32: 315-321.

173. Otson R, Williams DT, Bothwell PD. 1982. Volatile organic compounds in water at thirty Canadian potable water treatment facilities. *J Assoc Off Anal Chem.* 65: 1370-1374.

174. Pambrun V, Paul E, Spérandio M. 2006. Modelling the partial nitrification in a sequencing batch reactor for biomass adapted to high ammonia concentrations, *Biotechnol. Bioeng*. 95: 120–131.

175. Pavlostathis SG, Giraldo-Gomez E. 1991. Kinetics of anaerobic treatment: a critical review. *Crit Rev Environ Control*. 21:411-490.

176. Peters V, Janssen PH, Conrad R. 1998. Efficiency of hydrogen utilization during unitrophic and mixotrophic growth of *Acetobacterium woodii* on hydrogen and lactate in the chemostat. *FEMS Microbiol Ecol*. 26: 317–324.

177. Pirt SJ. Principles of Microbe and Cell Cultivation; Halsted Press: New York, 1976.

178. Ramos JL, Duque E, Gallegos MT, Godoy P, Ramos-Gonzalez MI, Rojas A,

Teran W, Segura A. 2002. Mechanisms of solvent tolerance in gram-negative bacteria. *Annu Rev Microbio* .56: 743–768.

179. Reeves GT, Narang A, Pilyugin SS. 2004. Growth of mixed cultures on mixtures of substitutable substrates: the operating diagram for a structured model. *J Theor Biol*. 226, 143-157.

180. Richardson RE, Bhupathiraju VK, Song DL, Goulet TA, Alvarez-Cohen L. 2002. Phylogenetic characterization of microbial communities that reductively dechlorinate TCE based upon a combination of molecular techniques. *Environ Sci Technol.* 36: 2652-2662.

181. Ritalahti KM, Löffler FE. 2004. Populations implicated in the anaerobic reductive dechlorination of 1,2-dichloropropane in highly enriched bacterial communities. *Appl Environ Microbiol*. 70:4088-4095.

182. Rittmann BE. 1982. Comparative performance of biofilm reactor types. *Biotechnology and Bioengineering*. 24: 1341-1370.

183. Rittmann BE. 1987. Aerobic biological treatment. *Environ Sci Technol*. 21: 128-136.

184. Robertson BK, Alexander M. 1996. Mitigating toxicity to permit bioremediation of constituents of nonaqueous-phase liquids. *Environ Sci Technol*. 30:2066–2070.

185. Robinson JA. 1985. Determining microbial kinetic parameters using nonlinear regression analysis. *Adv Microbiol Ecol*. 8: 61-114.

186. Robinson JA, Tiedje JM. 1982. Kinetics of hydrogen consumption by rumen fluid, anaerobic digestor sludge, and sediment. *Appl Environ Microbiol*. 44: 1374-1384.

187. Robinson JA, Tiedje JM. 1983. Nonlinear estimation of Monod hrowth kinetic parameters from a single substrate depletion curve. *Appl Environ Microbiol*. 45: 1453-1458.

188. Rogers JB, DuTeau NM, Reardon KF. 2000. Use of 16S-rRNA to investigate microbial population dynamics during biodegradation of toluene and phenol by a binary culture. *Biotechnol Bioeng*. 70: 436-445.

189. Rossetti S, Blackall LL, Majone M, Hugenholtz P, Plumb JJ, Tandoi V. 2003. Kinetic and phylogenetic characterization of an anaerobic dechlorinating microbial community. *Microbiology*. 149: 459-469.

190. Rutgers M, Teixeira De Mattos MJ, Postma PW, Van Dam K. 1987. Establishment of steady state in glucose-limited chemostat cultures of *Klebsiella pneumoniae*. *J gen Microbiol*. 133, 445-451.

191. Sahinkaya E, Dilek FB. 2007. Biodegradation kinetics of 2,4-dichlorophenol by acclimated mixed cultures. *J Biotechnol*. 127:716-726.

192. Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

193. Sanford RA, Cole JR, Löffler FE, Tiedje JM. 1996. Characterization of *Desulfitobacterium chlororespirans* sp. nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate. *Appl Environ Microbiol*. 62: 3800-3808.

194. Scholz-Muramatsu H, Neumann A, Meßmer M, Moore E, Diekert G. 1995. Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. *Arch Microbiol*. 163: 48-56.

195. Schumacher W, Holliger C, Zehnder AJ, Hagen WR. 1997. Redox chemistry of cobalamin and iron-sulfur cofactors in the tetrachloroethene reductase of *Dehalobacter restrictus*. *FEBS Lett*. 409:421-425.

196. Seagren EA, Kim H, Smets BF. 2003. Identifiability and retrievability of unique parameters describing intrinsic Andrews kinetics. *Appl Microbiol Biotechnol*. 61: 314-322.

197. Shen H, Sewell GW. 2005. Reductive biotransformation of tetrachloroethene to ethene during anaerobic degradation of toluene: experimental evidence and kinetics. *Environ Sci Technol*. 39: 9286-9294.

198. Shim H, Ryoo D, Barbieri P, Wood TK. 2001. Aerobic degradation of mixtures of tetrachloroethylene, trichloroethylene, dichloroethylene, and vinyl chloride by toluene-o-xylene monooxygenase of *Psuedomonas stutzeri* OX1. *Appl Microbiol Biotechnol*. 56:265-269.

199. Sikkema J, de Bont JAM, Poolman B. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol Rev*. 59:201–222.

200. Simkins S, Alexander M. 1984. Models for mineralization kinetics with variables of substrate concentration and population density. *Appl Environ Microbiol*. 47: 1299-1306.

201. Singh H, Löffler FE, Fathepure BZ. 2004. Aerobic biodegradation of vinyl chloride by a highly enriched mixed culture. *Biodegradation*. 15:197–204.

202. Sleep BE, Seepersad DJ, Kaiguo MO, Heidorn CM, Hrapovic L, Morrill PL, McMaster ML, Hood ED, Lebron C, Lollar BS, Major DW, Edwards EA. 2006. Biological enhancement of tetrachloroethene dissolution and associated microbial community changes. *Environ Sci Technol*. 40: 3623-3633.

203. Smatlak CR, Gossett JM, Zinder SH. 1996. Comparative kinetics of hydrogen utilization for reductive dechlorination of tetrachloroethene and methanogenesis in an anaerobic enrichment culture. *Environ Sci Technol*. 30:2850–2858.

204. Smidt HL, Akkermans AD, van der Oost J, de Vos WM. 2000. Halorespiring bacteria-molecular characterization and detection. *Enzyme Microb Technol.* 27: 812-820.

205. Smidt HL, de Vos WM. 2004. Anaerobic microbial dehalogenation. *Annu Rev Microbiol*. 58: 43-73.

206. Smith LH, McCarty PL, Kitanidis PK. 1998. Spreadsheet method for evaluation

of biochemical reaction rate coefficients and their uncertainties by weighted nonlinear least-squares analysis of the integrated Monod equation. *Appl Environ Microbiol*. 64: 2044-2050.

207. Smits HM, Devenoges C, Szynalski K, Maillard J, Holliger C. 2004. Development of a real-time PCR method for quantification of the three genera *Dehalobacter, Dehalococcoides*, and *Desulfitobacterium* in microbial communities. *J Microbiol Methods.* 57: 369–378.

208. Song X, Seagren EA. 2008. In Situ bioremediation in heterogeneous porous media: dispersion-limited scenario. *Environ Sci Technol*. 42: 6131 – 6140.

209. Stasinakis AS, Mamais D, Paraskevas PA, Lekkas TD. 2003. Evaluation of different methods for the determination of maximum heterotrophic growth rates. *Water Environment Research*. 75: 549-552.

210. Stuart SL, Woods SL, Lemmon TL, Ingle JD Jr. 1999. The effect of redox potential changes on reductive dechlorination of pentachlorophenol and the degradation of acetate by a mixed methanogenic culture. *Biotechnol Bioeng*. 63: 69-78.

211. Sung Y, Fletcher KE, Ritalahti KM, Apkarian RP, Ramos-Hernández N, Sanford RA, Mesbah NM, Löffler FE. 2006. *Geobacter lovleyi* sp. nov. strain SZ, a novel metal-reducing and tetrachloroethene-dechlorinating bacterium. *Appl Environ Microbiol*. 72: 2775-2782.

212. Sung Y, Ritalahti KM, Apkarian RP, Löffler FE. 2006. Quantitative PCR confirms purity of strain GT, a novel trichloroethene-to-ethene-respiring *Dehalococcoides* isolate. *Appl Environ Microbiol*. 72: 1980-1987.

213. Sung Y, Ritalahti KM, Sanford RA, Urbance JW, Flynn SJ, Tiedje JM, Löffler FE. 2003. Characterization of two tetrachloroethene-reducing, acetate-oxidizing anaerobic bacteria and their description as *Desulfuromonas michiganensis* sp. nov. *Appl Environ Microbiol*. 69: 2964-2974.

214. Suyama A, Iwakiri R, Kai K, Tokunaga T, Sera N, Furukawa K. 2001. Isolation and characterization of *Desulfitobacterium* sp. strain Y51 capable of efficient dehalogenation of tetrachloroethene and polychloroethanes. *Biosci Biotechnol Biochem*. 65: 1474–1481.

215. Suyama A, Yamashita M, Yoshino S, Furukawa K. 2002. Molecular characterization of the PceA reductive dehalogenase of *Desulfitobacterium sp*. strain Y51. *J Bacteriol*. 184: 3419-3425.

216. Tandoi V, DiStefano TD, Bowser PA, Gossett JM, Zinder SH. 1994. Reductive dehalogenation of chlorinated ethenes and halogenated ethanes by a high-rate anaerobic enrichment culture. *Environ Sci Technol*. 28: 973-979.

217. Templeton LL, Grady CPL. 1988. Effect of culture history on the determination of biodegradation kinetics by batch and fed-batch techniques. *J WPCF*. 60: 651–658.

218. Terzenbach DP, Blaut M. 1994. Transformation of tetrachloroethylene to

trichloroethylene by homoacetogenic bacteria. *FEMS Microbiol Lett*. 123: 213-218.

219. The Interstate Technology & Regulatory Council Bioremediation of DNAPLs Team. 2005. Overview of In Situ Bioremediation of Chlorinated Ethene DNAPL Source Zones. Washington, D.C.

220. Tobajas M, Garcia-Calvo E. 2000. Comparison of analysis methods for determination of the kinetic parameters in batch cultures. *World Journal of Microbiology and Biotechnology*. 16: 845-851.

221. Townsend GT, Suflita JM. 1996. Characterization of chloroethylene dehalogenation by cell extracts of *Desulfomonile tiedjei* and its relationship to chlorobenzoate dehalogenation. *Appl Environ Microbiol*. 62: 2850-2853.

222. Tros ME, Schraa G, Zehnder AJB. 1996. Transformation of low concentrations of 3-chlorobenzoate by *Pseudomonas* sp. strain B13: kinetics and residual concentrations. *Appl Environ Microbiol*. 62:437–442.

223. U.S. Environmental Protection Agency. 2005. Common Chemicals Found at Superfund sites; http://epa.gov/superfund/resources/chemicals.htm.

224. U.S. Environmental Protection Agency. Health Effects Assessment for Tetrachloroethylene. EPA/600/8-89-096. Environmental Criteria and Assessment Office, Office of Health and Environmental Assessment, Office of Research and Development, Cincinnati, OH. 1988a.

225. U.S. Environmental Protection Agency. Updated Health Assessment Document for Tetrachloroethylene. EPA/600/8-82/005B. Environmental Criteria and Assessment Office, Office of Health and Environmental Assessment, Office of Research and Development, Cincinnati, OH. 1988b.

226. U.S. Environmental Protection Agency. Integrated Risk Information System (IRIS) on Tetrachloroethylene. National Center for Environmental Assessment, Office of Research and Development, Washington, DC. 1999.

227. Utkin I, Woese C, Wiegel J. 1994. Isolation and characterization of Desulfitobacterium *dehalogenans* gen. nov., sp. nov., an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds. *Int J Syst Bacteriol*. 44: 612-619.

228. van Pee KH, Unversucht S. 2003. Biological dehalogenation and halogenation reactions. *Chemosphere.* 52: 299–312.

229. Vasiliadou IA, Siozios S, Papadas IT, Bourtzis K, Pavlou S, Vayenas DV. 2006. Kinetics of pure cultures of hydrogen-oxidizing denitrifying bacteria and modeling of the interactions among them in mixed cultures. *Biotechnol Bioeng*. 95: 513-525.

230. Verce MF, Ulrich RL, Freedman DL. 2000. Characterization of an isolate that uses vinyl chloride as a growth substrate under aerobic conditions. *Appl Environ Microbiol*. 66:3535–3542.

231. Verce MF, Ulrich RL, Freedman DL. 2001. Transition from cometabolic to

growth-linked biodegradation of vinyl chloride by a *Pseudomonas* sp. isolated on ethane. *Environ Sci Technol*. 35: 4242–4251.

232. Vermuë M, Sikkema J, Verheul A, Bakker R, Tramper J. 1993. Toxicity of homologous series of organic solvents for the gram-positive bacteria *Arthrobacter* and *Nocardia* Sp. and the gram-negative bacteria *Acinetobacter* and Pseudomonas Sp. *Biotechnol Bioeng*. 42: 747-758.

233. Villemur R, Lanthier M, Beaudet R, Lépine F. 2006. The *Desulfitobacterium* genus. *FEMS Microbiol Rev*. 30: 706–733.

234. Vogel TM, Criddle CS, McCarty PL. 1987. Transformation of halogenated aliphatic compounds. *Environ Sci Technol*. 21: 722-736.

235. Vogel TM, McCarty PL. 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl-chloride, and carbon-dioxide under methanogenic conditions. *Appl Environ Microbiol*. 49:1080–1083.

236. Wang X. 1988. The development of a nonlinear parameter estimation technique for use with biodegradation data. M. Engng Rep., Clemson Univ., S.C.

237. Waul C, Arvin E, Schmidt JE. 2008. Model description and kinetic parameter analysis of MTBE biodegradation in a packed bed reactor. *Water Res*. 42: 3122-3134.

238. Wayman M, Tseng MC. 1976. Inhibition-threshold substrate concentrations. *Biotechnol Bioeng*. 18: 383-387.

239. Westermann P, Ahring BK, Mah RA. 1989. Threshold acetate concentrations for acetate catabolism by aceticlastic methanogenic bacteria. *Appl Environ Microbiol*. 55: 514–515.

240. Wiedemeier TH, Rifai HS, Newell CJ, Wilson JT. 1999. Natural attenuation of fuels and chlorinated solvents in the subsurface; John Wiley & Sons: New York.

241. Wilhelm E, Battino R, Wilcock RJ. 1977. Low pressure solubility of gases in liquid water. *Chem Rev*. 77:219–262.

242. Wittich RM. 1998. Degradation of dioxin-like compounds by microorganisms. *Appl Microbiol Biotechnol*. 49: 489-499.

243. Yang Y, McCarty PL. 1998. Competition for hydrogen within a chlorinated solvent dehalogenating anaerobic mixed culture. *Environ Sci Technol*. 32: 3591-3597.

244. Yang Y, McCarty PL. 2000. Biologically enhanced dissolution of tetrachloroethene DNAPL. *Environ Sci Technol*. 34: 2979–2984.

245. Yang Y, McCarty PL. 2002. Comparison between donor substrates for biologically enhanced tetrachloroethene DNAPL dissolution. *Environ Sci Technol*. 36: 3400–3404.

246. Yang Y, Pesaro M, Sigler W, Zeyer J. 2005. Identification of microorganisms involved in reductive dehalogenation of chlorinated ethenes in an anaerobic microbial community. *Water Res*. 39: 3954–3966.

247. Yang Y, Zeyer J. 2003. Specific detection of *Dehalococcoides* species by fluorescence in situ hybridization with 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol*. 69: 2879-2883.

248. Yin H, Wood TK, Smets BF. 2005. Reductive transformation of TNT by *Escherichia coli* resting cells: kinetic analysis. *Appl Microbiol Biotechnol*. 69:326–334.

249. Yu S, Semprini L. 2004. Kinetics and modeling of reductive dechlorination at high PCE and TCE concentrations. *Biotechnol Bioeng*. 88: 451-464.

250. Yu, S, Dolan, ME, Semprini L. 2005. Kinetics and inhibition of reductive dechlorination of chlorinated ethylenes by two different mixed cultures. *Environ Sci Technol*. 39:195-205

251. Zawtocki C, Lieberman MT, Borden RC, Birk GM. 2004. Treatment of perchlorate and 1,1,1-trichloroethane in groundwater using edible oil substrate (EOS®), Proceedings of Remediation of Chlorinated and Recalcitrant Compounds-4th Internat. Conf., Monterey, CA.

252. Zenker MJ Borden RC, Barlaz MA, Lieberman MT, Lee MD. 2000. Insoluble substrates for reductive dehalogenation in permeable reactive barriers. In: G.B. Wickranayake, A.R. Gavaskar, B.C. Alleman and V.S. Magar, Editors, Bioremediation and Phytoremediation of Chlorinated and Recalcitrant Compounds, Battelle Press, Columbus, OH (2000), pp. 77–84.

253. Zhang JJ, Joslyn AP, Chiu PC. 2006. 1,1-dichloroethene as a predominant intermediate of microbial trichloroethene reduction. *Environ Sci Technol*. 40: 1830-1836.

254. Zheng D, Carr CS, Hughes JB. 2001. Influence of hydraulic retention time on extent of PCE dechlorination and preliminary characterization of the enrichment culture. *Biorem J*. 5: 159-168.

255. Zinder SH, Gossett JM. 1995. Reductive dechlorination of tetrachloroethene by a high rate anaerobic microbial consortium. *Environ Health Perspect*. 103: 5-7.