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Title: Inhibition, Kinetic and Modeling Studies of Acetylene and 1-chloro-1-fluoroethene on Reductive Dechlorination of TCE and Vinyl Chloride

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Lewis Semprini

Laboratory and modeling studies were performed with a mixed-anaerobic-culture obtained from the Evanite site in Corvallis, Oregon. The culture completely transforms trichloroethene (TCE) to *cis*-dichloroethene (*c*-DCE), vinyl chloride (VC), and finally to ethene. Acetylene inhibition studies were used to examine the culture's microbial activities. Kinetic studies determined the half-saturated constant (K_s), the maximum utilization rate ($k_{max}X$), and inhibition constants (K_I). The kinetic constants were used to model the results of inhibition studies using competitive and uncompetitive inhibition models.

Acetylene was found to function as a reversible inhibitor and was used to probe the activities of reductive dechlorination. Various acetylene concentrations were used to differentiate microbial processes, including methanogenesis, acetogenesis, and halorespiration. Acetylene concentrations of 48, 192, and 12

μM, respectively, were required to achieve 90% inhibition in the rates of methanogenesis, TCE and VC transformation. H_2-dependent acetate production was not inhibited by acetylene.

K_s values for TCE and VC were 12 μM and 63 μM, respectively. Model fitting of acetylene inhibition constants (K_{IC}) for TCE and VC transformations yielded the same value (0.4 μM) for a competitive inhibition model. However, for uncompetitive inhibition the estimated K_{IU} for TCE to *c*-DCE, TCE to 1,1-DCE and VC to ethene were 13.3, 14.1 and 2.2 μM, respectively. Competitive and uncompetitive inhibition models simulated experimental data equally well for results obtained at high TCE and VC concentrations. The models were further verified to fit transient data of acetylene inhibition at lower TCE and VC concentrations, and competitive inhibition resulted in a better fit to the experimental data.

1-chloro-1-fluoroethene (1,1-CFE) was found to track the rate of VC transformation well, since VC and 1,1-CFE had similar maximum transformation rates and K_s values. A competitive inhibition model with the measured K_s values, 63 and 87 μM, was used to predict the rates of VC and 1,1-CFE transformation, respectively. The similar rates and results of acetylene and compound inhibition studies indicated VC and 1,1-CFE were transformed by the same enzyme. 1,1-CFE transformation by three different cultures, clearly demonstrate that 1,1-CFE was an excellent surrogate to track rates of VC transformation.

Inhibition, Kinetic and Modeling Studies of Acetylene and 1-chloro-1-fluoroethene on Reductive Dechlorination of TCE and Vinyl Chloride

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CONTRIBUTION OF AUTHORS

Dr. Lewis Semprini, Dr. Michael Hyman, and Dr. Young Kim assisted in the design and editing of Chapters 3, 4, and 5.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF TABLES

Inhibition, Kinetic and Modeling Studies of Acetylene and 1-chloro-1-fluoroethene on Reductive Dechlorination of TCE and Vinyl Chloride

CHAPTER 1

INTRODUCTION

Chlorinated aliphatic hydrocarbons (CAHs), such as tetrachloroethene (PCE) and trichloroethene (TCE), are major groundwater contaminants (1). Anaerobic biotransformation of PCE and TCE sequentially generates *cis*-dichloroethene (*c*-DCE), vinyl chloride (VC), and finally ethene as a harmless end-product. Incomplete anaerobic biotransformation of PCE and TCE to *c*-DCE or VC through natural attenuation is frequently observed in CAH-contaminated groundwater (2). Among these products, VC is the most undesirable as it is a known human carcinogen and has the lowest allowable drinking water standard at 2 μg/L and is more toxic than its parent compounds (3).

Due to the generation of VC, anaerobic transformation was not considered a practical solution to degrade TCE until non-chlorinated products such as ethene and ethane were observed (4, 5, 6). These studies suggested that in situ bioremediation could be used as an alternative clean-up method (7). There have also been many reports of intrinsic in situ transformation of CAHs (8, 9). Measuring the reductive dechlorination of CAHs to harmless products has become a much studied topic in the last decade.

Much of the research has focused on the transformation of VC to ethene because it is the last and generally the most difficult step in the reductive dechlorination of CAHs. Previous studies found that high K_s values for *c*-DCE and VC may be a cause of the incomplete transformation of PCE and TCE to these two compounds (10). Alternative findings indicated that highly chlorinated CAHs, such as PCE, have inhibitory effects on lower chlorinated VC transformation (11). Also, Hardness, et al. (12) suggested that a missing critical bacterial population was often the cause of incomplete transformation, suggesting that bioaugmentation was necessary to complete reductive dechlorination to ethene. One of the most popular candidates for bioaugmentation is *Dehalococcoides ethenogenes strain 195*, which has the ability to transform PCE to ethene (13). However, another study found that the final step of VC to ethene is cometabolic in nature (14). Recent efforts have focused on VC halorespiration using mixed cultures to the isolation of a pure culture capable of using c-DCE and VC as electron acceptors (15, 16). This culture is unable to use PCE and TCE as electron acceptors.

The main focus of this study was on the use of inhibitors and inhibition modeling to analyze anaerobic reductive dechlorination of CAHs. Kinetic studies with a mixed anaerobic culture used acetylene as an inhibitor and 1-chloro-1-fluoroethene (1,1-CFE) as a reactive surrogate tracer of reductive dehalogenation of TCE and VC. The mixed culture was obtained from the Evanite site in Corvallis, OR and exhibited methanogenic, acetogenic, and reductive dehalogenation characteristics. The H_2-fed culture transformed TCE to ethene with *cis*-dichloroethene (*c*-DCE) and

VC as intermediates. Kinetic studies of concentration effects of acetylene and 1,1-CFE on microbial activities of the Evanite enrichment focused on the inhibition of TCE and VC transformation and transformation rate comparisons of 1,1-CFE and VC. Kinetic modeling of acetylene inhibition involved competitive and uncompetitive modeling of the inhibitors on TCE and VC transformation. Initial kinetic studies determined kinetic parameters, K_s and $k_{max}X$. These values were then used through modeling development to fit the transient data of TCE and VC transformation. These results of these studies are included in three manuscripts presented in Chapter 3, 4, and 5.

In the first manuscript (Chapter 3), acetylene is used as a reversible inhibitor of reductive dehalogenation of TCE and VC. This manuscript was published in Environmental Science and Technology (17). Batch kinetic studies showed that acetylene inhibited the reduction of both TCE and VC, and in both cases the levels of inhibition were strongly dependent on acetylene concentrations. To our knowledge, these were the first observation of using acetylene as an inhibitor to analyze reductive dechlorination. Different acetylene concentrations were also used to inhibit methanogenes and acetogenes. Details of the kinetic and modeling studies of acetylene inhibition, and the determination of the inhibition constant (K_I) are discussed in Chapter 4.

In the second manuscript (Chapter 4), inhibition type, kinetic parameters and inhibition constants (K_I) of acetylene inhibition on TCE and VC transformation were investigated. The main goal was to use kinetic and inhibition studies to investigate

basic mechanisms of acetylene inhibition on the reductive dechlorination of TCE and VC. Kinetic studies were used to determine the basic kinetic parameters of the substrates, K_s values for TCE and VC, and K_I for TCE and VC transformation. The K_I for TCE and VC transformation were obtained using competitive and uncompetitive modeling with non-linear regression fitting. The inhibition constants (K_I) for both competitive and uncompetitive inhibition modeling were then further used to model long-term transient data.

In the final manuscript (Chapter 5), the use of 1,1-CFE as a potential reactive tracer for VC transformation was investigated, since VC transformation is the critical step for complete anaerobic reductive dechlorination. 1,1-CFE was found to be the major transformation intermediate by the Evanite culture when trichlorofluoroethene (TCFE) was transformed through *cis*-1,1-chlorofluoro-2-chloroethene (*c*-DCFE). The fact that 1,1-CFE transformation co-existed with VC transformation provoked our interest in using 1,1-CFE as a reactive tracer to track VC transformation. Kinetic and inhibition studies were then used to examine the correlation between 1,1-CFE and VC transformation. The possibility of their being transformed by the same mechanism was also examined by using acetylene inhibition tests. Tests were also performed on VC and 1,1-CFE inhibition on each other, and results were modeled by using a competitive inhibition model. 1,1-CFE transformation was also tested under the influence of TCE and *c*-DCE transformation, to determine whether 1,1-CFE would be associated with the VC transformation step. Three different cultures that had different transforming abilities on CAH were used in this analysis.

OBJECTIVES

The main objectives of this study were:

1. To evaluate acetylene as an inhibitor on the biochemical processes of the Evanite mixed culture including methanogenesis, acetogenesis, and TCE and VC transformation.
2. To determine the kinetic parameters (K_s and $k_{max}X$) for the transformation of TCE, VC, and 1,1-CFE using non-linear regression fitting.
3. To evaluate different types of acetylene inhibition and the inhibition constants (K_I) on TCE and VC transformation via competitive and uncompetitive inhibition modeling using non-linear regression fitting.
4. To evaluate the potential use of 1,1-CFE as a reactive tracer on VC transformation and to model its transformation using competitive and uncompetitive inhibition modeling.
5. To model acetylene and 1,1-CFE inhibition on TCE and VC transformation.

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CHAPTER 2

Literature Review

BACKGROUND

The pathway for anaerobic reductive dechlorination of PCE and TCE to VC was proposed by Vogel and McCarty (1). Transformation of VC to ethene was reported by Freedman and Gossett (2). A complete anaerobic transformation pathway of PCE and TCE to ethene is shown in Figure 2.1 (3). Anaerobic biotransformation of PCE and TCE sequentially generates *cis*-dichloroethene (*c*-DCE), vinyl chloride (VC) with ethene as a harmless end-product. Each step of reductive dechlorination results in the removal of one chlorine atom and its replacement with a hydrogen atom.

However, complete anaerobic transformation of PCE and TCE to ethene is not always observed. Conditions required for complete transformation to proceed are not well understood. In soil groundwater microcosms from the Gilbert-Mosely site (4) and Lawrence Livermore Site 300 (5), TCE transformation stopped at c-DCE. In an enrichment from the Point Mugu Naval site in California, TCE was transformed rapidly to VC and slowly to ethene (6). Natural anaerobic transformation of chlorinated solvents is attractive as a low cost process, but undesirable intermediates, especially VC a known carcinogen, may be created that are more toxic than the parent compound. In order to practice natural attenuation in the field, the capacities and rate of VC transformation must be known.

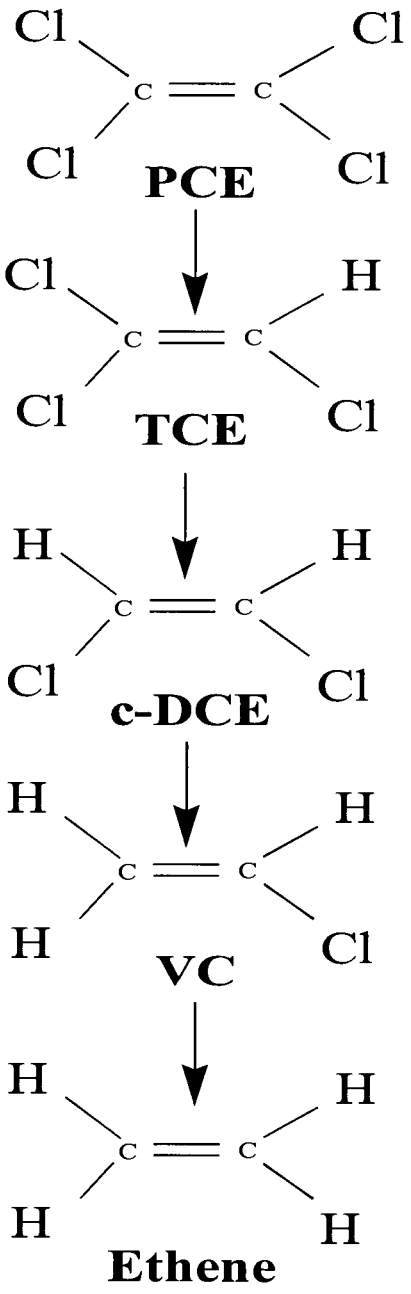

Figure 2.1. Sequential anaerobic transformation of TCE

Knowledge of anaerobic microbial transformation of chlorinated ethenes was generated during the last two decades. Cometabolic dechlorination and halorespiration were major biological mechanisms behind anaerobic transformation of chlorinated aliphatic hydrocarbons (CAHs). The difference between these two mechanisms is that halorespiration can use chlorinated solvents as electron acceptors for growth. Cometabolic dechlorination is fortuitous and may occur along with the biological processes, such as methanogenesis, and yields no energy for growth from the process. In previous studies, purified cultures of methanogens, acetogens, and sulfate-reducing bacteria that contain a reduced transition metal cofactor, were found to cometabolically reduce chlorinated solvents (7, 8, 9, 10).

The first isolated microorganism to use chlorinated solvents via halorespiration was *Dehalobacor restrictus*, a strict anaerobe, which reduced PCE to TCE and c-DCE, while using either hydrogen or formate as an electron donor (11). In another study, a partially purified culture capable of transforming PCE to ethene, was inoculated into a medium with H_2 and PCE (12). These purified cultures provided direct evidence of the existence of anaerobic dechlorination or halorespiration. Some researchers began to use electron transfer efficiency and hydrogen thresholds to identify halorespiratory physiology (13). They found experimentally that for halorespiration, the fraction of electron consumption ranges were between 0.63 and 0.7. The electron transfer efficiency is the fraction of electrons released during oxidation of the electron donor that is directed toward reduction of the terminal electron acceptor, excluding the energy for growth. For cometabolic dechlorination, the fraction is low and is near

0.01. In comparison, halorespiration is much more energy efficient than the cometabolic processes, and more than 50 percent of electron transfer is used for halorespiration, while only few percent of the transfer will go to the cometabolic processes. Thus, cometabolic dechlorination and halorespiration can be identified in terms of their electron transfer efficiency.

Several studies have examined the characteristics of enrichments that are capable of completely transforming PCE and TCE to ethene. Magnuson et al. (14) examined a methanol-PCE enriched culture and found that the TCE reductive dehalogenase could not utilize PCE and only the PCE reductive dehalogenase was capable of reducing PCE. The TCE reductive dehalogenase was capable of rapidly reducing TCE, c-DCE, and 1,1-DCE to VC, and then slowly reducing VC to ethene. Other scientists showed that the H_2-PCE grown enrichment "Dehalococcoides ethenogenes" 195, was capable using TCE, c-DCE, 1,1-DCE and 1,2-dichloroethane as electron acceptors for growth (15). However, the enrichment was not able to utilize VC or trans-DCE. The authors concluded that the reduction of these compounds was a cometabolic process. Maymo-Gatel et al. (15) showed that the enrichment was capable of utilizing multiple electron acceptors, but not all of their products, such as VC. Thus slow VC transformation to ethene was observed in their study.

Much research has focused on the VC transformation to ethene, which is the last and generally most difficult transforming step of the reductive dechlorination of chlorinated ethenes. Previous studies indicated high K_s values of *c*-DCE and VC

caused incomplete transformation of TCE and PCE to *c*-DCE or VC (16). PCE and TCE have also been shown to have inhibitory effects on VC transformation (17). Hardness et al. (18) also suggested that a missing critical bacterial population was one cause of incomplete transformation, suggesting bioaugmentation was necessary to complete reductive dechlorination to ethene (18). One of the most popular candidates for bioaugmentation is *Dehalococcoides ethenogenes strain 195* (19), which has the ability to transform PCE to ethene, however the final step of VC to ethene is cometabolic in nature (15). Recent efforts have focused on the VC halorespiration with mixed cultures (20, 21), which has led to isolation of pure culture (22) that is capable of using *c*-DCE and VC as electron acceptors, but not PCE or TCE (21, 22).

A variety of biochemical tools have been used to probe the complexity of reductive dehalogenation processes. Using molecular approaches, Flynn *et al.* (23) showed a microbial community shift in response to enrichment with different CAHs as chlorinated ethenes were successively transformed. Terminal restriction fragment length polymorphism (T-RFLP) demonstrated *c*-DCE- and VC-grown subcultures from PCE enrichments had distinct 16S rRNA genes, and subcultures grown on less chlorinated solvents lost their ability to degrade PCE. Molecular methods have been recently used to phylogenetically characterize anaerobic dechlorinating microbial communities (22, 24, 25). These molecular methods have been used to evaluate the success of bioaugmentation and biostimulation in field studies (26, 27). Carbon isotopic fractionation has also been used to provide qualitative evidence of the

occurrence and relative extent of biological reductive dechlorination of the chlorinated ethenes (28).

Inhibitors have also been widely applied as mechanistic probes for individual microbial activities in complex microbial mixtures (29). For example, the role of methanogens in a wide variety of mixed anaerobic cultures has previously been investigated using 2-bromoethanesulfonate (BES). This compound is a structural analog of coenzyme M and specifically inhibits methanogenesis. The inhibition results from coenzyme M competing in the terminal methylation reaction in methane formation (30). Although BES is water soluble and effective at low concentrations (< 1mM), it is often necessary to use much higher concentrations (10 mM) to fully inhibit methanogenesis, and to overcome possible degradation, transport, and resistance problems (29).

Acetylene is another compound that has been used to inhibit methanogenesis. Micromolar concentrations of acetylene have been shown to strongly inhibit methane production in lake sediments (31, 32), marine sediments (33), and fish intestines (34). Acetylene has also been shown to selectively inhibit acetate-dependent methanogenesis during acetone degradation by a mixed culture of an acetogenic eubacterium and a *Methanothrix* sp. (35). The effects of acetylene have also been examined with several pure cultures of methanogens (36). Growth of *Methanospirillum hungatei* was completely and reversibly inhibited by 8 μM of dissolved acetylene. Studies with cell free extracts indicated that acetylene did not inhibit several key enzyme activities including H_2 uptake by hydrogenases, NADP

reductase, methyl CoM reductase and ATP hydrolase. However, the presence of acetylene prevented cells of *M. hungatei* from maintaining a transmembrane pH gradient and this resulted in a loss of ATP synthesis activity and energy-dependent Ni^{2+} uptake (36).

Evaluation of transformation rates using a chemical surrogate analogue of TCE, such as trichlorofluoroethene (TCFE) can provide an alternative approach to track the transformation of TCE (18). The advantage of using a surrogate is that a unique analytical identification for in situ biotransformaion is possible, when background concentrations of the CAHs of interest are present. It is often difficult to demonstrate transformation is occurring when other complicated physical processes, such as sorption and desorption of the contaminant and nonaqueous phase liquid (NAPL) dissolution are occurring (37). Vancheeswaran et al. (37) observed when TCFE was transformed to three dichlorofluoroethene isomers (DCFEs), with *cis*-1,1-chlorofluoro-2-chloroethene (c-DCFE) was the main isomer formed. DCFEs can be potentially transformed to three chlorofluoroethene isomers (CFEs). Vancheeswaran et al. observed 1,2-CFE (E) being the main isomer (37), which can be further transformed to fluoroethene (FE). The fluorinated analogues are resistant to defluorination and fluorinated products are easily detected by standard gas chromatography methods to provide evidence of reductive dechlorination. The fluorinated analogues can be used in field studies of intrinsic anaerobic dehalogenation, such as push-pull tests described by Hageman *et al.* (38).

Several transformation models have been developed, and competitive inhibition is the most commonly used for modeling inhibition among CAHs. For the case of aerobic cometabolism of CAHs, Kim et al. (39) found inhibition was competitive in nature and could be well represented by the ratios of the measured K_s values (40). Several anaerobic modeling studies also used competitive inhibition models to describe the interference among multiple CAH transformations. Simulations demonstrated that competitive inhibition models fit well their experimental results (41, 42). In one modeling study, both competitive and non-competitive inhibition models were used to evaluate a sequential transformation of PCE through TCE, *c*-DCE and VC to ethene. The results showed competitive inhibition model fit experimental observations better than non-competitive inhibition (42).

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CHAPTER 3

Acetylene Inhibition of Trichloroethene and Vinyl Chloride Reductive Dechlorination

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ABSTRACT

Kinetic studies reported here have shown acetylene is a potent reversible inhibitor of reductive dehalogenation of trichloroethene (TCE) and vinyl chloride (VC) by a mixed dehalogenating anaerobic culture. The mixed culture was enriched from a contaminated site in Corvallis, OR, and exhibited methanogenic, acetogenic, and reductive dehalogenation activities. The H_2-fed culture transformed TCE to ethene *via* ***cis***-dichloroethene (*c*-DCE) and VC as intermediates. Batch kinetic studies showed acetylene reversibly inhibited reduction of both TCE and VC and the level of inhibition was strongly dependent on acetylene concentration in both cases. Acetylene concentrations of 192 μM and 12 μM, respectively, were required to achieve 90% inhibition in rates of TCE and VC transformation, at an aqueous concentration of 400 μM. Acetylene also inhibited methane production (90% inhibition at 48 μM), but did not inhibit H_2-dependent acetate production. Mass balances conducted during the studies of VC inhibition showed acetogenesis, VC transformation to ethene and methane production were responsible 52%, 47%, and 1% of the H_2 consumption, respectively. The results indicate halorespiration is the dominant process responsible for VC and TCE transformation and dehalorespiring organisms are the target of acetylene inhibition. Acetylene has potential use as a reversible inhibitor to probe the biological activities of reductive dechlorination and methanogenesis. It can be added to inhibit reactions, and then removed to permit reactions to proceed. Thus, it can be a powerful tool for investigating intrinsic and enhanced anaerobic remediation of

chloroethenes at contaminated sites. The results also suggest acetylene produced abiotically by reactions of chlorinated ethenes with zero valent iron (ZVI) could inhibit the biological transformation of VC to ethene.

INTRODUCTION

Chlorinated aliphatic hydrocarbons (CAHs), such as tetrachloroethene (PCE) and trichloroethene (TCE), are major groundwater contaminants (1). Anaerobic biotransformation of PCE and TCE generates *cis*-dichloroethene (*c*-DCE), vinyl chloride (VC) and ethene as products. Incomplete transformation of PCE and TCE to *c*-DCE or VC is frequently observed in CAH-contaminated groundwater and this limits the usefulness of anaerobic biotransformation as a natural attenuation process (2). Among these products, VC is the most undesirable as it is a human carcinogen and has the lowest drinking water standard (2 μg/L) (3).

A variety of biochemical tools have been used to probe the complexity of reductive dehalogenation processes in microbial systems. Using molecular approaches Flynn *et al.* (2000) showed that the structure of a dechlorinating mixed culture shifted as chlorinated ethenes were successively transformed. Terminal restriction fragment length polymorphism (T-FFLP) demonstrated *c*-DCE- and VC-grown subcultures from PCE enrichments had distinct 16S rRNA genes and subcultures grown on less chlorinated solvents lost their ability to degrade PCE. These results suggested that more than two dehalogenating microorganisms were responsible for the complete

transformation of PCE to ethene (4). Examining anaerobic biotransformation of CAHs at the biochemical level, Magnuson *et al.* (1998), partially purified two corrinoid-containing enzymes, PCE-reductive dehalogenase (PCE-RDase) and TCE-reductive dehalogenase (TCE-RDase), from *Dehalococcoides ethenogenes* 195 (5). The TCE-RDase was unable to transform PCE, a result that suggested multiple enzymes were required to complete transformation of PCE to ethene (6).

Inhibitors have also been widely used as mechanistic probes for individual microbial activities in complex microbial mixtures (7). For example, the role of methanogens in a wide variety of mixed anaerobic cultures has previously been investigated using 2-bromoethanesulfonate (BES). This compound is a structural analog of coenzyme M and specifically inhibits methanogenesis because it competes with coenzyme M in the terminal methylation reaction in methane formation (8). Although BES is water soluble and effective at low concentrations (< 1mM), it is often necessary to use much higher concentrations (10 mM) to fully inhibit methanogenesis and to overcome possible degradation, transport, and resistance problems (7).

The role of methanogens in PCE degradation has been investigated using BES. In the absence of BES an anaerobic sewage sludge system reduced PCE to TCE concurrently with methane production. In the presence of BES both PCE transformation and methane production were inhibited, suggesting that methanogens were responsible for PCE degradation (9). This conclusion was supported by evidence showing PCE reduction by pure cultures of methanogens (10). However, later studies

of the effects of BES on reductive dehalogenation have shown some dechlorinating microorganisms are not methanogenes (11). For example, DiStefano *et al.* (1991), found dechlorinators were not inhibited by BES.

Acetylene is another compound that has been used to inhibit methanogenesis. Micromolar concentrations of acetylene have been shown to strongly inhibit methane production in lake sediments (12, 13), marine sediments (14), and fish intestines (15). Acetylene has also been shown to selectively inhibit acetate-dependent methanogenesis during acetone degradation by a mixed culture of an acetogenic eubacterium and a *Methanothrix* sp. (16). The effects of acetylene have also been examined with several pure cultures of methanogens (17). Growth of *Methanospirillum hungatei* was completely and reversibly inhibited by 8 μM of dissolved acetylene. Studies with cell free extracts indicated that acetylene did not inhibit several key enzyme activities including H_2 uptake by hydrogenases, NADP reductase, methyl CoM reductase and ATP hydrolase. However, the presence of acetylene prevented cells of *M. hungatei* from maintaining a transmembrane pH gradient and this resulted in a loss of ATP synthesis activity and energy-dependent Ni^{2+} uptake (17).

While the precise mechanism of acetylene inhibition of methanogenesis remains unknown, the potency of the effects of acetylene on methanogenesis provoked our interest in evaluating this compound as a potential inhibitor and probe of reductive dechlorination activity in a mixed anaerobic culture. Acetylene is potentially useful as an inhibitor for this purpose because acetylene is highly water-soluble, but unlike BES

this inhibitor can be removed from incubations by sparging with an anaerobic gas. In this study acetylene was evaluated as an inhibitor of TCE and VC transformation by a mixed methanogenic/acetogenic/dehalogenatic culture. The effect of acetylene concentration on the dechlorinating activities was defined both in terms of concentration and the reversibility of the effects.

MATERIALS AND METHODS

Chemicals

PCE (99%) and *c*-DCE (97%) were purchased from Acros (Fisher Scientific, Pittsburgh, PA), and TCE (99.9%), 1,1-DCE (99%) and *trans*-DCE (98%) were purchased from Aldrich (Milwaukee, WI). Gas standards, ethene (1000 ppm ± 5%), VC (996 ppm ± 2%) in nitrogen were purchased from Scott Specialty Gases (Alltech Associates, Inc; Deerfield, IL). Methane (99%), CO_2 (99%) acetylene (99.6%) and H_2 (99 %) were obtained from AIRCO (Vancouver, WA). Yeast extract was purchased from Fisher Scientific (Fair Lawn, NJ).

Analytical Methods

Gas phase PCE, TCE, *c*-DCE, *trans*-DCE, 1,1-DCE, VC, acetylene, ethene, and methane concentrations were measured by injecting 100 μL of a headspace sample into a HP-5890 gas chromatograph (GC). The GC was equipped with a 30-m megabore GSQ-Plot column by (J&W Scientific, Folsom, CA), connected to a flame-

induced detector (FID). H_2 and CO_2 were analyzed by injecting 100 µL of headspace sample into a HP-5890 GC. Chromatographic separation was achieved using argon as a carrier gas, with a Supelco 60/80 Carboxen 100 column (15 ft X 0.125 in.; Bellefonte, PA), followed by quantification using a thermal conductivity detector (TCD).

Culture Growth

The mixed-anaerobic-culture was obtained from a TCE-contaminated groundwater and sediment at the Evanite site in Corvallis, Oregon. The area from which the samples were obtained had shown TCE transformation to VC, with methane present. The culture was first grown in batch serum bottles on benzoate and PCE in RAMM media (18). This mixed-culture is capable of completely transforming PCE through TCE, *c*-DCE and VC to ethene, with a conversion to ethene of nearly 100%. For the current study the cells were grown in a 4-liter batch reactor on benzoate, PCE, on the anaerobic RAMM mineral medium that contained added yeast extract. The media (400 ml) was exchanged weekly, resulting in a hydraulic residence time of about 70 days. The media contained 1.8 g of benzoate and 0.9 g of neat PCE and 0.02 g of yeast extract that contributed near 1 % of the carbon input of the benzoate addition. No additional vitamins were added as nutrients. Cells were harvested during the weekly batch exchange.

In order to achieve rapid VC transformation, the harvested cells were further enriched in a 1000-ml batch serum bottle reactor (Wheaton, Millville, NJ). The

harvested cells were purged with nitrogen to remove the residual CAHs. The purged gas was furnace treated to remove residual oxygen by in line contact with copper filings at 600°C. The cells were incubated in the 1000-ml batch reactor; with 5 ml H_2 (200 μ moles), 20 ml of VC (800 μ moles), and 20 ml of CO_2 added to the reactor headspace. Additional H_2 (5 ml) was added when H_2 levels fell below 0.5 % v/v gas phase. Ethene production was monitored to determine how well VC was transformed. The incubated cells were capable of transforming both TCE and VC. The VC concentration for cell incubation was double the concentration used in the inhibition studies (TCE and VC at 400 μM). This concentration was chosen to be much greater than reported K_S values (TCE, 1.4 μM and VC, 2.6 μM) of dehalogenated cultures (19), and to maintain high concentrations throughout the incubation. Cells were harvested when the batch reactor reached a VC removal rate of 80 μ moles per day.

Inhibition Tests

The tests were performed in 125-ml batch bottles (Wheaton; Millville, NJ) capped with butyl rubber septa (Wheaton; Millville, NJ) to allow temporal sampling. Cells (50 ml) were transferred from the VC-containing reactor to the 125-ml batch reactor in an anaerobic glove box. The reactors were purged with furnace-treated nitrogen for 10 minutes to remove the glove box H_2 and any residual VC or ethene. H_2 (5 ml), CO_2 (20 ml) (furnace-treated) and VC (2 ml) were then added to the reactor headspace. Alternatively, for studies following TCE reduction, TCE saturated liquid solution (2-ml) was added instead of VC. The initial H_2 and CO_2 concentrations in the

headspace of each reactor were about 5 % and 20% (v/v), respectively. The reactors were incubated at 20°C and shaken in an inverted-position at 200 rpm on a rotary shaker table. The H_2 and CO_2 concentrations were maintained at excess levels for this kinetic study. H_2 was re-supplied during the experiment when the headspace H_2 concentrations fell below 0.5 % (v/v), by adding 5 ml of H_2.

The acetylene inhibition experiments consisted of three stages: an initial rate determination stage, an inhibition stage, and a recovery stage. In the first stage the initial transformation rates of TCE or VC were determined in the absence of acetylene inhibition by monitoring their removal and accumulation of their transforming products (*c*-DCE and 1,1-DCE, and ethene). In the second stage the transforming rates were determined in the presence of the acetylene. Prior to addition, the acetylene was scrubbed with 10% (w/v) $Cu_2SO_4 \cdot 5H_2O$ to remove trace amounts of acetone, as described in Hyman *et al.* (20). Finally, in the third stage the rates were re-measured. The initial VC or TCE and H_2 concentrations were the same at the start of each stage, while the second stage had various amounts of acetylene added to the different reactors. Between stages, the reactors were purged with furnace treated nitrogen gas for 15 minutes to remove all the CAHs. This sequence enabled the determination of transformation rates with and without acetylene present and the recovery of the cells upon acetylene exposure.

Transformation rates for each CAH were determined for each stage by measuring product formation. The rate was determined by linear regression of at least 5 data points. The rates of product gain were determined by mass balances assuming

equilibrium between the gas and liquid phase based on Henry's law. Published Henry's law constants of Gossett (1987) were used for these determinations (21). The constant mixing of the reactors at 200 rpm, over the time scale of the experiments (hours) assured phase equilibrium was achieved.

The various experiments examining the effects of acetylene on VC and TCE reduction are summarized in Table 3.1. Variation of initial transforming rates was likely due to differences in concentration of the dechlorinating microorganisms. Test 1 investigated the reversibility of acetylene inhibition on VC transformation, while in Tests 2 and 3 the effect of acetylene concentration on VC transformation were studied. Acetylene inhibition of TCE transformation was studied in Tests 4 and 6. Test 6 was conducted over a broader range of acetylene concentrations and at a higher initial TCE concentration than Test 4. Only Test 4 and Test 5 were conducted with same set of harvested cells. Other studies were performed with cells harvested at different times.

Table 3.1. Concentration used in the inhibition experiments and initial rates of dehalogenation.

Experiment	Reactant (μM) Initial Conc.	Average Initial Rates of Product Production (μ mole /hr)	Acetylene Conc. (μM)
Test 1	VC (380)	Ethene (0.13)	120, 480, 1920
Test 2	VC (450)	Ethene (0.34)	0, 6, 12, 24, 48
Test 3	VC (440)	Ethene (0.23)	0, 0.75, 1.5, 3, 6, 12
Test 4 &	TCE (380)	c-DCE (0.85)	0, 12, 24, 48, 96, 192
Test 5	VC (470)	Ethene (0.16)	0, 2.4, 6, 12, 24, 48
Test 6	TCE (850)	c-DCE, (4.67)	2.4, 12, 48, 120, 180, 240

Test 4 and Test 5 were conducted with same set of harvested cells.

RESULTS

Acetylene Inhibition of VC Transformation

The initial study (Test 1) evaluated acetylene inhibition of VC transformation at three different acetylene concentrations, and investigated the reversibility of the inhibition. The experiments were conducted in duplicate at an aqueous VC concentration of 380 μM. Figure 3.1 shows the time course of ethene accumulation from VC transformation. Over the first 9 days small amounts of ethene accumulated in the reactors. The reactors were purged on day 10 then VC, H_2 and CO_2 were replenished. This procedure was repeated at day 14 and 17 and rapid rates of ethene generation were established after day 20. The increase in ethene production rates was consistent with exponential growth of a dehalogenating culture. On day 27 the reactors were again purged and replenished with H_2, CO_2 and VC. The rate of ethene production was ~83% of maximum rate prior to purging, a result that demonstrates purging did not substantially impact the culture's dehalogenating activity. When rapid dehalogenating activity had been confirmed, acetylene (480 μM) was added on day 31. Acetylene completely inhibited ethene production over a period of 4 days, while ethene production continued in control reactors with no acetylene added (data not shown). On day 36, acetylene was purged from the reactors, and VC, H_2 and CO_2 were again added. Ethene production resumed achieving a maximum rate of ~83% of that achieved prior to the acetylene inhibition test, indicating acetylene inhibition was largely reversible. A higher concentration of acetylene (1920 μM) was added to the

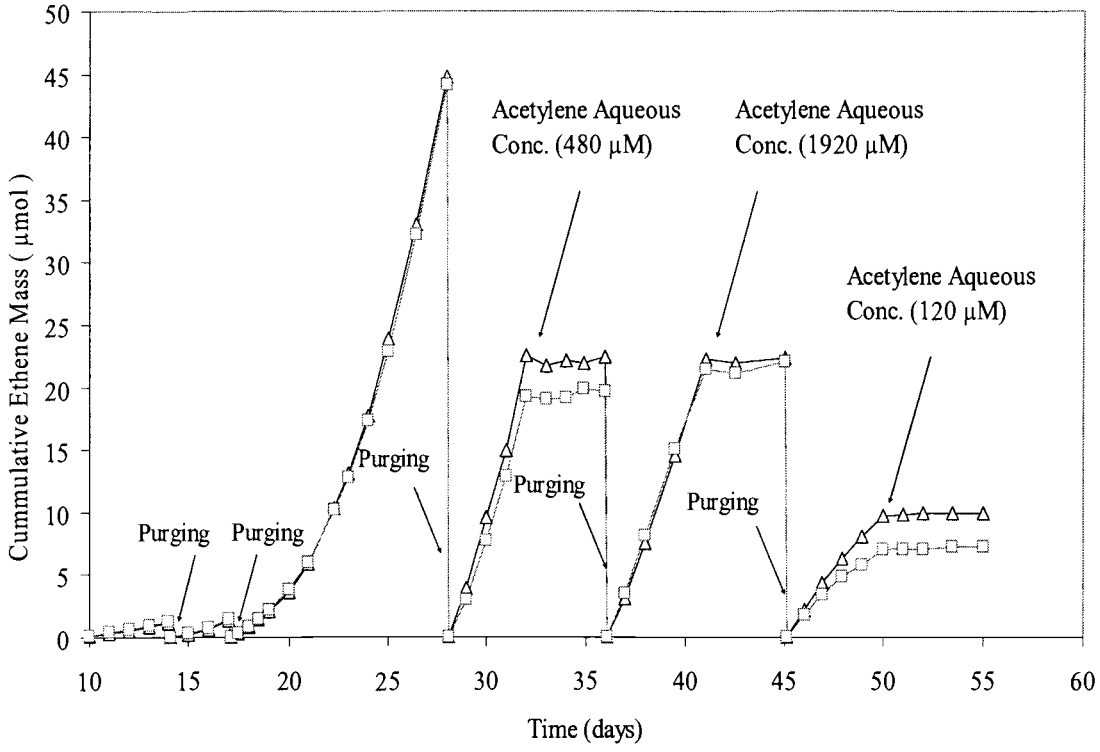

Figure 3.1. Successive inhibition of VC transformation to ethene, at three different aqueous acetylene concentrations, and recovery upon removal of acetylene.

reactors on day 40 to determine whether the effects of acetylene were irreversible at higher concentrations. As expected, ethene production was completely inhibited by acetylene, but slower rates of ethene production were subsequently observed. The maximal rate of ethene production on day 40 was about 30% of the maximal rate observed prior to the first addition of acetylene. We do not know whether the rate decrease is associated with the exposure to the high acetylene concentration or other processes, such as the competition for hydrogen by acetogens as a result of the 45 day incubation. A third test with 120 µM acetylene showed VC transformation was fully inhibited at this lower concentration.

The second set of experiments examined the inhibitory effect of lower acetylene concentrations on VC transformation and methane production by the mixed culture. For these and all subsequent tests, cell mass was increased from that achieved in the mother reactor by an additional growth step in a batch reactor fed VC, as described in the Methods section. Figure 3.2 shows the effect of varying acetylene concentrations (0 to 48 μM) on VC transformation to ethene and methane production. Initial ethene production rates in stage I were similar in all the batch reactors. In stage II, varying concentrations of acetylene were introduced into duplicate reactors. Ethene production was completely inhibited with the highest acetylene concentration tested (48 μM) and was strongly inhibited (>75%) at the lowest acetylene concentration tested (6 μM). The effects of acetylene on methane production were distinctly different than those observed for VC transformation. While methane production was essentially completely inhibited at the highest concentration of acetylene tested (48 μM), it was only inhibited by ~50% with 24 μM acetylene. Little or no inhibition of methane production occurred with the lower concentrations of acetylene tested (6 and 12 μM). In stage III, both ethene and methane production were rapidly reinstated after the removal of acetylene. As shown in earlier experiments, the ethene-generating activity was generally constant during stage III and the rate of ethene-production from most acetylene-treated reactors was indistinguishable from the untreated control reactors. The stage III recovery phase verified the reversibility of VC transformation over a range of the acetylene inhibitions. In contrast, the methane-generating activity

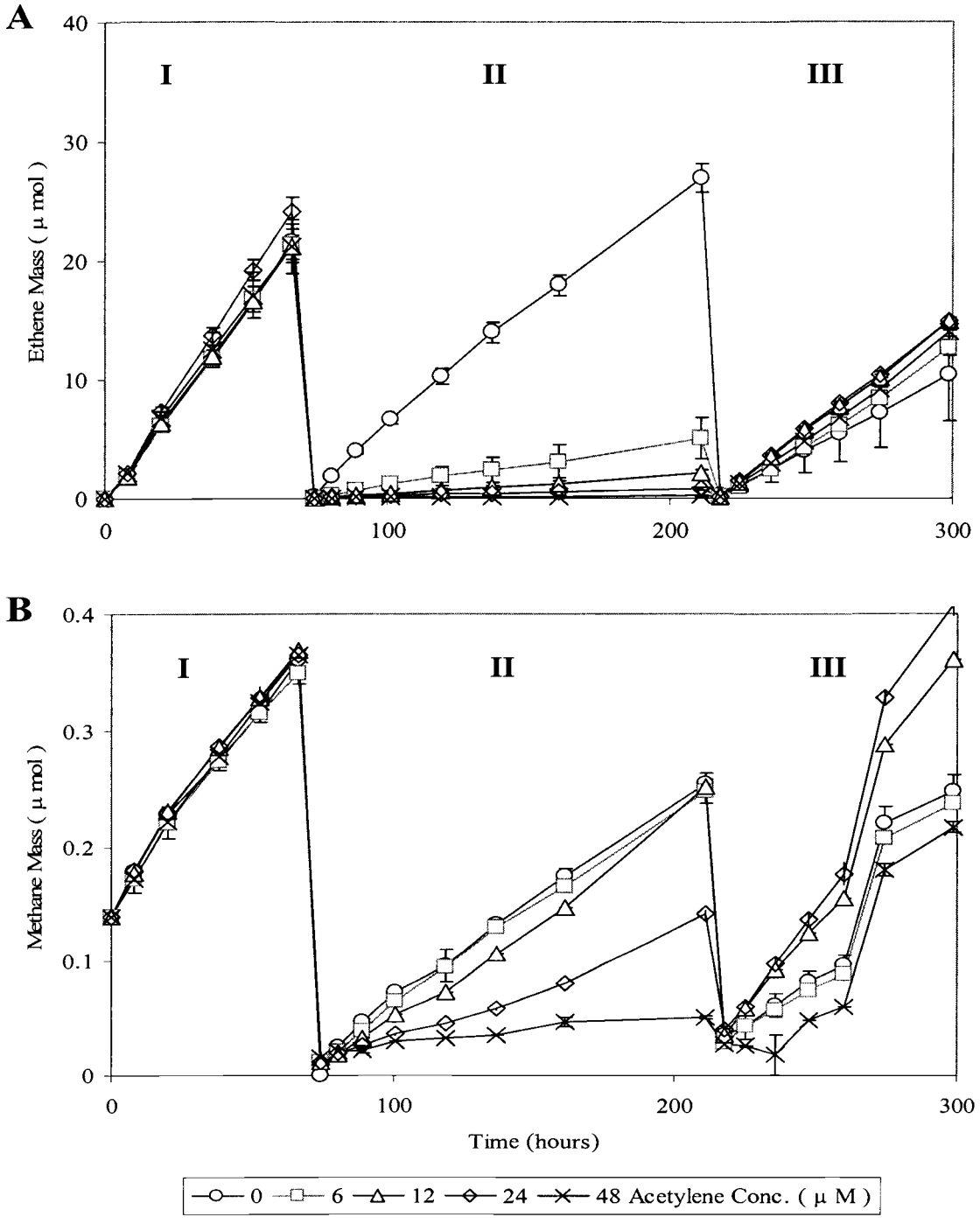

Figure 3.2. Figure 3.2A Inhibition of VC transformation to ethene at acetylene concentrations ranging from 0 to 48 µM. Methane inhibition results are shown in Figure 3.2B. The experiments were carried out in three stages: initial (I), inhibited (II), and recovery (III) of methane and ethene production. (The average and range of duplicates tests are presented)

was not constant over time and there is evidence that the effects of the higher concentrations of acetylene were not fully reversible after removal of the inhibitor.

The results presented in Figure 3.2 showed VC reduction was more acetylene-sensitive than methanogenesis. We therefore investigated the effects of a lower range of acetylene concentrations (0-12 μM) on VC transformation (Figure 3.3).

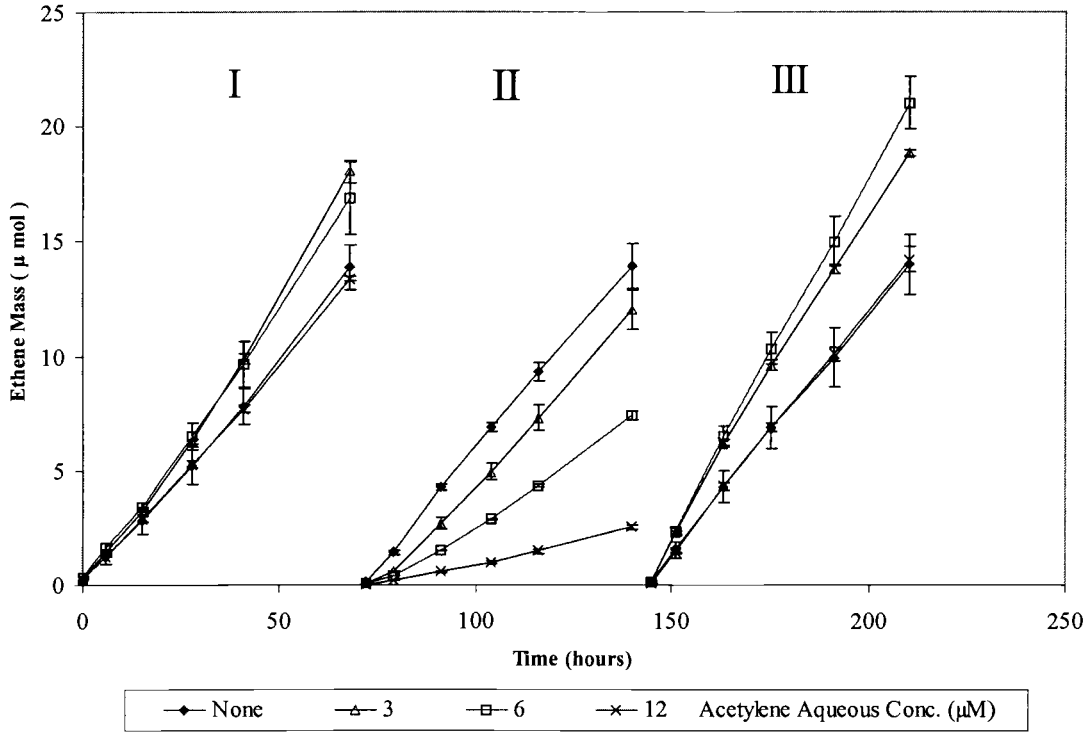

Figure 3.3. Inhibition of VC transformation to ethene at acetylene concentrations ranging from 0 to 12 μM. The results are shown in three stages: initial (I), inhibited (II), and recovery (III). (The average and range of duplicates tests are presented)

All batch tests had similar initial rates of VC transformation in stage I. In stage II, the rate of ethene production decreased with increasing acetylene concentration. Duplicate values showed nearly identical results. An acetylene concentration of 6 μM inhibited

the rate of VC transformation by greater than 50%. The reversibility of acetylene inhibition was again demonstrated in stage III after acetylene was removed. The results clearly demonstrated the dependence of the inhibition of the rates of VC transformation on acetylene concentration.

To determine which microbial process were inhibited by acetylene in the reactions described in Figure 3.3, mass balances for H_2 utilization, methane production, and acetate formation were performed for each reactor during stage II of the tests. Average values for all the reactors indicated methane production was responsible for about less than 1 % of the H_2 consumed; VC transformation to ethene about 47 %; and the acetogenesis about 52 %. Figure 3.4 shows measured H_2 utilization *versus* acetylene concentration for each reactor during the stage II period of reactions (Figure 3.3). Higher acetylene concentrations resulted in a substantial reduction in H_2 utilization for reductive dechlorination, while H_2 utilization mainly for acetogenesis was largely unaffected by concentrations of acetylene that fully inhibited VC reduction. H_2 consumption decreased at high acetylene concentrations due to the inhibition of VC transformation. The amount of acetate produced during the three stages of the experiment was a factor of three greater than that predicted for stage two, indicating acetogenesis was occurring throughout the experiment.

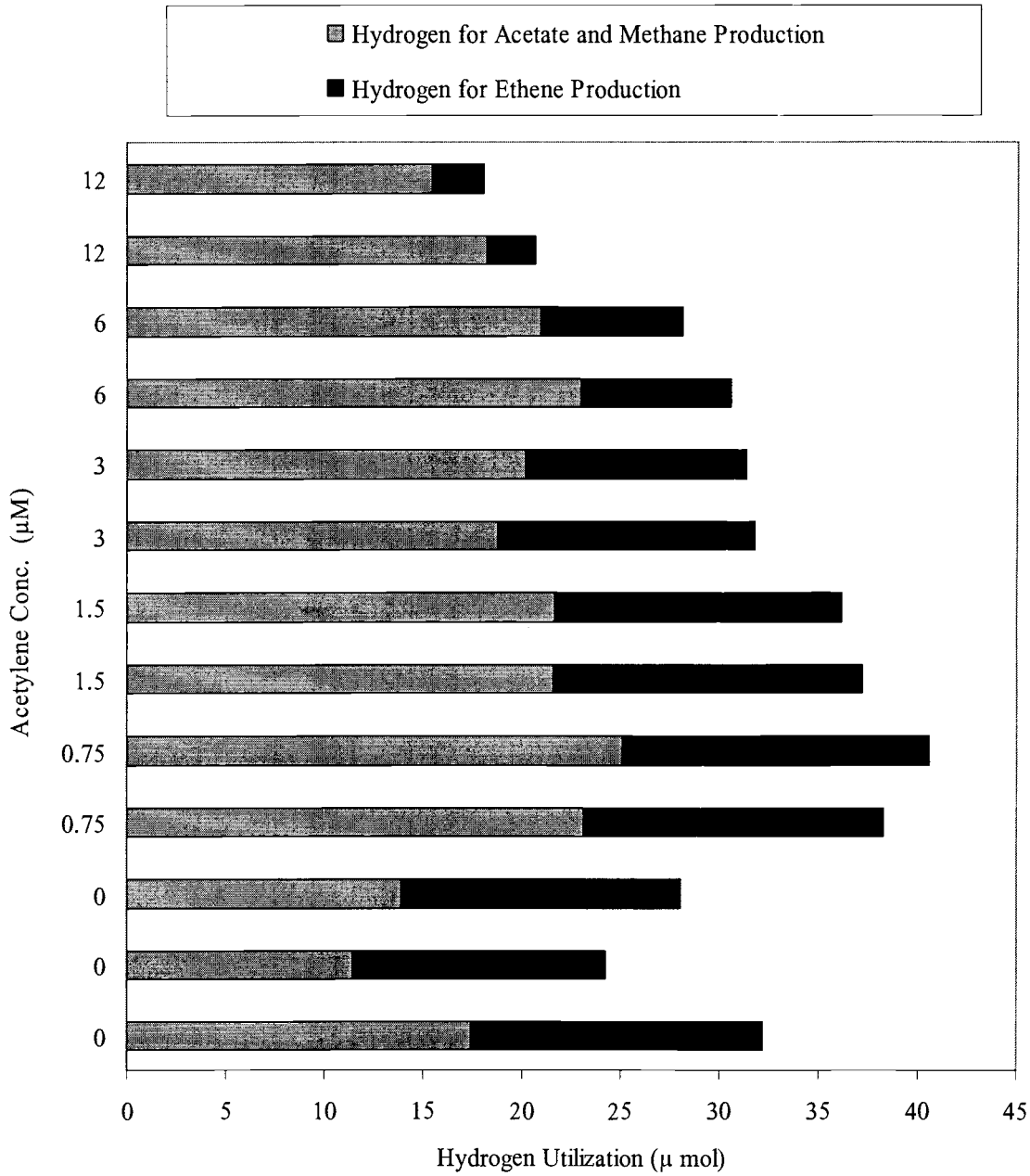

Figure 3.4. Mass balances of hydrogen utilization for reductive dechlorination, acetate, and methane production during stage II of Figure 3.3. Hydrogen utilization for acetate production was estimated based on the total hydrogen consumed minus that used for methane production and reductive dechlorination.

Acetylene Inhibition on TCE Transformation.

Acetylene inhibition of TCE transformation by the mixed culture was also investigated. Since the culture transformed TCE at a faster rate than VC, it was hypothesized that higher acetylene concentrations might be required to inhibit TCE transformation. Inhibition of TCE transformation was therefore studied at concentrations of acetylene up to 192 μM. The aqueous TCE concentration for these tests was 400 μM.

Figure 3.5 shows the concentration history of TCE and its transformation products over the range of acetylene concentrations from 0 to 192 μM for three stages of the inhibition tests. Both *c*-DCE and 1,1-DCE were produced from TCE transformation, with *c*-DCE, accounting for more that 99 % of the DCE products formed. Some VC was also formed and its production increased after significant *c*-DCE accumulated. The controls (no acetylene added) showed similar DCE production rates for all stages. In stage II, the production of *c*-DCE and 1,1-DCE decreased proportionally with increasing acetylene concentration. VC production was essentially completely inhibited at all acetylene concentrations. The results indicated *c*-DCE transformation was more susceptible to acetylene inhibition than TCE transformation. After the removal of acetylene, similar rates of TCE transformation and *c*-DCE and 1,1-DCE product accumulation occurred as in stage I, indicating that TCE inhibition by acetylene was reversible for all acetylene concentrations tested.

The change in production rates of *c*-DCE and 1,1-DCE upon inhibition with acetylene were similar. The result possibly indicates the same enzyme is responsible

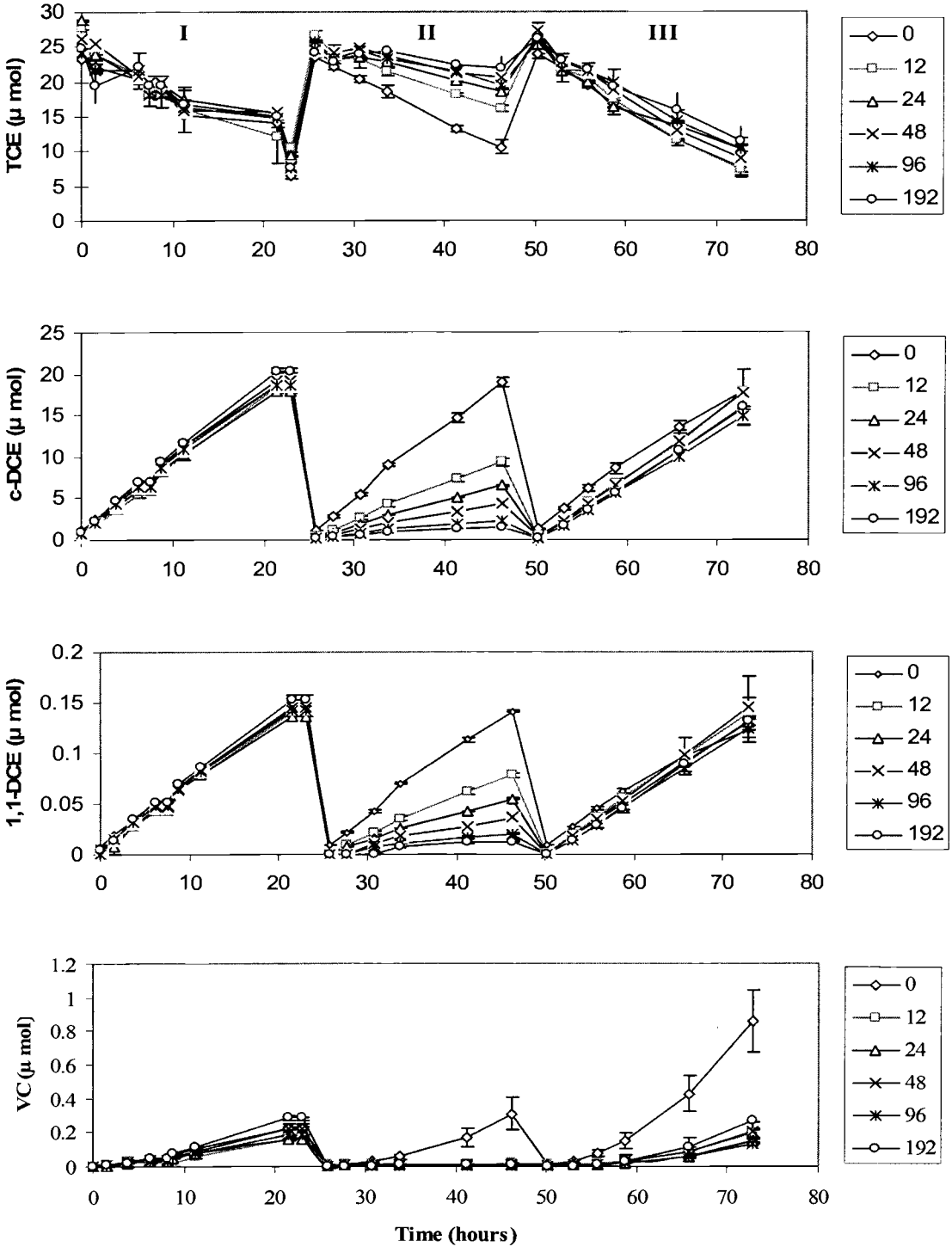

Figure 3.5. TCE transformation and *c*-DCE, 1,1-DCE, and VC production at acetylene concentrations ranging from 0 to 192 µM. (The average and range of duplicates tests are presented)

for the production of both compounds. An interesting observation is that rates of VC production appear to increase through the test, with controls (no acetylene) accelerating at a faster rate in stage III, while rates of DCE accumulation are similar. The results might indicate growth of a dehalogenating population that transforms *c*-DCE to VC, with a faster rate of growth in controls that were not inhibited by acetylene. The results could indicate growth of a DCE-dehalogenating culture was inhibited by acetylene during stage II.

Inhibition Patterns

Rates of TCE and VC transformation were determined based on product production rates prior to and after acetylene inhibition. To normalize each reactor for small differences in the initial rates, the measured rates were normalized by taking a ratio of the inhibited rate ($V_{I=I}$) in stage II to the initial rate ($V_{I=0}$) in stage I. The normalized ratio represents the fractional decrease in rate due to acetylene inhibition. The ratio was then used to calculate the percent inhibition of the initial rate ($V_{I=0}$) as given in Equation 3.1:

$$\%\ Inhibition = \left(1 - \frac{V_{I=I}}{V_{I=0}}\right) \times 100 \qquad (3.1)$$

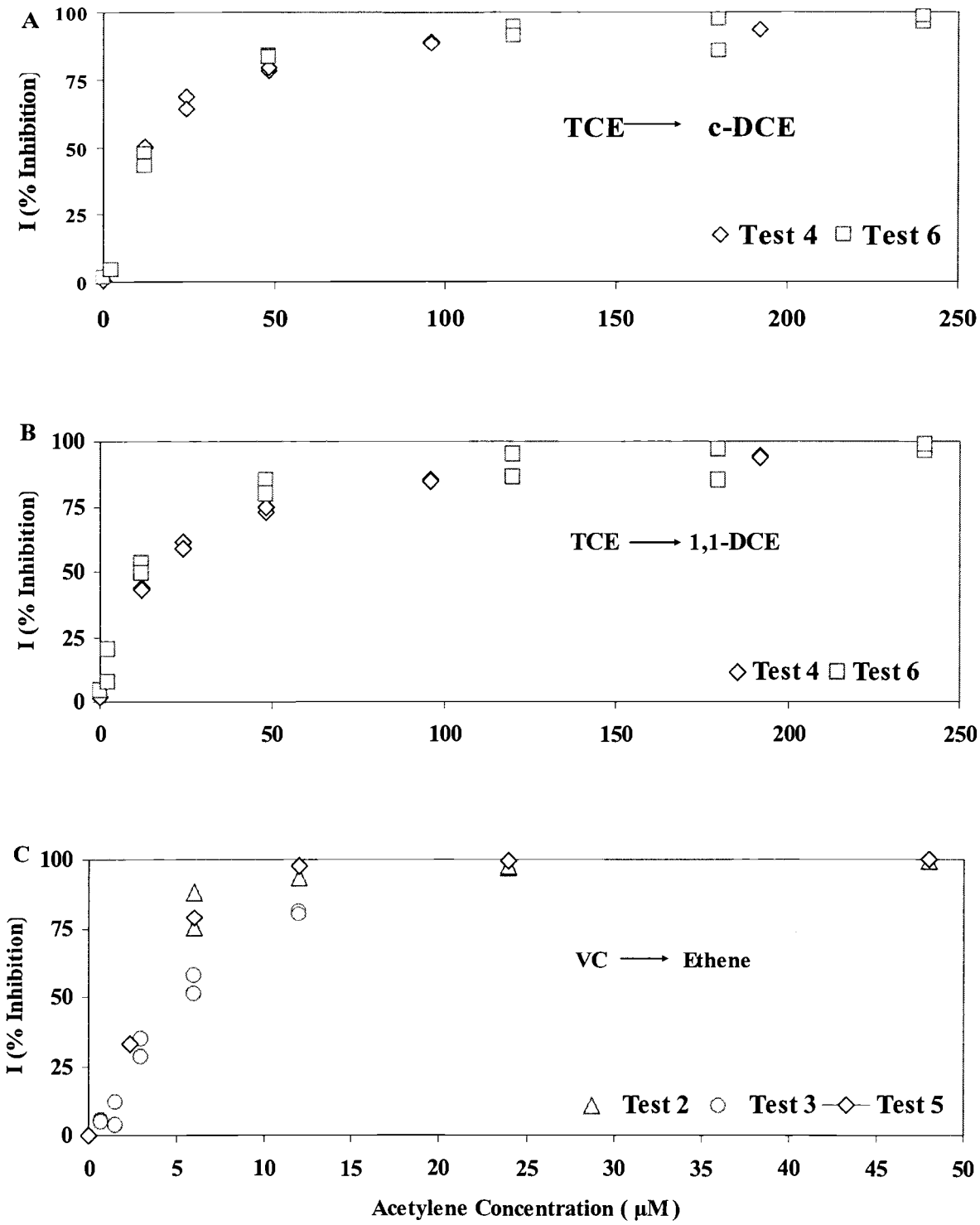

Figure 3.6. Percent inhibition as defined in equation 3.1, as a function of acetylene concentration. Data from multiple experiments are present for TCE conversion to *c*-DCE and 1,1-DCE (A+B), and VC to ethene (C).

The percent inhibition was then plotted versus acetylene concentration, thus permitting a comparison between the overall inhibition patterns for TCE and VC transformation.

Figure 3.6 summarizes the overall patterns of acetylene inhibition of TCE transformation to *c*-DCE (6A), and 1,1 DCE (6B), and on VC transformation to ethene (6C) for the repeated tests (Table 1). The normalization to the initial rates permits a comparison for cultures with different transformation activities. The tests show very reproducible effects of acetylene on TCE transformation for both *c*-DCE and 1,1-DCE (Figure 3.6 A, B) and VC transformation to ethene (Figure 3.6C). Essentially identical inhibition patterns were observed for TCE transformation to *c*-DCE and 1,1-DCE, possibly indicating the same enzyme is involved in their production. The stronger inhibition of acetylene on VC transformation is apparent, as indicated by the lower acetylene concentrations.

DISCUSSION

This study demonstrated that acetylene is a potent and reversible inhibitor of several reductive dechlorination reactions catalyzed by organisms in a mixed dehalogenating culture. Our results confirm several previous reports concerning the inhibitory effects of acetylene on methanogenesis (22, 23). For example, the experiment described in Figure 3.2B demonstrated an almost complete inhibition of methane production with 24 μM acetylene. We do not know whether both acetoclastic and H_2 utilizing methanogens are present in the culture. VC reduction was

considerably more sensitive to acetylene inhibition than methanogenesis, with more than 90% inhibition of VC reduction achieved with 12 μM acetylene, a concentration which achieved less that 10% inhibition of methanogenesis. The selectivity of the effects of low concentrations of acetylene (<12 μM) towards VC reduction rather than either methanogenesis or acetogenesis was further confirmed by the results presented in Figure 3.4. Given the characteristically large flux of H_2 utilization coupled to VC reduction (Figure 3.4) and the apparent lack of involvement of methanogenesis or acetogenesis in VC reduction (Figure 3.2), our results suggest that organisms capable of using chlorinated alkenes and likely VC, as terminal electron acceptors, are the main target of inhibition by low concentrations of acetylene.

Two different groups of organisms capable of dehalorespiration with chlorinated alkenes are currently recognized. One group of organisms, including, among others, *Dehalospirrillum multivorans* and *Desulfitobacterium* strains, can use PCE as an electron acceptor during H_2-dependent growth. These organisms can reduce PCE to TCE and DCE, but do not reduce DCE further. The second group of chlorinated alkene-respiring organisms, currently containing only *Dehalococcoides ethenogenes,* can reduce PCE further to ethene (5). Both groups of organisms possess cobalamin- and Fe-S-containing reductase enzymes responsible for chlorinated alkene reduction. All PCE-respiring-organisms contain a PCE reductase responsible for PCE reduction to TCE. These enzymes also reduce TCE further to DCE. The extended substrate range of *D. ethenogenes* is due to the activity of a second reductase enzyme,

TCE reductase that reduces TCE primarily to *c*-DCE and can also reduce *c*-DCE to VC and VC to ethene (6).

The mode of action of acetylene as an inhibitor of the reductive dechlorination reactions described here remains unclear. Acetylene has many effects on microbial processes and typically inhibits redox-active metalloenzymes (24). The inhibitory mechanisms underlying these effects are diverse and occur over a wide range of acetylene concentrations (24). Acetylene is perhaps best known as an inhibitor of Mo and V-containing nitrogenases. In this instance acetylene acts as poor alternative substrate for these enzymes (K_m = 0.12 - 0.8 mM) and undergoes reduction to yield either ethene or ethane (25, 26). The potential for acetylene reduction was also monitored during our experiments and in control reactions containing acetylene and no TCE or VC additions. No significant ethene production was observed. In addition, in the test shown in Figure 3.1, ethene production was not evident when VC transformation was blocked at high acetylene concentration (1920 μM), indicating acetylene was not being reduced. Acetylene concentrations also remained constant in all the tests (data not shown). Limited ethene inhibition studies where also performed along with the acetylene studies report here. Ethene aqueous concentration of 140, 350 and 575 μM showed minimal inhibition of VC transformation. These results suggest ethene accumulation during VC transformation tests did not affect VC transformation rates.

Acetylene also inhibits many Ni/Fe-containing uptake hydrogenases, and the inhibition process conforms to many of the characteristics of slow, tight-binding

inhibition (27). While an effect of acetylene on hydrogenase activity could potentially account for the effects we have observed in this study, several factors suggest this is unlikely. First, acetylene is a relatively weak inhibitor of Ni/Fe uptake hydrogenases (K_I ~ 0.3 M) and these enzymes are not expected to be impacted by the low concentrations of acetylene shown to be effective in our experiments (27). Second, H_2 is an extremely potent competitive protecting agent against acetylene inhibition (K_D ~0.4 μM) (27). The experiments described here were all conducted with high H_2 concentrations, conditions that would be expected to minimize acetylene inhibition of hydrogenases. Third, our results suggest that H_2 consumption coupled to either methanogenesis or acetogenesis was not inhibited by the low acetylene concentrations shown to be effective against VC reduction. It is also important to recall that, as outlined in the Introduction, acetylene inhibition of methanogenesis has previously been studied and could not be correlated with effects on hydrogenase activity (16).

Our study has provided evidence for an unusually potent inhibition of reductive dechlorination reactions. One possibility is acetylene is directly inhibiting chlorinated ethene reductases required for TCE and VC reduction in this mixed culture. Like all other acetylene-inhibited enzymes, these are metalloenzymes and, unlike many other acetylene-sensitive enzymes, acetylene is a close structural analog of the reductase substrates. It is also notable that as corrinoid enzymes these enzymes contain a cofactor that can readily undergo alkylation reactions (28). It would clearly be very interesting to examine the effects of acetylene on either pure cultures of

dehalorespiring organisms or the purified reductases to test the validity of these conclusions and further define the inhibition mechanism and its kinetic characteristics.

Irrespective of the precise site or mode of action of acetylene discussed above, it is important to recognize that the effects of acetylene we have characterized here have potentially broad implications. For example, acetylene is known to be produced from the abiotic reduction of TCE and PCE to chloroacetylene and acetylene (29, 30). The results of our work would indicate the abiotically produced acetylene could potentially inhibit the biotic transformation of TCE and VC in a combined process, such as in-situ treatment using zero valent iron (ZVI) (31, 32). Lamprom, *et al.* (2001) showed changes in the product distribution of TCE transformation resulting from microbial transformation around a permeable iron barrier (31). The result indicated a combination of abiotic and biotic transformations might result in VC accumulation. Luo and Sewell (2001) found PCE was completely degraded to ethene and ethane in a ZVI system that produced ethene and ethane, but not acetylene (32). However, in systems where acetylene was produced, only partial transformation of PCE was observed. It may be that the production of acetylene inhibited these systems. If abiotic acetylene is produced, and is a stable product, our results indicate the biotic transformation of VC could be inhibited.

Since acetylene is a gaseous chemical, physically it can be easily applied and removed by purging, unlike 2-bromoethane-sulfonate (BES) that is not volatile. Acetylene can be easily purged by sparging with an anaerobic gas, as was demonstrated here, and dehalogenation was rapidly reinitiated with an addition of

either TCE or VC, and H_2. Acetylene is a naturally produced gas and thus regulatory approval might be obtained for its use in field studies of intrinsic or enhanced anaerobic dehalogenation. For example, it might be used in push-pull tests described by Hageman *et al.* (33). Acetylene could be used to inhibit transformation rates of TCE and an added analogue, such as trichlorofluoroethene (TCFE), to provide indirect evidence of in situ transformation (34). Being a reversible inhibitor it can be added to the subsurface to inhibit transformations, and upon its removal transformations should again proceed. The inhibition of transformation of other CAHs including PCE and *c*-DCE is also of interest. Future studies are also needed with pure cultures such as *Dehalococcoides ethenogenes* (5).

ACKNOWLEDGEMENTS

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CHAPTER 4

Kinetic Measurements and Modeling of Acetylene Inhibition On Reductive Dechlorination of TCE and VC

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ABSTRACT

Acetylene was found to be a reversible inhibitor of reductive dehalogenation of trichloroethene (TCE) and vinyl chloride (VC) by a mixed dehalogenating anaerobic culture. The H_2-fed mixed culture was enriched from a contaminated site in Corvallis, OR, and is capable of reductive dehalogenation, from perchloroethene (PCE) and TCE, through *cis*-dichloroethene (*c*-DCE) and VC to ethene. Half-velocities (K_s) values of TCE and VC were determined using a multiple equilibration method, yielding values of 12 and 63 μM, respectively. Acetylene inhibition constants (K_I) for TCE and VC transformation were determined using competitive and uncompetitive inhibition models by non-linear regression fitting of data presented by Pon et al. (2003). Both inhibition models were used to simulate non-steady state transformation results. The acetylene (K_I) inhibition constants, based on the independently measured K_s values and the competitive inhibition model, were essentially the same value of 0.4 μM for results of both TCE and VC transformation. Uncompetitive inhibition modeling resulted acetylene inhibition constants for transformation steps of, TCE to c-DCE, TCE to 1,1-DCE and VC to ethene of 13.3 ± 0.9, 14.1± 0.8 and 2.2 ± 0.4, respectively. Both the competitive and uncompetitive models fit these data equally well when the experiments were conducted at high TCE and VC concentrations near 400 μM, well above the measured K_s values. The K_s values and K_I values for competitive or uncompetitive inhibition were used to simulate non-steady state experimental data generated at lower TCE and VC concentrations. At these

concentrations competitive inhibition model fit the results better than the uncompetitive inhibition model. The model results suggest that acetylene is binding at reactive sites of TCE and VC transformation.

INTRODUCTION

Chlorinated aliphatic hydrocarbons (CAHs), such as tetrachloroethene (PCE) and trichloroethene (TCE), are major groundwater contaminants (1). Anaerobic biotransformation of PCE and TCE sequentially generates *cis*-dichloroethene (*c*-DCE), vinyl chloride (VC) and ethene. Incomplete transformation of PCE and TCE to *c*-DCE or VC is frequently observed in CAH-contaminated groundwater and this limits the usefulness of anaerobic biotransformation as a natural attenuation process (2). Among these products, VC is the most undesirable, as it is a known human carcinogen, and has the lowest drinking water standard (2 μg/L) (3). The completion of reductive dechlorination of CAHs is dependant on the final step of VC transformation.

To ensure complete transformation of the CAHs, researchers have focused on the VC transformation, which is the last and generally most difficult transformation step of the reductive dechlorination. Previous studies indicated high K_s values of *c*-DCE and VC could cause kinetic limitations for transforming *c*-DCE and VC at low concentration levels (4). One of the most studied microorganisms is *Dehalococcoides ethenogenes strain 195* (5), which has the ability to transform PCE completely to

ethene by halorispiration (6,7), with the final step of VC to ethene being slow and cometabolic in nature (6).

Several transformation models have been developed, and competitive inhibition is most commonly used to model inhibition among CAHs. For the case of aerobic cometabolism of CAHs, Kim et al. (8) found inhibition was competitive in nature and could be well represented by the ratios of the measured K_s values (9). Several anaerobic modeling studies also used competitive inhibition models to describe the interference among multiple CAH transformations. Model simulations demonstrated that a competitive inhibition model fit well to experimental data than uncompetitive and noncompetitive inhibition models (10, 11). In one modeling study, both competitive and non-competitive inhibition models were used to evaluate a sequential transformation of PCE through TCE, *c*-DCE and VC to ethene. The results showed competitive inhibition model fit experimental observations better than non-competitive inhibition (11).

In a previous study, we found acetylene to be a reversible inhibitor for reductive dechlorination of TCE and VC (12). Two different inhibition patterns for TCE and VC were observed (12). In the study presented here, the inhibition patterns were further investigated using these published data to gain a better understanding of the inhibition mechanism. Kinetic parameters K_s and $k_{max}X$, of TCE and VC were evaluated for the same anaerobic dehalogenating culture used in (12). Inhibition constants (K_I) were determined using competitive and uncompetitive inhibition models and the independently determined K_s values. The kinetic parameters and inhibition

constants were used to simulate acetylene inhibition of TCE and VC transformation with a non-linear regression modeling program. Both inhibition models were simulated and then used to further evaluate non-steady data of TCE and VC inhibition by acetylene.

EQUATIONS DERIVATION

Non-linear Regression for $k_{max}X$ and K_s Determination.

The experiments were conducted in batch reactors contained both gas (headspace) and aqueous phases. Monod kinetics which have bee previously demonstrated for reductive dehalogenation are presented in equation (Equation 4.1), where v is substrate degradation rate (μmol/hr), $k_{max}X$ and K_s are maximum utilization rate at given cell mass, and half-velocity coefficient, respectively. Equation 4.1 can be modified to meet the experimental conditions of the batch reactors by substituting equation 4.2 into equation 4.1 where V_L and V_G are the volumes of liquid and gas phases of the batch reactor. The M_s is the total mass of substrate. The H_{CS} is the unitless Henny's constant of the substrate. (see appendix for complete equation symbols nomenclatures and units)

$$v = \frac{-k_{\max} X S_L}{K_s + S_L} \tag{4.1}$$

$$S_L = \left(\frac{M_s}{V_L + V_G H_{cs}} \right) \tag{4.2}$$

Based on the results of mass transfer experiments, the assumption of equilibrium is valid over the time scale of the kinetic experiments.

Non-linear Regression for K_I determination

Equations were derived to analyze the acetylene inhibition data (12). using non-linear regression fitting. A modified Monod equation with competitive inhibition kinetics including batch reactor total mass balances is shown in equation 4.3. Equation 4.3 represents the rate of total mass change in a batch reactor for any given substrate concentration, as a function of time.

$$\frac{dM_S}{dt_{(com)}} = \frac{-k_{\max} X S_L V_L}{K_S(1+\frac{I_L}{K_{IC}}) + S_L} \qquad (4.3)$$

$$I_L = \left(\frac{M_I}{V_L + V_G H_{CI}} \right) \qquad (4.4)$$

where dM_s/dt is the total mass transformation rate (μ moles / day / liter); k_{max} is the maximum utilization rate (μ moles / mg of cell/ day); X is the biomass (mg of cell /liter); S_L is the substrate concentration (μ M); K_s is the half-saturation coefficient (μ M); I_L is the inhibitor concentration (μ M), and K_{IC} is the inhibition constant (μ M). Inhibitor partitioning between a two phase batch reactor in given in equation 4.4. M_I is

the total mass of the inhibitor. The H_{CI} is the unitless Henny's constant of the inhibitor.

The inhibition tests measured acetylene concentration effects on the rates of reductive dechlorination of TCE and VC. Pon et al. (12) presented the acetylene concentration dependence on a percent inhibition basis, as shown in equation 4.5. The tests compared rates of dehalogenation of the inhibited ($\frac{dM_I}{dt}$) versus the non-inhibited ($\frac{dM}{dt}$) reactors at the same CAH concentration. Equation 4.5 is a function of the mass transformation

$$\%Inhibition = (1 - \frac{\frac{dM_I}{dt}}{\frac{dM}{dt}}) \times 100 \qquad (4.5)$$

rates defined in equation 4.3. The non-inhibited rate equation has inhibitor concentration (I_L) set equal to zero. By substituting equations 4.1, 4.2, 4.3, and 4.4 into equation 4.5, then simplifying and rearranging, equation 4.6 can be derived for the competitive inhibition case. For simplification, the cell mass is assumed equal in all the batch reactors. The $k_{max}X$ term in equation 4.3 cancels each other for the inhibited and non-inhibited reactors. Equation 4.6 is the resulting equation that can be used in non-linear regression to determine the competition inhibition constant, K_{Ic} with a known K_s value. α_I is the constant used to convert the total mass to aqueous

concentration of the batch reactor. H_{CI} is the dimensionless Henny's constant of the inhibitor.

$$\%\ \ Inhibition_{(com)} = (1 - \frac{K_S + M_S}{K_S\left(1 + \frac{M_I}{K_{IC}\alpha_I}\right) + M_S}) \times 100 \tag{4.6}$$

$$\alpha_I = V_L + V_G H_{CI} \tag{4.7}$$

For the uncompetitive inhibition model, a modified Monod equation for a batch reactor including the total reactor mass balance is shown as equation 4.8:

$$\frac{dM_S}{dt}_{(uncom)} = \frac{-\frac{k_{\max} X S_L V_L}{(1 + \frac{I_L}{K_{IU}})}}{\frac{K_S}{(1 + \frac{I_L}{K_{IU}})} + S_L} \tag{4.8}$$

Equation 4.8 gives the transformation rate in the presence of an inhibitor for the case of uncompetitive inhibition. By substituting equation 4.1, 4.2, 4.7, and 4.4 into equation 4.5, and simplifying and rearranging, equation 4.9 can be derived for non-linear regression fitting.

$$\%\ Inhibition_{(uncom)} = (1 - \frac{K_S + S_L}{(1 + \frac{M_I}{K_{Iu}\alpha_I})(\frac{K_S}{1 + \frac{M_I}{K_{Iu}\alpha_I}} + S_L)}) \times 100 \tag{4.9}$$

Inhibition constants, K_{Ic} and K_{Iu} for competitive inhibition (equation 4.6) and uncompetitive inhibition (equation 4.9), respectively, were determined by non-linear regression analysis of the experimental data using the S-Plus program (Insightful Corporation). The independently determined K_s values for TCE and VC were set as constants for the regression analysis.

Model Simulations

A non-steady-state model was constructed and implemented in STELLA Research 5.0 (Harvard Business School Publishing Corporation), and used to simulate the non-steady-state operation of the batch reactors. The model solved equations 4.3 and 4.8 as function of time for competitive and uncompetitive inhibition models, respectively. Pulse inputs of TCE, VC and acetylene were simulated with independently measured kinetic parameters and inhibition constants. The cell mass was assumed constant during the simulations. Equilibrium mass transfer between the aqueous and gaseous phases was incorporated in the model for the CAHs and ethene using published Henny's constants (14). A small time step (dt), of 0.0005 h, was used to avoid numerical instabilities.

MATERIALS AND METHODS

Chemicals

PCE (99%) and *c*-DCE (97%) were purchased from Acros (Fisher Scientific, Pittsburgh, PA), TCE (99.9%), VC (99.5%) and 1,1-DCE (99%) were purchased from Aldrich (Milwaukee, WI). Gas standards, ethene (1000 ppm ± 5%), VC (996 ppm ± 2%) in nitrogen were purchased from Scott Specialty Gases (Alltech Associates, Inc; Deerfield, IL). Methane (99%), CO_2 (99%), acetylene (99.6%) and H_2 (99 %) were obtained from AIRCO (Vancouver, WA). Yeast extract was purchased from Fisher Scientific (Fair Lawn, NJ).

Analytical Methods

Gas phase PCE, TCE, *c*-DCE, 1,1-DCE, VC, acetylene, ethene, and methane concentrations were measured by injecting a batch reactor headspace sample (100 µL) into a HP-5890 gas chromatograph (GC). The GC was equipped with a 30-m megabore GSQ-Plot column (J&W Scientific, Folsom, CA), connected to a flame-ionized detector (FID). H_2 and CO_2 were analyzed by injecting 100 µL of headspace sample into a HP-5890 GC. Chromatographic separation was achieved with a Supelco 60/80 Carboxen 100 column (15 ft X 0.125 in.; Bellefonte, PA), followed by quantification using a thermal conductivity detector (TCD). The detection limit of analysis was 1 ppm (vol/vol) for H_2 in the gas phase.

Growth of the Culture

The Evanite mixed-anaerobic-culture was obtained from a TCE-contaminated groundwater and sediment at the Evanite site in Corvallis, Oregon. The culture was first grown in batch serum bottles on benzoate and PCE in RAMM media (15). This mixed-culture is capable of completely transforming PCE through TCE, *c*-DCE and VC to ethene, with a conversion to ethene of nearly 100%. Cells were harvested when the batch reactor reached a TCE or VC removal rate of 80 μ moles per day. The details of the growth conditions of the Evanite enrichments were previously described (12).

Batch Reactor Construction

All the kinetic and inhibition tests were performed in 125-ml batch bottles (Wheaton; Millville, NJ) capped with butyl rubber septa (Wheaton; Millville, NJ) to allow temporal sampling. Cells present in 50 ml of reactor media were transferred in an anaerobic glove box from the VC-incubated reactor to 125-ml batch reactors. The reactors were purged with furnace-treated nitrogen for 10 minutes to remove the glove-box H_2 and any residual VC or ethene. Furnace-treated H_2 (5 ml), CO_2 (20 ml) were then added to the reactor headspace for VC and TCE kinetic studies. The reactors were incubated at 20°C and shaken in an inverted-position at 200 rpm on a rotary shaker table. The H_2 and CO_2 concentrations were maintained at excess levels for the kinetic studies. H_2 was re-supplied during the experiment when the headspace H_2 concentrations fell below 0.5 % (v/v), by adding 5 ml of H_2.

$k_{max}X$ and K_s Determinations

A multiple equilibration method in a single batch reactor was used to measure TCE and VC transformation rates over a broad range of concentrations to develop a Monod kinetic curve. The transformation rate was first determined at the lowest substrate concentration. The concentration was then increased through TCE or VC addition and the rate was determined at the next desired concentration. Concentrations were then successively increased until a maximum rate was achieved. At each given substrate concentration the transformation rate of TCE or VC was measured based on product formation, *c*-DCE and ethene, respectively. The transformation rates within the reactor were determined using mass balances for the liquid and gas phases and Henry's Law equilibrium between phases. Rates were determined by linear regression of five product formation data points versus time. Kinetic parameters of $k_{max}X$ and K_s of the TCE and VC transformation were determined by non-linear regression fitting of the Monod equation using the S-Plus program (Insightful Corporation). The transformation test was completed within a 12 hour period so that cell growth would be limited. An estimated doubling time of the Evanite culture grown on VC was approximately 60 hours, based observed exponential increases in ethene production reported by Pon et al. (12).

Acetylene Inhibition Test

The non-steady-state acetylene inhibition studies were conducted at lower TCE and VC concentrations using procedures described by Pon et al. (12). These tests represent conditions where acetylene was added as the reactions proceeded. The experimental concentration ranges were 0 to 20 μM and 0 to 100 μM for TCE and VC, respectively. Batch reactors constructed using the same procedures and had the same initial cell concentration as the kinetic tests. The inhibition study consisted of two stages: an initial stage without acetylene and an inhibition stage. No purging was performed between the two stages. The initial stage was used to estimate the cell mass ($k_{max}X$) in each reactor by trial and error with STELLA simulations. Then, at the beginning of stage two various acetylene amounts were added to the different reactors and the changes in transformation rates were determine.

RESULTS

Determination of $k_{max}X$ and K_s values for TCE and VC

The kinetic studies focused on determining $k_{max}X$ and K_s values for TCE and VC. Rates of product formation were measured over a wide range of substrate concentrations (0 to 1000 μM) using the multiple equilibration method. The TCE test presented in Figure 4.1A shows the successive increases in TCE concentration, and *c*-DCE production data used to determine rates. Substrate concentrations were successively increased until rates close to the maximum transformation rates were achieved. The resulting Monod plot for TCE is shown in Figure 4.1B along with the non-linear regression fit to the Monod equation along with 95% confidence intervals. The kinetic tests were performed at three different cell concentrations ($k_{max}X$). The estimated $k_{max}X$ values of 24 ±2, 275 ±10, and 540 ±18 μM per day yielded K_s values were 4.2 ±1.3, 15 ±2, and 15 ±2, μM, respectively. The K_s values were reasonably close over the factor of 20 difference in $k_{max}X$ values.

The same procedure was used to determine the $k_{max}X$ and K_s values for VC, with estimated values of 334 ±11 μM per day and 63 ±7 μM, respectively. These results were published in a previous study (13). The K_s value for VC was a factor of six greater than that of TCE. These determined K_s values were later used in the non-linear regression analysis to determine the acetylene inhibition constant (K_I).

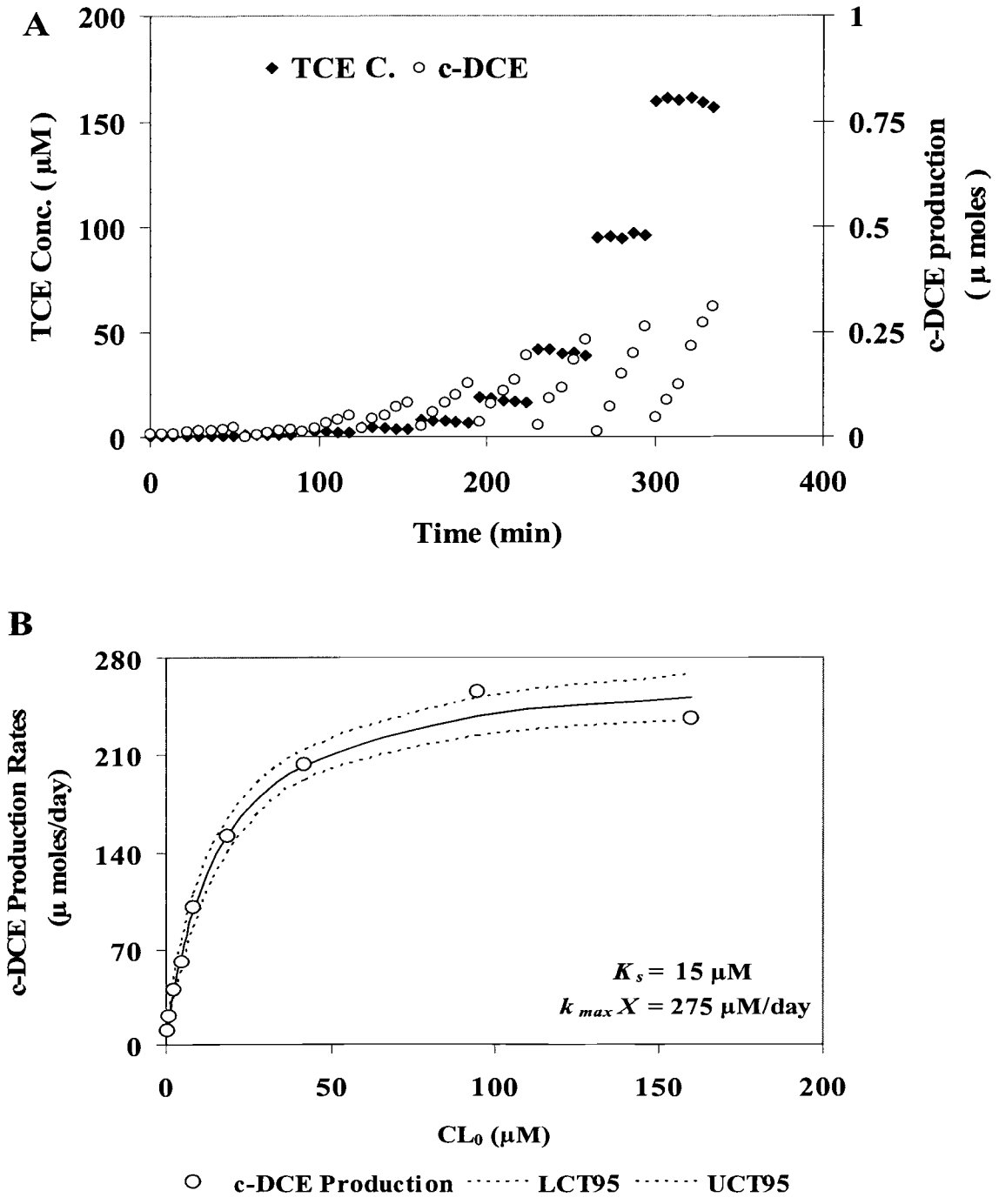

Figure 4.1. (A) *c*-DCE production rates at various TCE concentrations using the multiple equilibration method. The *c*-DCE production rates were determined by linear regression, immediately after the successive addition of TCE. (B) Monod curve used to determine K_s and $k_{max}X$ for TCE by non-linear regression.

Acetylene Inhibition Constants (K_I) Determinations

Our previous work reported acetylene to be a potent, reversible inhibitor of reductive dehalogenation of TCE and VC. In that kinetic study, levels of inhibition were strongly dependent on acetylene concentration. The dependence of the extent of inhibition on acetylene concentration was presented on a percent inhibition basis (12). These data were further analyzed with competitive and uncompetitive inhibition models by non-linear regression analysis of equations 4.6 and 4.9.

The results are presented in Figure 4.2. The lines in Figure 4.2 and values in Table 4.1 represent results of the fit to the competitive and uncompetitive inhibition models. The data presented in Figure 4.2 A-C were used to predict K_{IC} and K_{IU}, with the independently determined K_s values input into the model. K_s values for the transformation of TCE to *c*-DCE and 1,1-DCE were 12 μM (average value of the three measured TCE K_s values), while the K_s value for VC transformation to ethene was 63 μM. The product of $k_{max}X$ was assumed constant between the non-inhibited and inhibited tests, and thus their input was not needed. Both inhibition models showed good fits to the acetylene inhibition data for TCE transformation to *c*-DCE and 1,1-DCE, and a reasonable fit for VC transformation to ethene. There was no significant difference between the models fits to the data, as indicated by the identical residual standard errors, as shown in Table 4.1. An interesting result is that the K_I values were almost identical for all three reactions with competitive inhibition modeling, as shown

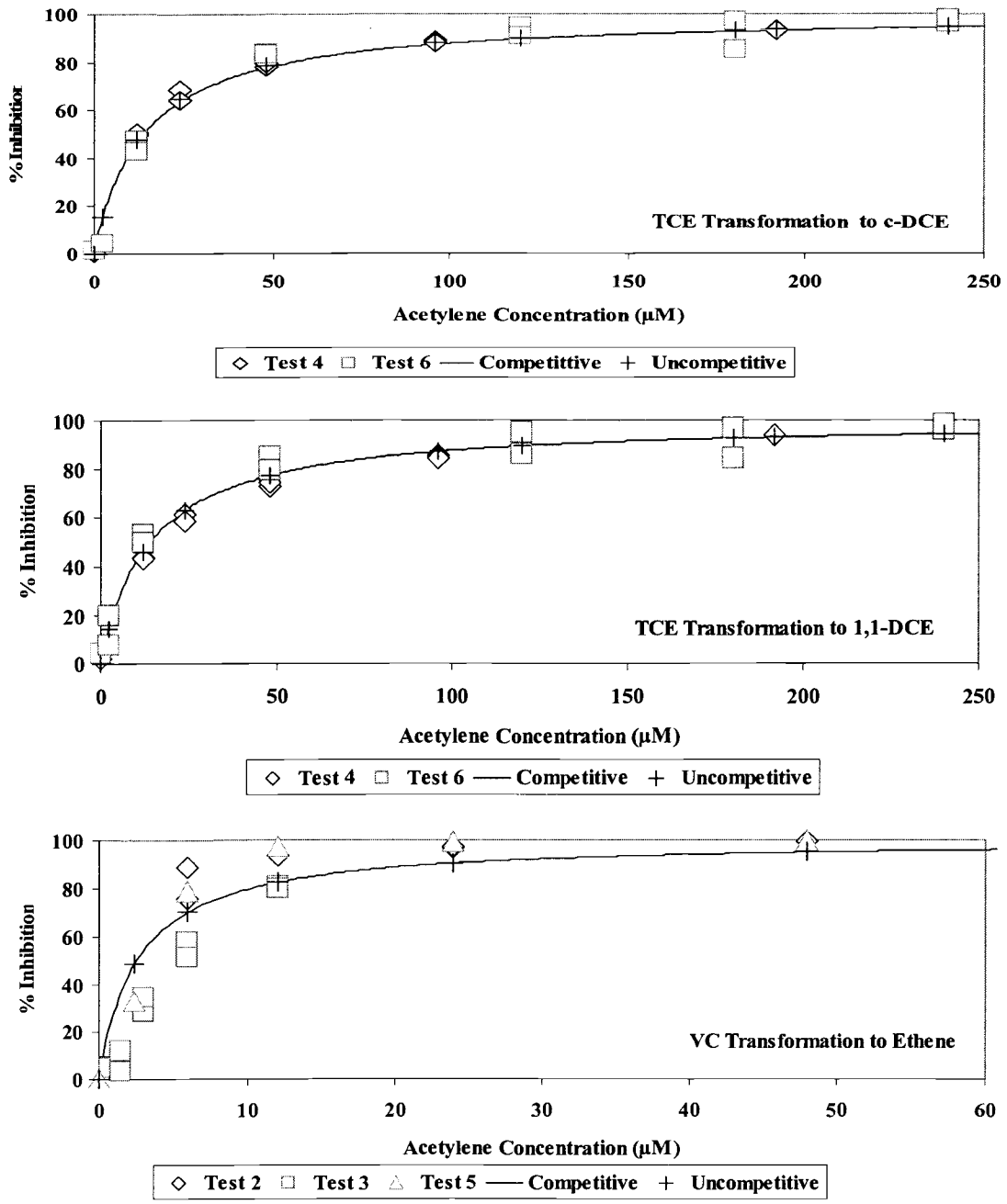

Figure 4.2. Percent inhibition as defined in equation 4.4, as a function of acetylene concentration. Data from multiple experiments are present for TCE conversion to *c*-DCE and 1,1-DCE (A+B), and VC to ethene (C). The solid line represents the model fit to competitive inhibition (equation 4.3), converted to rate changes given by equation 4.6. The + sign represents the model fit to uncompetitive inhibition (equation 4.9).

in Table 4.1. The relatively low K_{IC} values in comparison with the measured K_s values indicate acetylene is a strong inhibitor of TCE and VC transformation. However, with uncompetitive inhibition modeling, the determined coefficients of TCE are 6 times larger than that for VC. For transformation of TCE to *c*-DCE and transformation of TCE to 1,1-DCE the inhibition constants were essentially equal.

Table 4.1. Acetylene inhibition constant K_I for TCE and VC transformation using competitive and uncompetitive inhibition modeling

	TCE to c-DCE (μM)	TCE to 1,1-DCE (μM)	VC to ethene (μM)
Competitive Inhibition Modeling	K_{IC} = 0.39 ± 0.03 (4.93)*	K_{IC} = 0.41 ± 0.02 (4.36)*	K_{IC} = 0.35 ± 0.07 (12.52)*
Uncompetitive Inhibition Modeling	K_{IU} =13.32 ± 0.87 (4.92)*	K_{IU} =14.07 ± 0.81 (4.36)*	K_{IU} =2.18 ± 0.42 (12.52)*

*Residual Standard Error (μM)

Model Simulations

Computer simulations were performed of the transient acetylene inhibition of TCE and VC transformations with the STELLA® program. The model was used to solved equations 4.3 (competitive inhibition) and equation 4.8 (uncompetitive inhibition). The estimated kinetic parameters (K_s) and inhibition constants (K_{IC} and K_{IU}) were used in the simulation. These transient experimental data were used to construct percent inhibition plot shown in Figure 4.2.

Figure 4.3A shows the experimental data of ethene accumulation from VC transformation. The first ethene accumulations were used to determine the initial VC

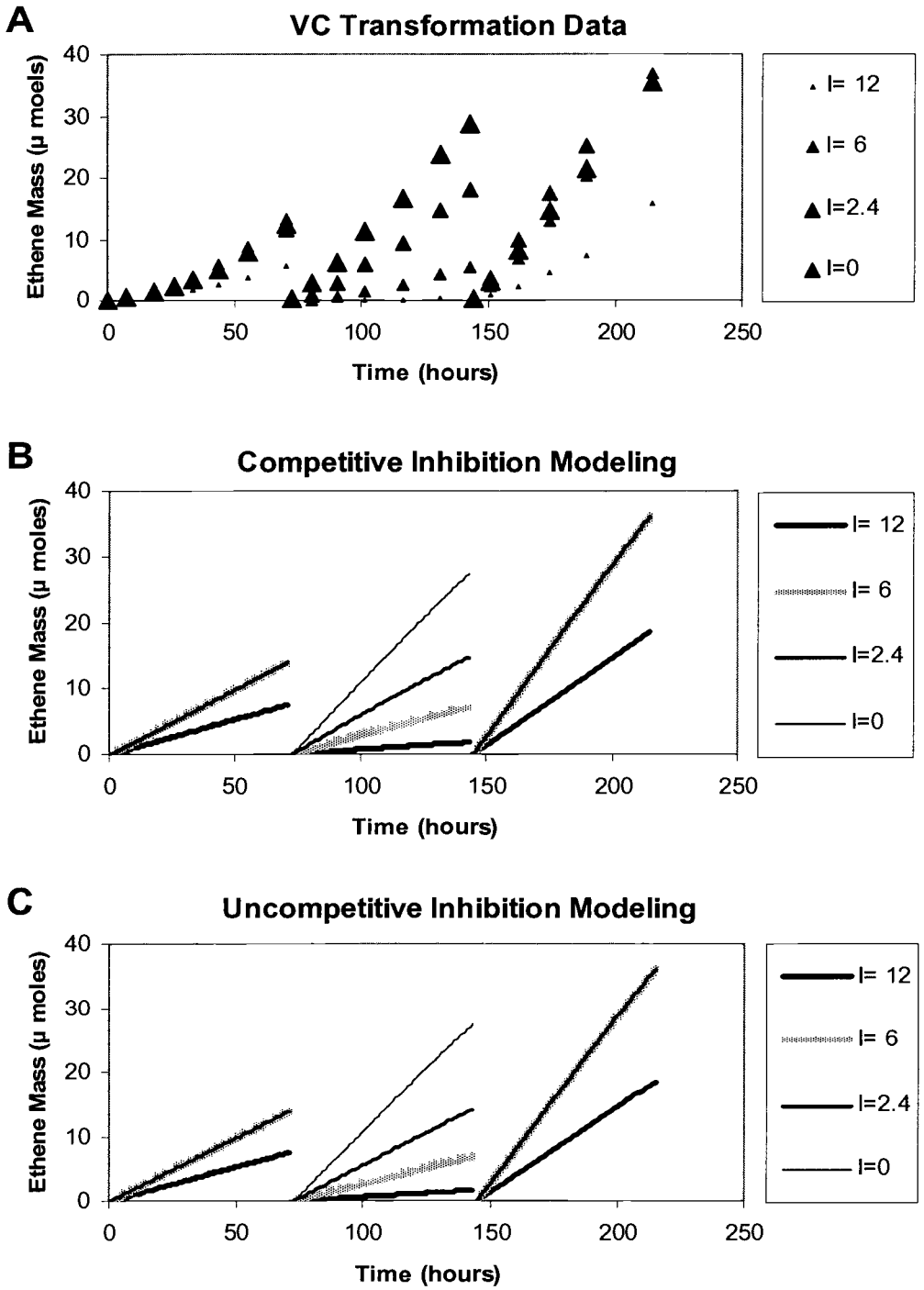

Figure 4.3. (A) Inhibition of VC transformation to ethene at acetylene concentrations ranging from 0 to 48 µM. (B) STELLA modeling of the acetylene inhibition using competitive inhibition. (C) STELLA modeling of the acetylene inhibition using uncompetitive inhibition. The experiments were carried out in three stages: initial (I), inhibited (II), and recovery (III).

transformation rate in the absence of acetylene inhibition. The second ethene accumulation showed various rates of ethene production due to acetylene inhibition (0 to 48 μM), while the third set of ethene accumulations measured the recovery of VC transformation after the inhibition. In this experiment there were two sets of initial transformation rates, with three of the six reactors having faster initial transformation rates. Reactors with the fast rate were used for three lower acetylene inhibition concentrations (0, 12, 24 μM), while the other three were applied with higher acetylene inhibition concentrations. Figure 4.3B and 4.3C showed simulated results using competitive and uncompetitive inhibition modeling, respectively, along with the two different initial rates designated by $k_{max}X$ in the model. The simulated data successfully reconstructed the acetylene inhibition patterns resulting from the acetylene doses. The results showed that both inhibition models predicted equally well the transient response of acetylene inhibition on VC transformation. These model simulations show the determined constant values (K_s and K_I) obtained by the non-linear regression fitting (Figure 4.2), matched the transient experimental data well.

The recovery of VC transformation rates were about double the initial rates (Figure 4.3 III). Results indicated the potential growth of the VC dehalogenator during the course of the experiment. A control that had no acetylene addition showed successive increase in ethene production ($M_I = 0$ μM). For the recovery simulations adjustments were made to the $k_{max}X$ values according to the control observations. Cell masses were estimated from the ethene production rates in regions I and III. The average of cell masses of regions I and III were used for region II simulations.

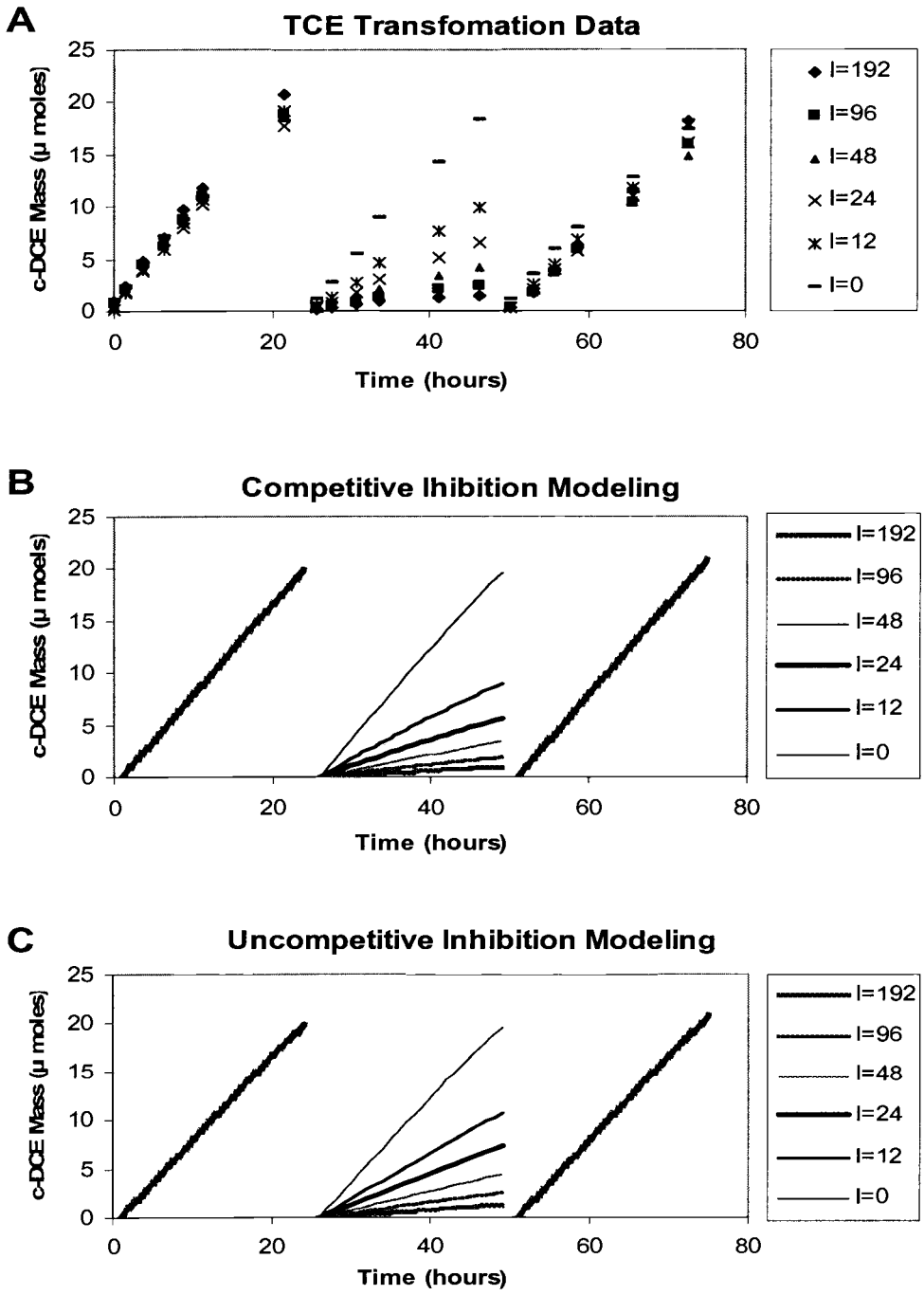

Figure 4.4. (A) Inhibition of TCE transformation to *c*-DCE at acetylene concentrations ranging from 0 to 192 µM. (B) STELLA modeling of the acetylene inhibition using competitive inhibition. (C) STELLA modeling of the acetylene inhibition using uncompetitive inhibition. The experiments were carried out in three stages: initial (I), inhibited (II), and recovery (III)

Simulations were also performed of acetylene inhibition on TCE transformation. The results are shown in Figure 4.4. Both competitive and uncompetitive inhibition models simulated equally well the experimental data. The experiment was conducted over a short period of time. Simulations with the same $k_{max}X$ values were used over the course of the three tests, indicating no significant cell growth had occurred.

Simulation of Acetylene Inhibition for Lower VC and TCE Concentration

This study extends the previous model simulations to inhibition tests conducted at lower VC and TCE concentrations. Since previous experiments were performed at high VC and TCE concentrations (400 μM), these tests determined how the models and estimated parameters fit at lower concentrations. Acetylene was added at VC and TCE concentrations near the measured K_s values (65 μM and 12 μM for VC and TCE, respectively), where the inhibition should be more sensitive to the inhibitor according to the competitive inhibition model. The desired acetylene concentrations for these tests were predicted by competitive inhibition simulations and were moderately lower than the previous experimental conditions. The results are shown in Figure 4.5 and Figure 4.6 for VC and TCE inhibitions, respectively.

The VC inhibition studies were conducted in separate batch reactors that were given differing amounts of acetylene after initial rates were established, as shown in Figure 4.5. The acetylene aqueous concentrations ranged from 0 to 0.67 μM.

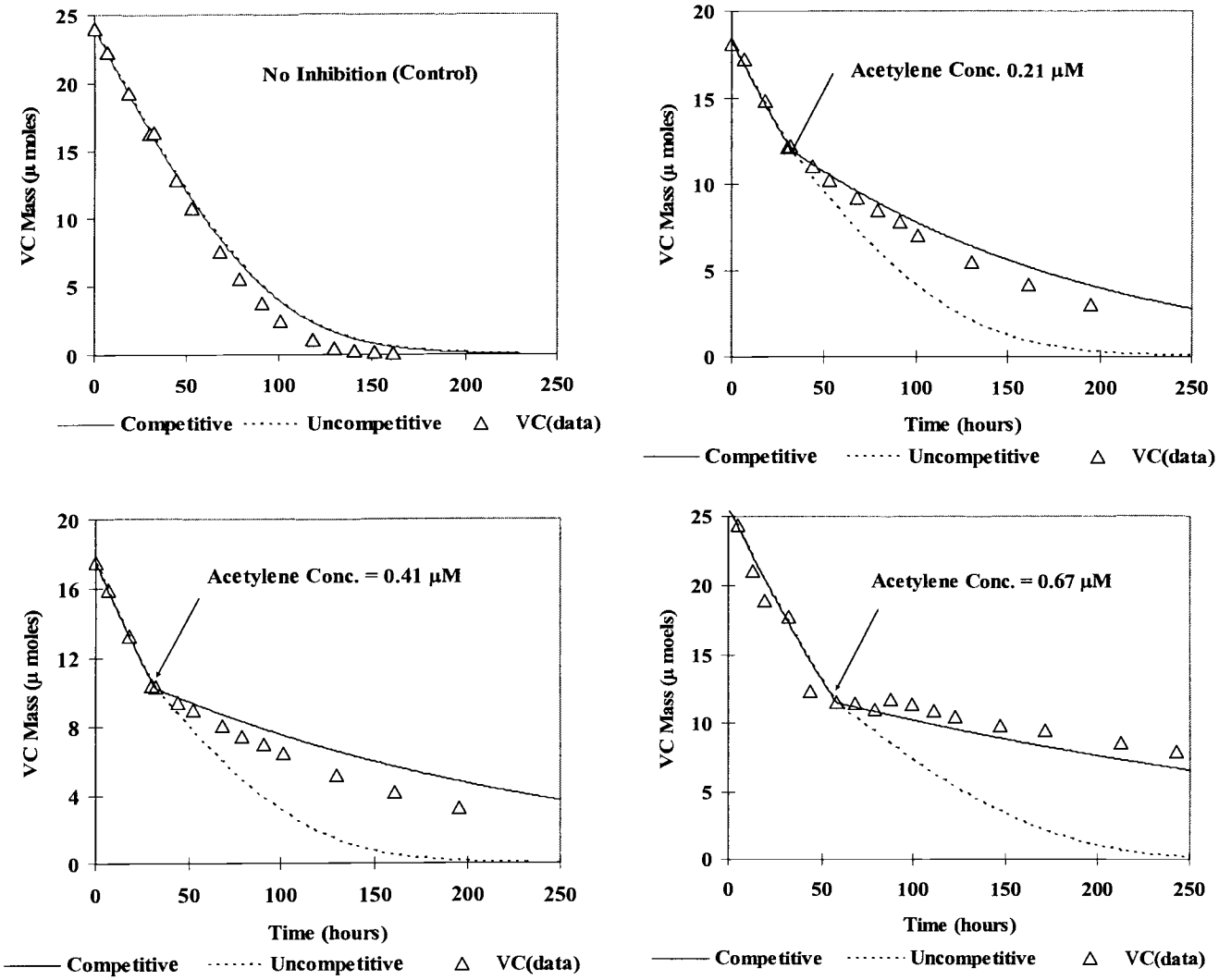

Figure 4.5. Inhibition of VC transformation at acetylene concentrations ranging from 0 to 0.67 µM. The solid line represents the simulation using the competitive inhibition model, and the dotted line represents the simulation using the uncompetitive inhibition model.

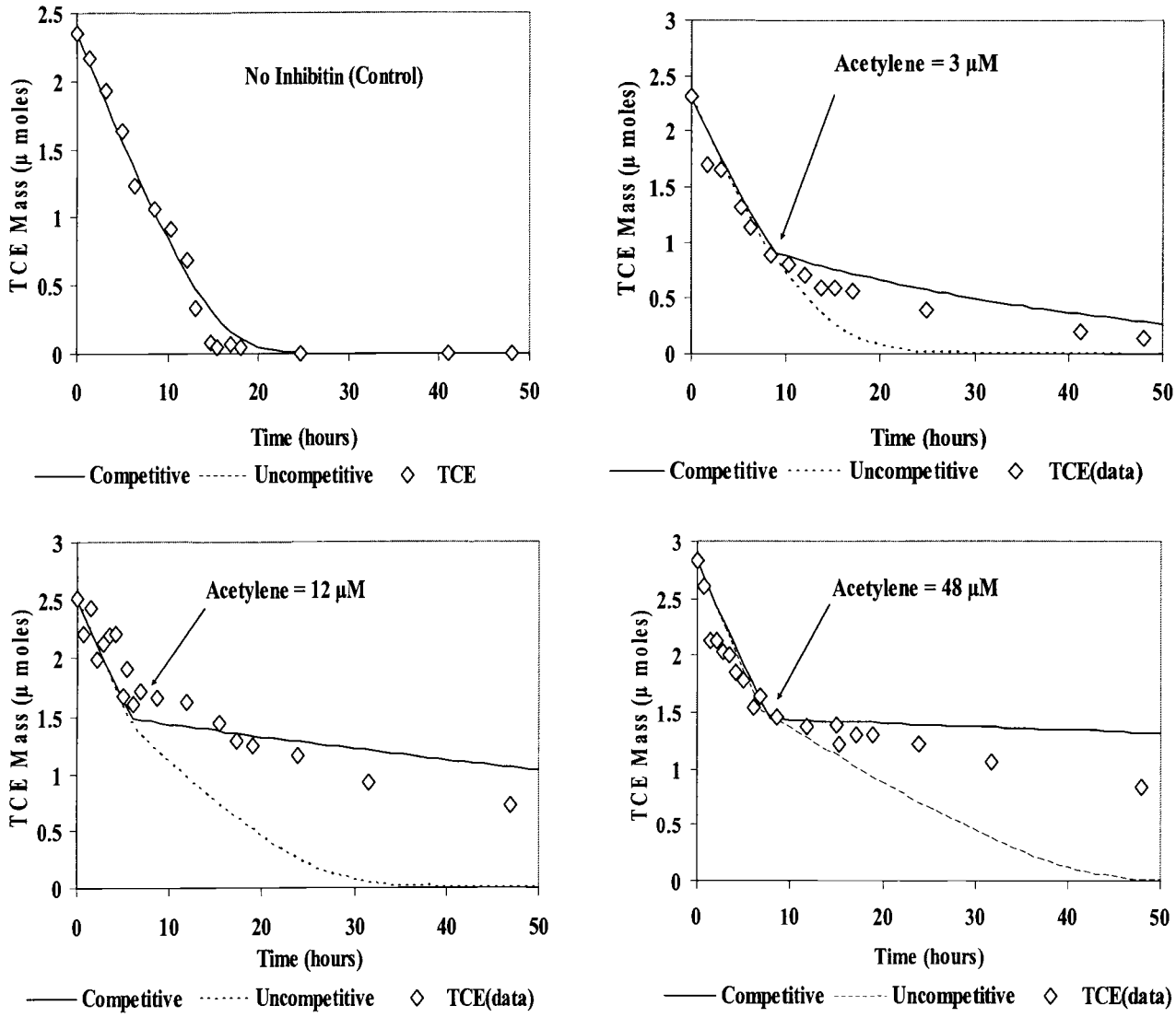

Figure 4.6. Inhibition of TCE transformation at acetylene concentrations ranging from 0 to 48 µM. The solid line represents the simulation using the competitive inhibition model, and the dotted line represents the simulation using the uncompetitive inhibition model.

As VC concentrations were reduced to near the K_s values (63 μM), varying amount of acetylene were applied to inhibit VC transformation, as shown in Figure 4.5. The VC transformations were inhibited to varying degrees related to the acetylene concentration. The dehalogenating cell concentrations X estimated from the initial rate data are shown in Table 4.2. Since TCE transformation was faster than VC transformation, the cell masses were diluted to achieve in a slower rate transformation for TCE transformation. Both competitive and uncompetitive inhibition simulations are also shown along with the experimental data. The competitive inhibition model shows a reasonably good fit to the experimental data, while the uncompetitive inhibition model yielded a poorer fit.

Table 4.2. Estimated cell masses from the initial rate data.

VC Transformation		TCE Transformation	
Acetylene Conc. (μM)	Estimated Cell Masses (X=mg/L)	Acetylene Conc. (μM)	Estimated Cell Masses (X=mg/L)
0	25	0	13
0.21	27	3	13
0.41	26	12	14
0.67	25	48	15

Similar experimental procedures were applied to the TCE inhibition. The results are shown in Figure 4.6. The applied acetylene liquid concentrations ranged from 0 to 48 μM. The competitive inhibition model showed a reasonably good fit to the experimental data. The uncompetitive inhibition model failed to simulate the experimental results. In this TCE inhibition study, TCE was transformed rapidly

through c-DCE to VC, with VC accumulation (data not shown). VC accumulation was expected from the previous study, since VC transformation to ethene was inhibited at lower acetylene concentrations (12). *c*-DCE transformation was not as sensitive as VC transformation to acetylene inhibition, as previously observed (Pon et al., 2003).

DISSCUSSION

In our previous study (12) acetylene was found to be a reversible inhibitor for reductive dechlorination of TCE and VC. Acetylene's molecular structure is similar to TCE and VC. Acetylene can be reduced as an electron acceptor. Thus acetylene is a good candidate as a transition-state analog for TCE and VC. Experimental results showed no product formation from acetylene and the inhibition was reversible, making acetylene an ideal inhibitor for probing dechlorination reactions.

The main goal of the inhibition study was to probe acetylene inhibition of TCE and VC transformations. Two major inhibitions models, competitive and uncompetitive, were investigated. The competitive inhibition model indicates the substrate (TCE or VC) and inhibitor (acetylene) are competing for a common active site that is responsible for TCE and VC transformation. An alternative possibility is uncompetitive inhibition, where the inhibitor binds to the substrate and enzyme complex. Uncompetitive inhibition is often found in enzymatic reactions that have two or more substrates involvement (17).

A non-linear regression method was used to determine inhibition constants (K_I) for acetylene inhibition on VC and TCE transformation, for competitive or

uncompetitive inhibition. The inhibition constants are presented in Table 4.1. Uncompetitive inhibition constants (K_{IU}) are much larger than the competitive inhibition constants (K_{IC}). Competitive inhibition models yielded similar inhibition constants (0.41 μM) between the TCE and VC transformation. However, the uncompetitive inhibition constants for TCE transformation are 5 times larger than the one for VC transformation. Both inhibition models resulted in an equal amount of residual standard errors, indicating both models fit the experimental data equally well. Simulations of the transient data using these constants were also performed with a STELLA program. Regression analysis showed little difference between the competitive and uncompetitive inhibition models (Figure 4.2). The inability to distinguish between the types of inhibition may be related to the experimental conditions. The experimental data were obtained by applying various acetylene concentrations to inhibit TCE or VC transformations at initial concentrations of 400 μM. These concentrations were much higher than the measured K_s values. The TCE and VC were supplied in excess levels, as to not limit these reactants during the kinetic studies. Results suggest that more inhibition studies need to be performed over a range of TCE and VC concentrations at a fixed acetylene concentration. By performing the experiments in this manner the results can then be plotted using Lineweaver-Burk methods to determine the inhibition type (17).

The transient experiments were performed at lower VC and TCE concentrations in an attempt to resolve the indifference found in the earlier inhibition modeling simulations (Figure 4.5 and Figure 4.6). Results show that the degree of

inhibition is dependent on the acetylene concentration used, which is consistent with the previous study (12). The results showed that the competitive inhibition model fit the experimental data for both TCE and VC transformation much better than the uncompetitive model.

This study does not, however, provide sufficient evidence to confirm that competitive inhibition is the correct model of acetylene inhibition on TCE and VC transformation. The results indicate that competitive inhibition model is better than the uncompetitive inhibition model at lower TCE and VC concentrations. This result is consistent with previous competitive inhibition studies among the chlorinated ethenes (8, 9, 10).

It is noteworthy that the K_s values we have determined for TCE and VC reduction are also in reasonable agreement with previously published values for chlorinated ethene reduction by dehalorespiring organisms (4). Our kinetic analysis based on a competitive model (Figure 4.2 and Table 4.1) also suggests acetylene has the same inhibition constant, K_{IC} value, as an inhibitor of all three of the reactions we have examined (TCE reduction to *c*-DCE and 1,1-DCE and VC reduction to ethene). One possible interpretation is that acetylene inhibits a process common to all of these reactions. The site of inhibition could potentially include a critical metabolic step unrelated to the reduction of chlorinated ethenes. Alternatively, given the broad substrate range of enzymes like TCE reductase or VC reductase and the close structural similarity of acetylene to this enzyme's substrates, the constant K_{IC} value could reflect the direct inhibition of the reductase responsible for chlorinated ethene

reduction. Acetylene potentially inhibits the same functional site for both TCE and VC transformations.

Although these tests were performed with an anaerobic mixed culture, the enrichment step of growth on VC prior to kinetic tests likely selected for microorganisms capable of growth of VC. In other tests when the culture was fed PCE or TCE, $k_{max}X$ values for TCE were on order of magnitude greater than VC, Yu et al. (18), and K_s values were a factor of 10 lower for TCE, than those reported here. K_s values of VC reported here were approximately equal those of Yu et al. (18). Thus TCE transformation may have been initiated primarily by VC degrading microorganisms, which could explain the competitive inhibition and the same K_{IC} values that were obtained.

Mode of Action of Acetylene: The mode of action of acetylene as an inhibitor of the reductive dechlorination reactions described here remains unclear. The inhibition appears to be a rapid process. We did not observed any time dependency during the establishment of the inhibition consistent with the transient simulations. Short-term kinetic tests, however, were not performed to evaluate the kinetics of the inhibition. The inhibition process also appears to be reversible in the short-term experiments.

Hydrogenases are inhibited by acetylene (19). This could be important in the case of dehalorespiration where the respiratory chain is limited to an uptake hydrogenase coupled to a chlorinated ethene reductase. Acetylene inhibits many Ni/Fe-containing uptake hydrogenases, and the inhibition process conforms to many

of the characteristics of slow, tight-binding inhibition (19). While acetylene inhibition on hydrogenase activity could potentially account for the effects we have observed, several factors suggest this is unlikely. Acetylene is a relatively weak inhibitor of uptake by hydrogenases (K_I ~ 0.3 M) and these enzymes are not expected to be impacted by the low concentrations of acetylene. Our inhibition constant is 0.41 μM is very low. Second, H_2 is an extremely potent competitive protecting agent against acetylene inhibition (K_D ~0.4 μM) (19). The experiments described here were all conducted with high H_2 concentrations, conditions that would be expected to minimize acetylene inhibition of hydrogenases.

It is possible that the effects we have observed here are directed at the chlorinated ethene reductases required for TCE and VC reduction. Like all other acetylene-inhibited enzymes, these are metalloenzymes and, unlike many other acetylene-sensitive enzymes, acetylene is a close structural analog of the reductase substrates. It is also notable that as corrinoid enzymes these enzymes contain a cofactor that can readily undergo alkylation reactions (20). It would be very interesting to examine the effects of acetylene on either pure cultures of dehalorespiring organisms or the purified reductases to further define the inhibition mechanism and its kinetic characteristics.

ACKNOWLEDGEMENTS

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CHAPTER 5

Anaerobic Reductive Dechlorination of 1-Chloro-1-fluoroethene to Track the Transformation of Vinyl Chloride

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Submitted to

Environmental Science and Technology

ABSTRACT

1-chloro-1-fluoroethene (1,1-CFE) was studied as a reactive tracer to quantify the anaerobic transformation of vinyl chloride (VC). Batch kinetic studies of 1,1-*CFE* and VC transformation were performed with an enrichment culture, obtained from the Evanite Site in Corvallis, OR. The culture is capable of completely transforming trichloroethene (TCE) through *cis*-dichloroethene (*c*-DCE) and VC to ethene. The culture also transforms fluorinated analogues, such as trichlorofluoroethene (TCFE) to fluoroethene as a final product. The transformation sequence of the fluorinated analogue was correlated with that achieved for the CAH, with the same degree of chloride substitution. For example, the production of 1,1-CFE, the major CFE isomer formed from TCFE transformation was correlated with the production of VC from TCE transformation. Since the 1,1-CFE and its product, fluoroethene (FE), have a distinct analytical signature, 1,1-CFE can be potentially used as a reactive tracer in field evaluations to determine the potential of VC transformation to ethene. Kinetic studies quantified the transformation rates of VC and 1,1-CFE by the Evanite culture. The half-saturated constants (K_s) of VC and 1,1-CFE were 63 and 87 μM, respectively, while similar maximum utilization rates ($k_{max}X$), of 334 and 350 μM/day, were achieved. Acetylene inhibition studies also indicated that VC and 1,1-CFE were likely transformed by the same enzyme. A competitive inhibition model with the independently measured K_s values used as the inhibition constants predicted rates of transformation of both VC and 1,1-CFE when both compounds were present in batch

kinetic tests. 1,1-CFE transformation was also tested with three different cultures during sequential transformation of TCE. The Point Mugu culture more slowly transforms VC to ethene than the Evanite culture, while the Lawrence Livermore culture essentially stops at VC. With all the cultures 1,1-CFE transformation was associated with VC transformation to ethene, and rates of transformation were comparable. The results demonstrated that 1,1-CFE was a good surrogate analogue for evaluating the rates of VC transformation.

INTRODUCTION

Chlorinated aliphatic hydrocarbons (CAHs), such as tetrachloroethene (PCE) and trichloroethene (TCE), are major groundwater contaminants (1). Anaerobic biotransformation of PCE and TCE sequentially generates *cis*-dichloroethene (*c*-DCE), vinyl chloride (VC), with ethene as a harmless end-product. Incomplete transformation of PCE and TCE to *c*-DCE or VC is frequently observed in CAH-contaminated groundwater and this limits the usefulness of anaerobic biotransformation as a natural attenuation process (2). Among these products, VC is the most undesirable as it is a known human carcinogen and has the lowest drinking water standard (2 μg/L) (3).

Researchers have focused on the VC transformation to ethene, which is the last and generally most difficult transforming step of the reductive dechlorination of chlorinated ethenes. High K_s values of *c*-DCE and VC caused incomplete

transformation of TCE and PCE to *c*-DCE or VC (4), that and highly chlorinated CAHs, such as PCE, have inhibitory effects on the lower chlorinated transformation products (5). Hardness et al. indicate that a missing critical bacterial population was a cause for incomplete transformation, suggesting bioaugmentation was necessary to complete reductive dechlorination to ethene (6). One of the most studied dehalogenators is *Dehalococcoides ethenogenes strain 195* (7), which has the ability to transform PCE to ethene, however the final step of VC to ethene is cometabolic in nature (8). VC halorespiration by mixed cultures (9, 10) has lead to the isolation of a pure culture (11) that is capable of using *c*-DCE and VC as electron acceptors, but not PCE and TCE (10, 11).

A variety of biochemical tools have been used to probe the complexity of reductive dehalogenation processes. Using molecular approaches Flynn *et al.* (12), showed a microbial community shift in response to enrichment as different chlorinated ethenes were successively transformed. Terminal restriction fragment length polymorphism (T-RFLP) demonstrated *c*-DCE- and VC-grown subcultures from PCE enrichments had distinct 16S rRNA genes and subcultures grown on less chlorinated solvents lost their ability to degrade PCE. Molecular methods have been recently used to phylogenetically characterize anaerobic dechlorinating microbial communities (11, 13, 14), and evaluate the success of bioaugmentation and biostimulation in field studies (15, 16). Carbon isotopic fractionation has also been used to provide qualitative evidence of the occurrence and relative extent of biological reductive dechlorination of the chlorinated ethenes (17).

Using a chemical surrogate analogue of TCE, such as trichlorofluoethene (TCFE), is an alternative approach for tracking the transformation of TCE (18). The advantage of using a transformation analogue is that a unique analytical identification for in situ biotransformation is possible when background concentrations of the CAHs of interest are present. It is often difficult to demonstrate transformation is occurring when other complicated physical processes, such as sorption and desorption of the contaminant and nonaqueous phase liquid (NAPL) dissolution are occurring. Fluorinated analogues, are resistant to defluorination and fluorinated products are easily detected by standard gas chromatographic methods. The fluorinated analogues can be use in field studies of intrinsic anaerobic dehalogenation, such as push-pull tests described by Hageman *et al.* (19).

In a previous study, Vancheeswaran *et al.* observed TCFE was transformed to three dichlorofluoroethene isomers (DCFEs), with 1-dichloro-2-fluoroethene (*c*-DCFE) being the main isomer. The DCFE isomers can be potentially transformed to three chlorofluoroethene isomers (CFEs), which can be further transformed to fluoroethene (FE). Vancheeswaran et al. observed limited DCFEs transformation to CFE, thus the major DCFE transformation product was not clearly determined (20). In our study, we identified the major transformation intermediate of TCFE through c-DCFE as 1,1-chlorofluoroethene (1,1-CFE). This led us to evaluate 1,1-CFE as a potential analogue for the transformation of VC.

Kinetic and inhibition studies were performed in our study to exam the correlation of 1,1-CFE with VC transformation. Kinetic values of $k_{max}X$ and K_s for VC

and 1,1-CFE were determined. The potential for their being transformed by the same mechanism was also examined through inhibition studies. In a previous study with the Evanite culture we found acetylene was a reversible inhibitor of the reductive dechlorination (21). Acetylene inhibition of 1,1-CFE and VC transformations were therefore investigated. Tests were also performed of VC and 1,1-CFE inhibition on each other, and results were evaluated using a competitive inhibition model. 1,1-CFE transformation was also tested concurrent with TCE and *c*-DCE transformation to determine whether TCE and *c*-DCE transformations could possibly induce 1,1-CFE transformation. These tests were conducted with three different cultures, enriched from different contaminated sites, had different transforming abilities. The Point Mugu culture can completely transform PCE and TCE to ethene, but at low concentrations transforms VC to ethene more slowly than the Evanite culture, while the Lawrence Livermore culture transforms PCE and TCE to VC.

The objectives of this study were to: 1) identify 1,1-CFE as the main isomer formed by TCFE transformation by the Evanite Culture; 2) determine transformation kinetic parameters ($k_{max}X$ and K_s) for VC and 1,1-CFE with the Evanite culture; 3) study inhibition of VC and 1,1-CFE on the transformation of each other, and acetylene inhibition on the transformation both compounds; and 4) evaluate how 1,1-CFE tracks the transformation of VC with different dehalogenating cultures.

MATERIALS AND METHODS

Chemicals

PCE (99%) and *c*-DCE (97%) were purchased from Acros (Fisher Scientific, Pittsburgh, PA) and TCE (99.9%), was purchased from Aldrich (Milwaukee, WI). TCFE (97%), *c*-DCFE (98%) (consisting of 1 to 2% of *t*-DCFE), 1,1-CFE (98%), and 1-chloro-2-fluoro-CFE (98%) (E/Z mixture consisting of 50% (E) and 50% (Z) isomers), were purchased from SynQuest Labs., Inc. (Alachua, FL). Gas standards, ethene (1000 ppm ± 5%), VC (996 ppm ± 2%) in nitrogen were purchased from Scott Specialty Gases (Alltech Associates, Inc; Deerfield, IL). Methane (99%), CO_2 (99%), acetylene (99.6%) and H_2 (99 %) were obtained from AIRCO (Vancouver, WA). Yeast extract was purchased from Fisher Scientific (Fair Lawn, NJ).

Analytical Methods

Gas phase TCE, TCFE, *c*-DCE, *c*-DCFE, 1,1-CFE, VC, acetylene, ethene, and methane concentrations were measured by injecting a batch reactor headspace sample (100 µL) into a HP-5890 gas chromatograph (GC). The GC was equipped with a 30-m megabore GSQ-Plot column by (J&W Scientific, Folsom, CA), connected to a flame-ionized detector (FID). H_2 was analyzed by injecting 100 µL of headspace sample into a HP-5890 GC. Chromatographic separation was achieved using argon as a carrier gas, with a Supelco 60/80 Carboxen 100 column (15 ft X 0.125 in.; Bellefonte, PA), followed by quantification using a thermal conductivity detector (TCD).

Growth of the Cultures

The Evanite anaerobic culture was obtained from TCE-contaminated groundwater and sediment at the Evanite site in Corvallis, Oregon. The growth conditions for the Evanite culture were described in Chapter 3 (21). This mixed-culture is capable of completely transforming PCE through TCE, *c*-DCE and VC to ethene, with nearly 100% conversion to ethene. For the VC and 1,1-CFE kinetic parameter measurements, the culture was further enriched in batch growth reactors on VC and H_2. The cells were incubated in the 1000-ml batch reactor with 5 ml H_2 (200 μ moles), 20 ml of VC (800 μ moles), and 20 ml of CO_2 added to the reactor headspace. Additional H_2 (5 ml) was added when gas phase H_2 levels fell below 0.5 % v/v. Cells were harvested when the batch reactor reached a VC removal rate of 80 μ moles per day.

The Point Mugu culture was maintained using the same incubation procedures as the Evanite culture, except TCE was used as the electron acceptor instead of PCE. The Point Mugu culture was capable of completely transforming TCE through *c*-DCE and VC to ethene. It required a substantial longer time to transform VC to ethene than the Evanite culture.

The original Lawrence Livermore groundwater microcosm showed transformation of TCE mainly to *c*-DCE (18), with some production of VC. The cultures growth conditions are presented by Yu and Semprini (22).

All three cultures were examined by using a PCR assay developed to detect 16S Ribosomal RNA gene (rDNA) sequence with *Dehalococcoides*-specific primers Dhg 728F and 1155 R (23) and Dhc 1F and 1377R (24) and *Desulfuromonas*-specific primers Dsm 205F and 1015R (23). Specific PCR results showed all three cultures contained *Dehalococcoides*-like microorganisms, however *Desulfuromonas*-like microorganisms were not found.

The Kinetic Tests

The kinetic tests were performed in 125-ml batch bottles (Wheaton; Millville, NJ) capped with butyl rubber septa (Wheaton; Millville, NJ) to allow temporal sampling. Cells present in 50 ml of reactor media were transferred in an anaerobic glove box from the VC-incubated reactor to 125-ml batch reactors. The reactors were purged with furnace-treated nitrogen for 10 minutes to remove the glove box H_2 and any residual VC or ethene. H_2 (5 ml), CO_2 (20 ml) (furnace-treated) were then added to the reactor headspace for VC and CFE kinetic studies. The reactors were incubated in at 20°C and shaken in an inverted-position at 200 rpm on a rotary shaker table. The H_2 and CO_2 concentrations were maintained at excess levels for the kinetic studies. H_2 was re-supplied during the experiment when the headspace H_2 concentrations fell below 0.5 % (v/v), by adding 5 ml of H_2.

$k_{max}X$ and K_s Determinations

A multiple equilibration method in a single batch reactor was used to measure VC and CFE transformation rates over a broad range of concentrations to develop a Monod kinetic curve. The transformation rate was first determined at the lowest substrate concentration. The concentration was then increased through VC or CFE addition and the rate determined at the next desired concentration. Concentrations were then successively increased until a maximum rate was achieved. At each given substrate concentration the transforming rate of 1,1-CFE or VC was measured based on product formation, FE and ethene, respectively. The transformation rates within the reactor were determined using mass balances for the liquid and gas phases and Henry's Law equilibrium between phases. Rates were determined by linear regression of five product formation data points versus time. Kinetic parameters of $k_{max}X$ and K_s of the 1,1-CFE and VC transformations were determined by non-linear regression of the Monod equation using S-Plus program (Insightful Corporation). The transformation test was completed with a 12 hour period so that cell growth would be limited. An estimated doubling time of the Evanite culture with grown on VC was approximately 60 hours, based observed exponential increases in ethene production reported by Pon et al. (Chapter 3) (21).

Acetylene Inhibition Test

The inhibition experiments consisted of three stages: an initial rate determination stage, an inhibition stage, and a recovery stage. Details were described

in a previous study (Chapter 3)(21). In the first stage, the initial transformation rates of 1,1-CFE and VC were determined in the absence of acetylene inhibition by monitoring their removal and accumulation of their transforming products (FE and ethene). In the second stage sufficient acetylene was added as an inhibitor. Prior to addition, the acetylene was scrubbed with 10% (w/v) $Cu_2SO_4 \cdot 5H_2O$ to remove trace amounts of acetone, as described in Hyman *et al.* (25). In the third stage the reactors were purged with furnace treated nitrogen gas for 15 minutes to remove all the CAHs, and the rates were re-measured upon VC or 1,1-CFE and H_2 addition.

The Transformation-Induction Tests of 1,1-CFE by Three Culture Enrichments

The goal of these tests was to verify that the 1,1-CFE transformation solely tracks the transformation of VC and is not induced by the transformation of TCE and *c*-DCE. The experiments were conducted separately with the three different cultures that were previous described. The preparation of the tested reactors adopted the same procedures of the kinetic tests, except 1,1-CFE was added along with TCE.

The aqueous-phase concentrations and total mass balances of PCE, TCE, *c*-DCE, *c*-DCFE, VC, ethene, 1,1-CFE, and FE were determined by measuring gas-phase concentrations and applying appropriate Henry's law constants. Henry's law constants for PCE, TCE, *c*-DCE, VC and ethene were those published by Gossett (26). The dimensionless Henry's constants for 1,1-CFE and FE were determined at 20 °C, using the successive air-water equilibrations method (27). Equilibrations were made in a 50 ml gastight syringe (Hamilton; Reno, NV) containing a 25 ml aqueous solution

containing 1,1-CFE or FE and an equal volume of nitrogen gas. The syringe was vigorously shaken by hand to establish an equilibrium partitioning between the gas and aqueous phases. The concentration in the gas phase was determined by the GC method described above. The gas phase was expelled by advancing the plunger, and replaced with a same volume of nitrogen gas re-equilibrated using the same procedure describe above and gas phase concentration was measured. The procedure was repeated several times. The Henry's constant was determined by plotting the logarithm of the gas phase concentration versus the number of successive equilibration. The slope of the plot is equal to $\log(\frac{V_w}{H_{cc} * V_g + V_w})$ where V_g is the volume of the gas phase and V_w is the volume of the liquid phase and H_{cc} is the dimensionless Henry's constant.

RESULTS

PCE and TCE Transformation

An initial study was performed to determine the transformation pathway of TCFE. The Evanite culture transformed PCE and TCFE simultaneously when present in the same reactor. The culture transformed PCE through TCE, *c*-DCE, and VC to ethene. TCFE was transformed through *c*-DCFE and 1,1-CFE to FE, as shown in Figures 5.1A and B. PCE was transformed through TCE, with *c*-DCE accumulating during the first 70 days of the incubation (Figure 5.1A). TCFE was transformed to *c*-DCFE over a similar time interval (Figure 5.1B). VC and 1,1-CFE were produced at similar rates from the transformation of *c*-DCE and *c*-DCFE, respectively. The results indicated *c*-DCFE was a good surrogate for *c*-DCE transformation. A similar transforming trend was also observed for the subsequent transformation of VC and 1,1-CFE to ethene and FE, respectively. This initial study demonstrated the fluorinated analogues, *c*-DCFE and 1,1-CFE followed *c*-DCE and VC transformation patterns, respectively.

The transformation correlations of TCE and TCFE are parallel in dechlorination steps as shown in Figure 5.2. The major TCFE transformation pathway is shown by dark arrows. TCFE is potentially transformed to three DCFE isomers (DCFEs), with *c*-DCFE being the main isomer formed. *c*-DCFE is mainly transformed to 1,1-CFE, finally transformed to FE as an end-product. The Evanite culture transformed TCE through *c*-DCE and VC to ethene.

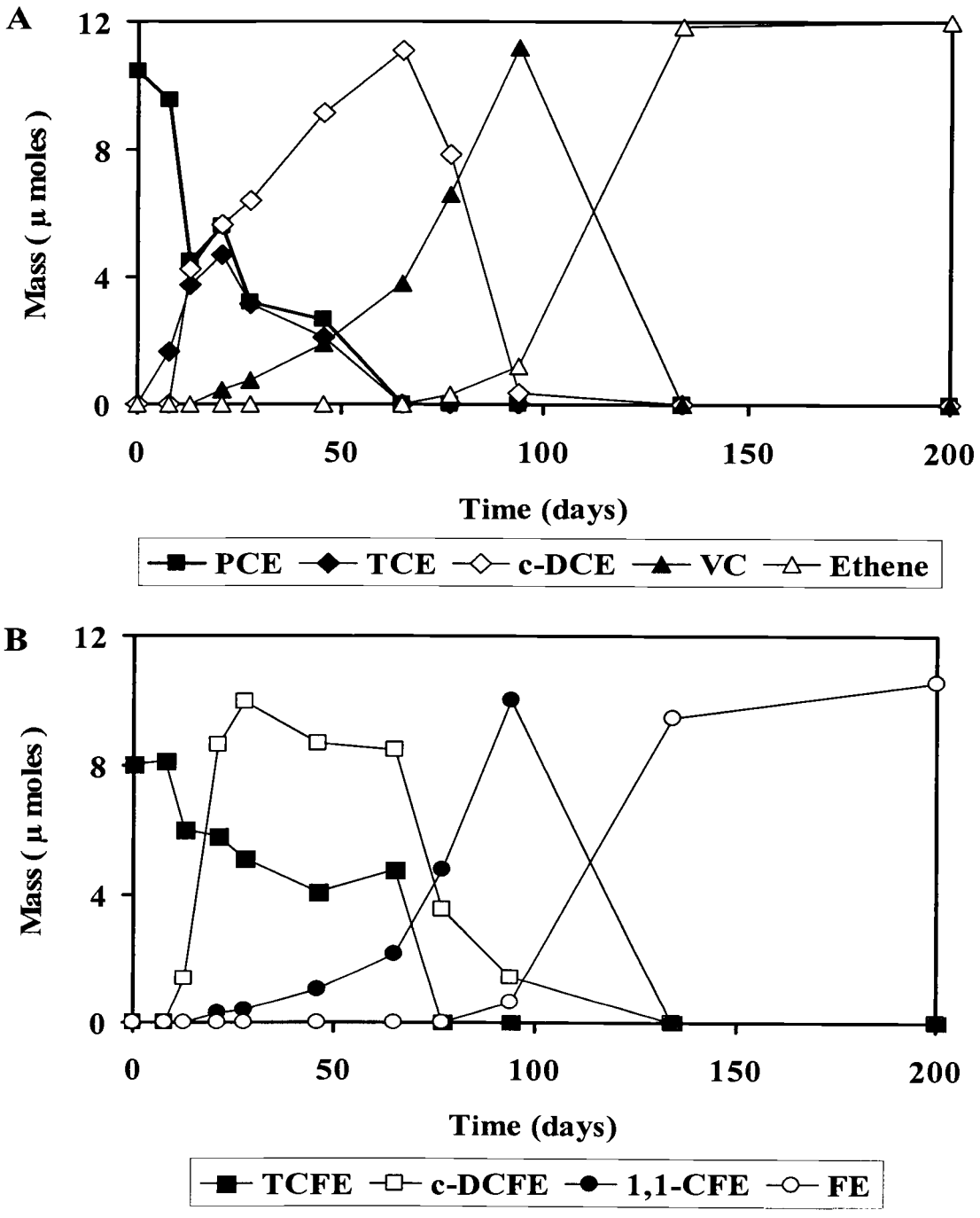

Figure 5.1. Simultaneous transformation of PCE and TCFE performed in a batch reactor by the Evanite enrichment. Results are plotted separately for comparison purposes. The enrichment transformed (A) PCE through TCE, *c*-DCE, and VC to ethene, and (B) TCFE through c-DCFE and 1,1-CFE to FE.

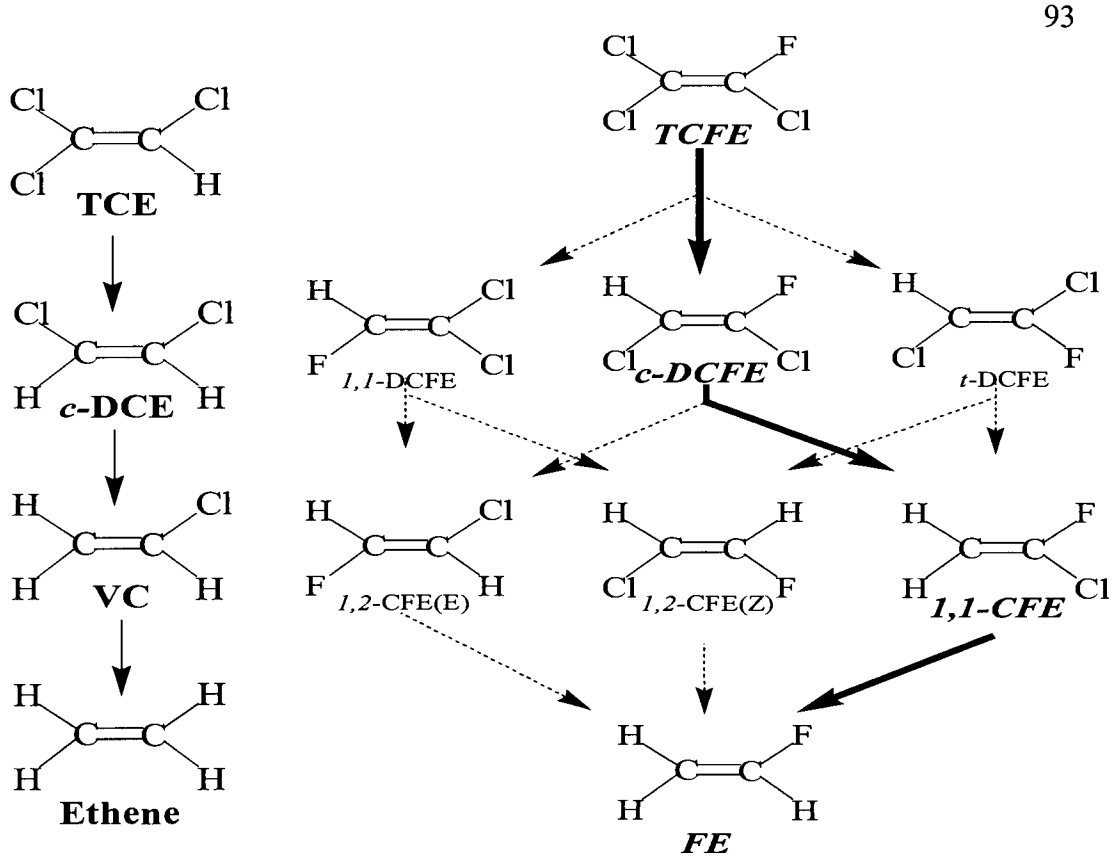

Figure 5.2. Major TCFE and TCE transformation pathways observed with the Evanite culture.

One objective was to determine the major CFE isomer produced from TCFE transformation. In order to evaluate the transformation pathway of TCFE to FE differ isomers of DCFE and CFE had to be identified. Vancheeswaran et al. (20) proposed a transformation pathway for TCFE. Their microcosm study showed incomplete transformation beyond *c*-DCFE that limited the ability accurately to predict the major CFEs intermediate. In our study, TCFE was transformed mainly to *c*-DCFE with some *t*-DCFE (two GC peaks had relative percent areas of 96% and 4%). A peak corresponding 1,1-dichloro-2-fluoroethene (1,1-DCFE) was not observed. *c*-DCFE

further was transformed mainly to 1,1-CFE, base on the GC retention time of 5.0 minute, corresponding to the authentic standard (Figure 5.2). In contrast, a 50/50 standard of 1,2-CFE (E) and 1,2-CFE (Z) had two peaks with retention times of 5.3 and 5.6 minutes, that indicated they were not the major intermediates. A small GC peak was observed at 5.3 minutes, that potentially corresponded to 1,2-CFE(E) or 1,2-CFE (Z). The small peak was likely 1,2-CFE (E), since it is more likely produced from *c*-DCFE transformation due to the location of the fluoride substitute (see Figure 5.2). These fluorinated CAHs were identified by their retention times during GC analysis. The retention times of TCFE, *t*-DCFE, c-DCFE, 1,2-CFE(Z), 1,2-CFE(E), 1,1-CFE, and FE were 8.3, 7.3, 7.1, 5.6, 5.3, 5.0, 2.3, minutes, respectively. The two major intermediates of TCFE transformation were c-DCFE and 1,1-CFE, and the main pathway is linked with bolded arrows, as shown in Figure 5.2.

Kinetic Study for 1,1-CFE and VC

The kinetic studies focused on determining $k_{max}X$ and K_s values for 1,1-CFE and VC. Rates of product formation were measured over wide range of substrate concentrations (0 to 1000 μM) using the multiple equilibration method. The 1,1-CFE test presented in Figure 5.3A shows the successive increase in 1,1-CFE concentration, and FE concentration data used for rate determinations. Substrates concentrations were successively increased until rates close to the maximum transformation rate were reached. The resulting Monod plot for 1,1-CFE is shown in Figure 5.3B along with the

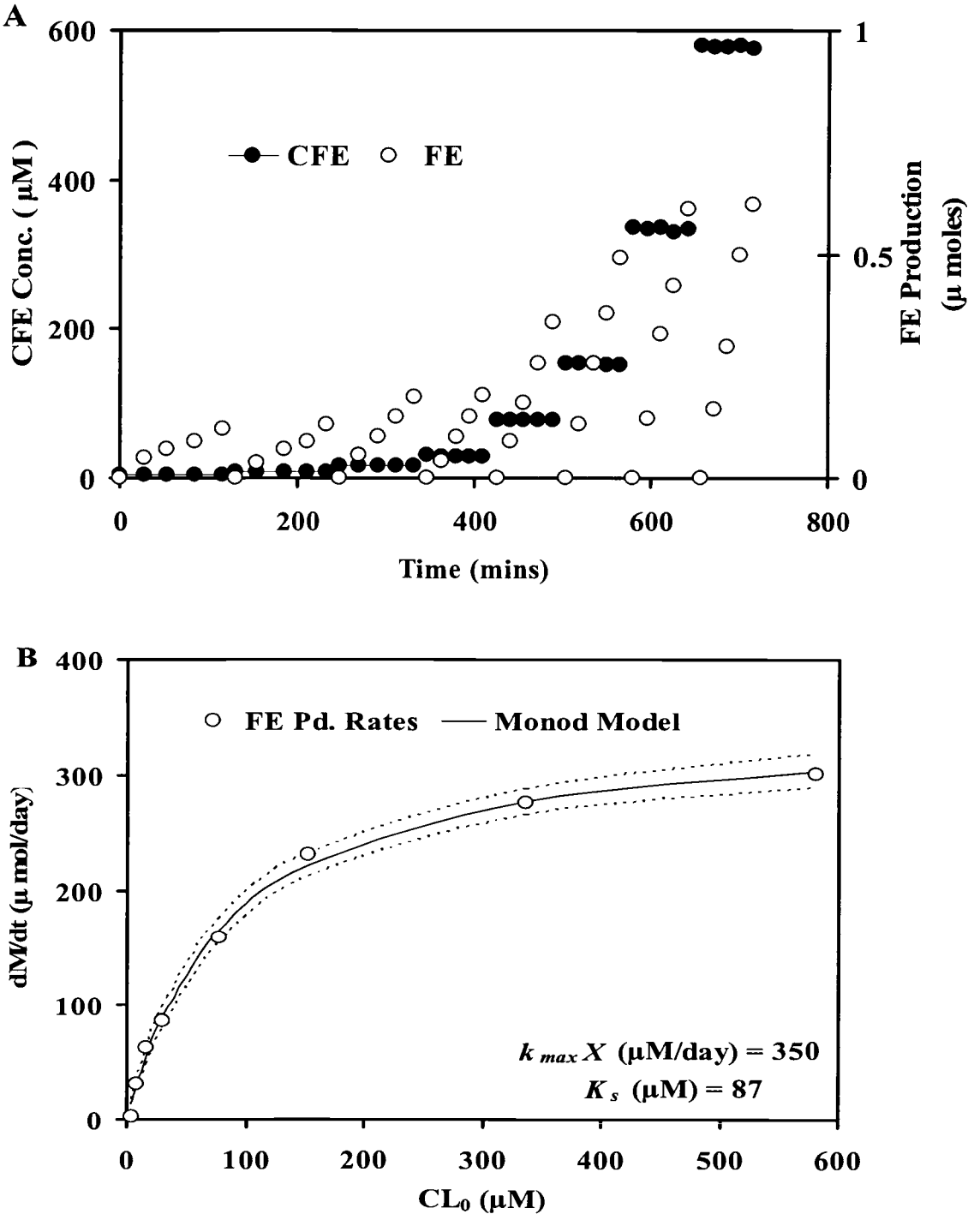

Figure 5.3. (A) FE production rates at various 1,1-CFE concentrations using the multiple equilibration method. The FE production rates were determined by linear regression, immediately after the successive addition of 1,1-CFE. (B) Monod curved used to determine K_s and $k_{max}X$ for 1,1-CFE by non-linear regression.

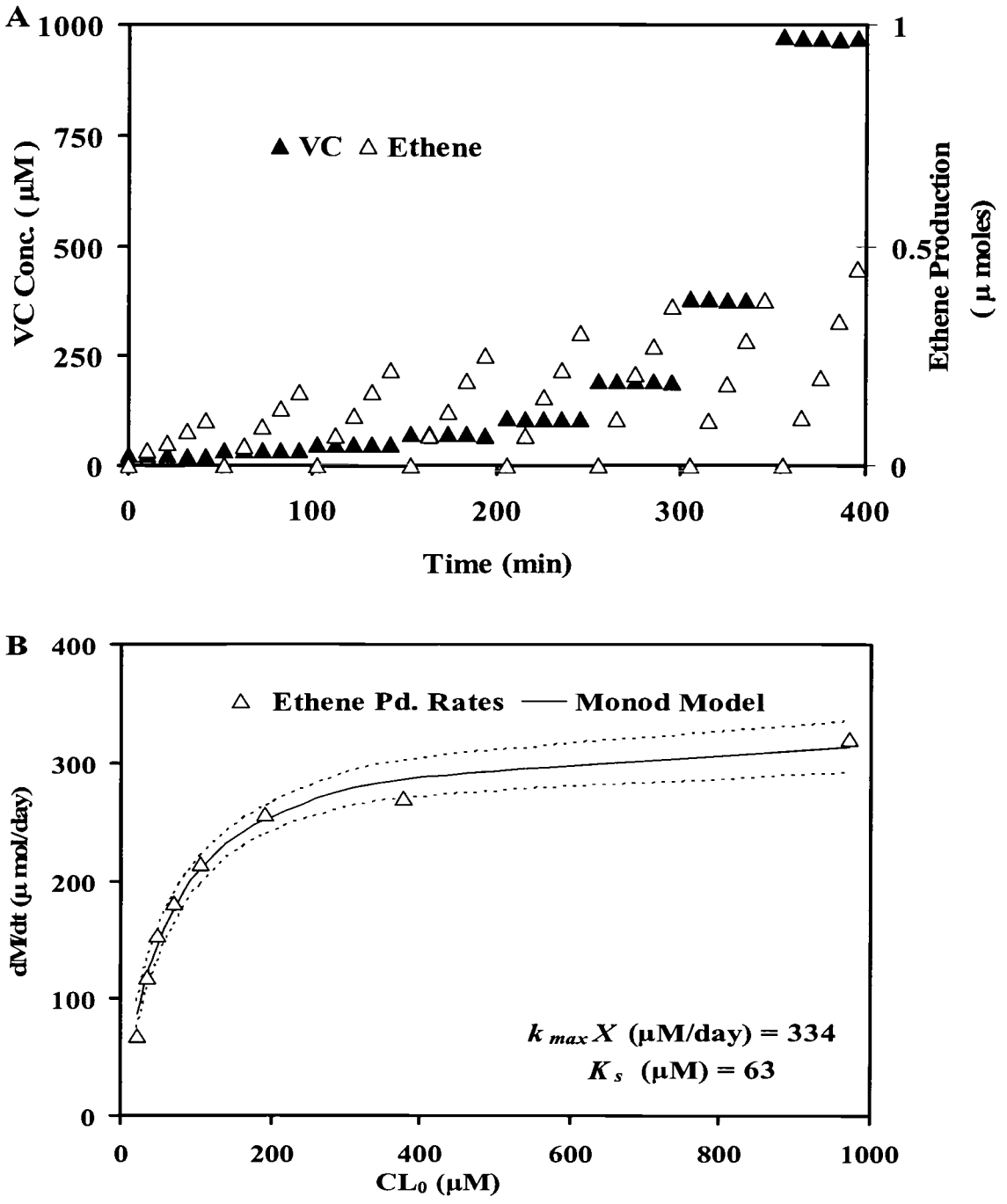

Figure 5.4. (A) Ethene production rates at various VC concentrations using the multiple equilibration method. The ethene production rates were determined by linear regression, immediately after the successive addition of VC. (B) Monod curved used to determine K_s and $k_{max}X$ for VC by non-linear regression.

non-linear regression fit to the Monod equation and the 95% confidence intervals. The regression yields estimates of $k_{max}X$ and K_s with low standard deviations of about 3% and 10%, respectively of the estimated values. The $k_{max}X$ and K_s were 350 ± 10 μM per day and 87 ± 8 μM, respectively, for the 1,1-CFE transformation. The same procedure was used to determine the $k_{max}X$ and K_s values for VC transformation (Figure 5.4A and B), with estimated values of 334 ±11 μM per day and 63 ±7 μM, respectively. The results showed 1,1-CFE and VC had similar $k_{max}X$ and K_s values and therefore similar transformation rates.

VC and acetylene inhibition studies on the 1,1-CFE transformation

Experiments were performed to study inhibition between VC and 1,1-CFE and how both compounds responded to acetylene as an inhibitor. Varying amounts of VC were used as an inhibitor of 1,1-CFE transformation. Acetylene inhibition was used to provide addition evidence that VC and 1,1-CFE transformation likely shared the same enzymatic pathway. For initial transformation rate measurements, all reactors were given the same amount of 1,1-CFE. Different amounts of VC or 1,1-CFE were then added to perturb the VC and 1,1-CFE aqueous concentration ratios (Table 5.1). 1,1-CFE was completely transformed to FE in a non-inhibited control reactor (data not shown).

Figure 5A shows fairly constant rate of FE production until VC was added at 85 hours. VC addition inhibited the 1,1-CFE transformation, as indicated by the

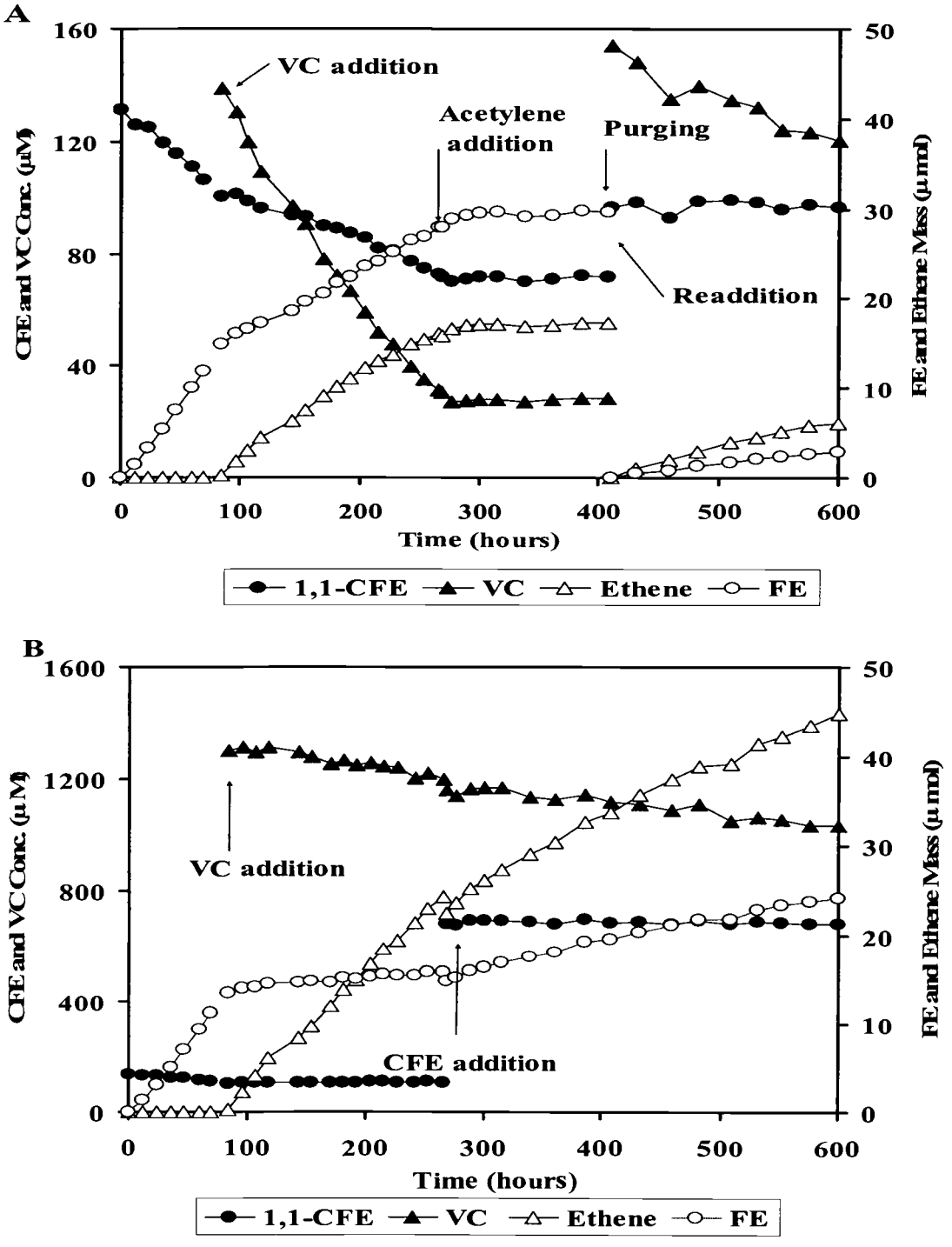

Figure 5.5. (A) VC inhibition of FE transformation and acetylene (24 µM) inhibition on 1,1-CFE and VC transformation (The initial VC/1,1-CFE aqueous concentration ratio was 1:1). (B) Ethene/FE production at VC/1,1-CFE aqueous concentration ratios of VC/CFE concentration ratios 12:1 and 2:1.

decrease in FE production rate. Ethene and FE were produced at similar rates over the period of 85 to 270 hours, as would be expected based on the measure kinetic values and the aqueous concentrations, potentially by a competitive inhibition kinetics, as will be discussed. When acetylene was added at 270 hours, both VC and CFE transformation were inhibited, as indicated by the cessation of FE and ethene formation. The acetylene aqueous concentration of 24 μM was twice that used in previous studies to completely block the VC transformation by the Evanite culture (Chapter 3) (21). Inhibition was removed by purging the acetylene with anaerobically treated nitrogen gas at 410 hours. VC and 1,1-CFE transformation to ethene and FE were then observed upon their re-addition. The rates upon recovery were about a third of the rates observed prior to acetylene inhibition (Figure 5.5A and Table 5.1).

The second batch reactor was subjected to two different levels of VC inhibition on 1,1-CFE transformation. Aqueous concentration ratios of VC to 1,1-CFE of 12 to 1 and 2 to 1, were used to demonstrate inhibition between these two substrates. Aqueous concentrations for these tests were higher than the measured K_s values. 1,1-CFE was initially added and FE production rate was measured prior to the addition of VC (Figure 5.5B). VC was then added to the reactor to achieve a 12 to 1 (VC: 1,1-CFE) aqueous concentration ratio. After the addition of VC, the FE production rate decreased dramatically, and the ethene production rate from VC transformation was 11 times higher than the rate of FE production (Table 5.1). At 269 hours, 1,1-CFE was added to achieve a VC to 1,1-CFE concentration ratios of 2 to 1. This resulted in an increase in FE production rate and a decrease in ethene production.

The rates of ethene and FE production from VC and 1,1-CFE transformation were estimated for the different stages of the experiments show in Figure 5.5A and 5.5B. The analysis assumed competition for the same enzyme and used a competitive inhibition model. Using the previous determined K_s values for VC and 1,1-CFE, the ethene and FE production rates were predicted using a competitive inhibition model (28) shown in the following equation:

$$\frac{d[C_{substrate}]}{dt} = \frac{k_{\max} X * C_{substrate}}{C_{substrate} + K_{S(substrate)} + \frac{K_{S(substrate)}}{K_{S(Inhibitor)}} * C_{Inhibitor}} * V_L \qquad (5.1)$$

The maximum transformation rate for the estimates ($k_{max}X$) was determined from the initial 85 hours of FE production (Figure 5.5A and B) in the absence of VC as an inhibitor. Both VC and 1,1-CFE production rates are a function of substrate and inhibitor concentrations. VC and 1,1-CFE were complimentary substrate and inhibitor pairs as defined in equation 1. The inhibition was based on the ratio of the measured K_s values as proposed by Haston (29).

The measured and predicted rates of ethene and FE production and ratio of ethene and FE production rates (measured / predicted) are presented in Table 5.1. There is good agreement between the measured and predicted ratios. The changes in production rates are shown to be correlated to the aqueous concentrations, as expected in a competitive inhibition model. The results indicate VC and1,1,1-CFE transformation

Table 5.1. Comparison of measured and predicted rates of 1,1-CFE and VC transformations for results presented in Figure 5. 5.

Initial VC Cl_{iq} (μM)	Initial CFE Cl_{iq} (μM)	Ethene Production Rate Measured/Predicted (μ moles/hr)	FE Production Rate Measured/Predicted (μ moles/hr)	Ethene/FE Production Rate Ratio Measured/Predicted
139	101	0.091/0.127	0.082/0.067	1.1/1.9
154	96.6	*0.033/0.140	*0.015/0.061	2.2/2.3
1300	103	0.132/0.227	0.012/0.012	11.0/18.9
1160	680	0.075/0.170	0.032/0.073	2.3/2.3

$k_{max}X$ is (5.01 μ moles/hr/L) estimated from the initial 85 hours of FE production from Figure 5 A and B.

*rate measured after the acetylene inhibition test.

rates could be predicted reasonable well using the measured K_s values in a competitive inhibition model. Differences in predicted and measured rates could result from culture growth and decay during the course of the experiment. The ratios however of measured to predicted rates, which would normalize of growth and decay, are in very good agreement. Note the greatest difference between measured and predicted rates for both ethene (0.033 μmole/hr) and FE (0.015 μmole/hr) occurred after the acetylene inhibition test.

Acetylene inhibited both VC and 1,1-CFE transformation and the inhibitions were partly reversible after acetylene was removed (Figure 5.5A). Both VC and 1,1-CFE transformation rates recovered to only 36 to 18 % of the rate achieve prior to acetylene inhibition. The reason for the loss of activity is not known. Microbial decay over the 125 hours (5.2 days), without electron acceptor utilization is one potential factor causing a loss in activity. Acetylene toxic on the microbial community could be another factor.

1,1-CFE transformation studies with three different enrichment cultures

Experiments performed with the three different cultures further explored 1,1-CFE as a reactive surrogate that tracks the rate of VC transformation. The experiments were conducted in batch reactors to which 1,1-CFE was added along with TCE to determine the step of TCE transformation at which 1,1-CFE was transformed.

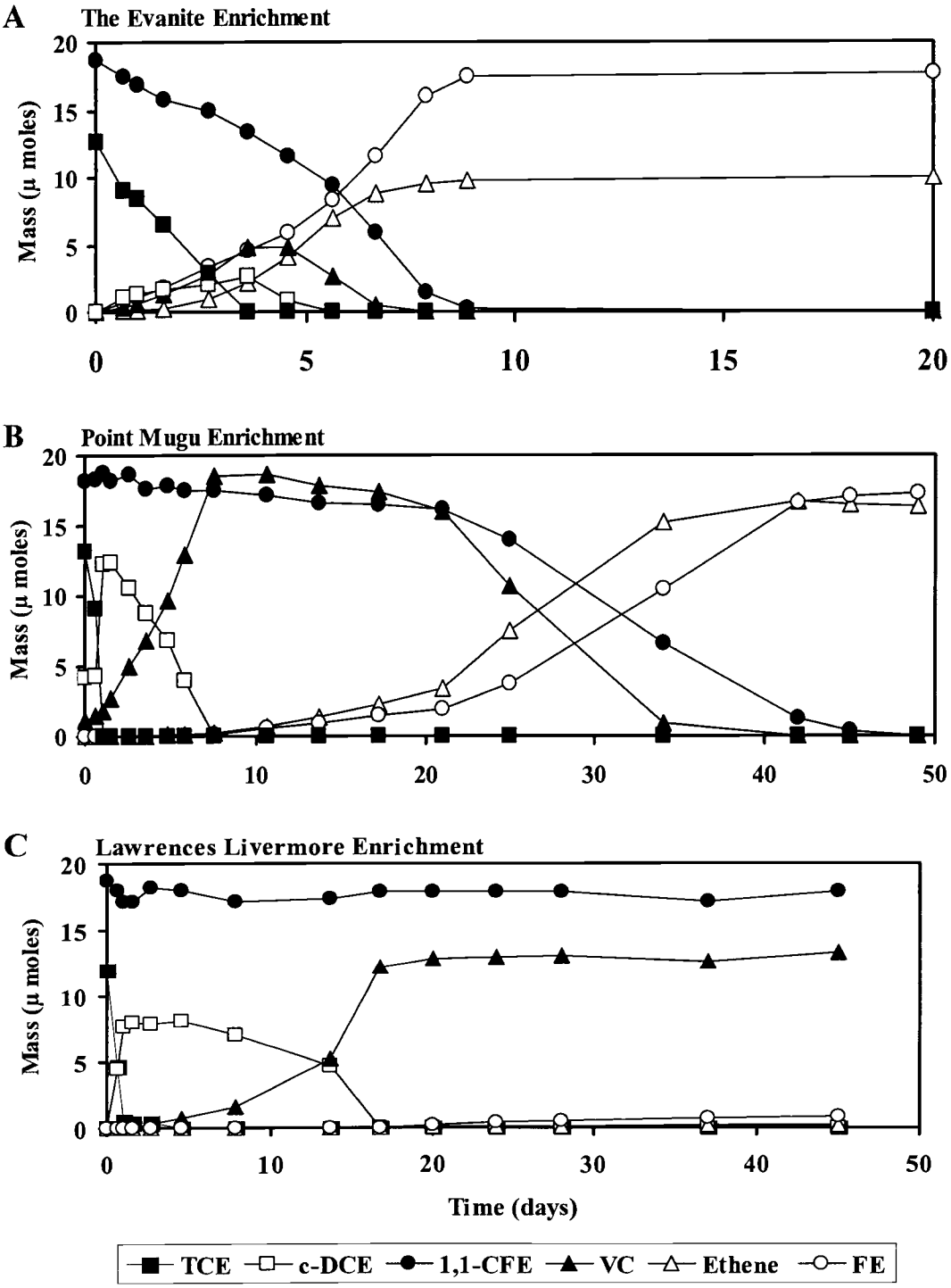

Figure 5.6. 1,1-CFE transformation in batch reactors that are transforming TCE to VC and ethene. The enrichment cultures include the Evanite (A), the Point Mugu (B), and the Lawrence Livermore (C).

With the Evanite culture, 1,1-CFE and VC were both transformed in the presence of TCE and *c*-DCE. Ethene and FE were produced at similar rates, and essentially complete conversion of 1,1-CFE and VC was achieved within 10 days (Figure 5.6A). Results are consistent with those shown in Figure 5.1, where TCFE and PCE were initially present. The Pt. Mugu culture results are shown in Figure 5.6B. Both VC and 1,1-CFE transformation occurred after the *c*-DCE concentration was reduced to a low level. 1,1-CFE transformation tracked both the initial slow and then later accelerated rates of VC transformation. The correlation of 1,1-CFE with VC transform was further demonstrated with the Lawrence Livermore culture, as shown in Figure 5.6C. The culture transformed TCE through *c*-DCE to VC. 1,1-CFE was very slowly transformed to FE while VC was very slowly transformed to ethene. In this case CFE tracked the very slow transformation of VC to ethene. The three cultures showed 1,1-CFE tracked VC transformation well in the presence of higher chlorinated CAHs, despite the different transformation abilities of the cultures.

DISCUSSION

The Evanite culture transformed TCFE to *c*-DCFE and *t*-DCFE, with *c*-DCFE being the main isomer formed. *c*-DCFE was then transformed to 1,1-CFE. The initial transformation step is consistent with that observed by Vancheeswaran et al. in a groundwater microcosm study (20). However, there was a difference in the subsequent transformation step. The Evanite culture transformed *c*-DCFE to 1,1-CFE as the major

intermediate. In the study of Vancheeswaran et al., *c*-DCFE was transformed to 1,2-CFE(E) as the main intermediate, when TCFE transformation essentially stopped at *c*-DCFE with a trace amount of CFEs formed. Thus the major CFE intermediate was determined based very limited formation of the CFE isomers. Our studies showed the Evanite culture completely transformed TCFE to FE.

The FE was not defluorinated to ethene by the Evanite culture. The transformation pathway shows the fluorinate substitute acts more like a hydrogen substitute than a chloride substitute. This might result from combined effect of it being poorer leaving group than chloride, and its size being smaller than chloride. Thus the CFE dechlorination step is associated with the VC step, rather than the DCE dechlorination step, as indicated in Figures 5.1 and 5.6.

This study used a single batch reactor multiple equilibration method to obtain more precise kinetic parameters than previous studies. The K_s and $k_{max}X$ determined by non-linear regression of the Monod curves had standard deviations of less than 20% of the measured values. Previous studies using the traditional multi-reactor method resulted in large standard deviations of approximated 50 % of K_s value and 20% of the $k_{max}X$ values (4, 29). The multi-reactors method assume all the reactors having same initial conditions, which for sensitive redox VC dehalogenation reactions, may be hard to achieve. The multiple equilibration method provides a simple and more precise approach to obtain kinetic parameters, and is less labor intensive than the traditional method.

VC and 1,1-CFE had very similar K_s and $k_{max}X$ values, indicating VC and 1,1-CFE E were transformed at similar rates by the Evanite culture. The kinetics values support the same enzymatic pathway for both compounds. The transformation of both compounds sharing the same enzymatic pathway is also supported by data shown in Figure 5.5, that shows VC inhibiting 1,1-CFE transformation. Predicted rates and measured rates matched well when the measured K_s values were used in the competitive inhibition model, for different VC and 1,1-CFE concentrations (Figure 5.5B). The ratios of the K_s values modeled the competitive inhibition well, consistent with earlier reports of Haston (29) for *c*-DCE on VC transformation, providing addition evidence the two compound likely shares the same transformation mechanism (4).

Acetylene was previously reported to be a reversible inhibitor of VC transformation by the Evanite culture (Chapter 3) (21). Acetylene inhibited both VC and 1,1-CFE transformation to similar extends and the transformation rate partially recovered upon removal of acetylene (Figure 5.5A). The inhibition by acetylene provide addition evidence that the VC and 1,1-CFE transformation likely share a common enzymatic pathway.

1,1-CFE transformation tracked well VC transformation with three different cultures. No false-positive results were observed, where 1,1-CFE was transformed when VC was not. The results suggest 1,1-CFE is an excellent surrogate for determine rates and extents of VC transformation.

FE was a stable product of 1,1-CFE transformation, within our ability to measure FE concentration or ethene production. Thus, FE production rates can be used to estimate 1,1-CFE transformation rates. Rates of VC transformation can be estimated from the known kinetic parameters of VC and 1,1-CFE for the Evanite culture and reactive concentrations of VC and 1,1-CFE. VC transformation rates observed upon its addition to batch reactors could be estimated from 1,1-CFE rates as illustrated in Figure 5.5 and Table 5.1. The results illustrate the importance of both 1,1-CFE and VC concentration when estimating transformation rates in field studies when 1,1-CFE is added as a surrogate.

Vinyl bromide (VB) has also been evaluated as an indicator of VC transformation potential (30). In these studies VB reduction to ethene resulted of a bromide ion release which might also tracked the VC transformation. Vinyl bromide transformation was much more rapid than VC transformation, and is potentially is a tool rapidly screen for VC transformation potential. However, 1,1-CFE transformation rate more closely tracks those of VC when they are present at similar aqueous concentrations. Thus there is potential for using VB to rapidly screen for VC transformation potential, and for 1,1-CFE to help quantify VC transformation rates.

1,1-CFE was demonstrated here to be an excellent surrogate to track VC transformation and to estimate rates of intrinsic or enhanced in situ transformation. Thus 1,1-CFE could be added in field studies, such as push-pull tests described by Hageman *et al.* (19) to help determine rates of VC transformation.

ACKNOWLEDGEMENTS

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CHAPTER 6

Engineering Significance and Conclusions

ENGINEERING SIGNIFICANCE

CAHs are often present as dense non aqueous phase liquids (DNAPLs) in many groundwater aquifers. These DNAPL phases can slowly dissolve creating large dissolved contaminant plumes. Pump-and-treat remediation is considered effective for removing contaminants at high concentration levels. However slow dissolution of the DNAPLs and sorbed contaminants result in prolonged and expensive treatment to reach low concentration levels. Natural attenuation and enhanced bioremediation, therefore, have become attractive methods for site remediation.

Methods are needed for evaluating whether biological transformations are occurring in-situ and at what rates. This study focused on developing biological probes which can help in evaluating the biological activities of reductive dechlorination and the rates of VC transformation. Through the combination of kinetic and modeling studies the reductive dechlorination processes can be identified and the rates of transformation can be estimated. Thus, engineering strategies can be developed for long-term and low cost bioremediation.

Batch kinetic studies were performed with an enrichment culture obtained from the Evanite Site in Corvallis, OR. The culture is capable of completely

transforming TCE through *c*-DCE and VC to ethene. This study focused on VC transformation because it is a known carcinogen and the slowest to biotransform of the chlorinated ethenes. Since VC transformation is slow, it accumulates in groundwater. TCE transformation was studied for comparison purposes. Inhibition, kinetic, and modeling studies presented in this dissertation used inhibitors and surrogate compounds to evaluate the reductive dechlorination of TCE and VC. Inhibitors have great potential for evaluating biological activities of reductive transformations. Surrogate compounds have potential for estimating rates of transformation.

Acetylene was found to be a reversible inhibitor and mechanistic probe for evaluating the activities of TCE and VC transformations. Applying acetylene inhibition provides indirect evidence of the biotransformation then the activities were recovered after the removal of the inhibitor. Acetylene is also a useful inhibitor, since it is a non-regulated chemical and regulatory approval may be obtained for its use in field studies. In addition, since acetylene is a gaseous chemical, it can be easily applied and removed by purging, unlike 2-bromoethane-sulfonate (BES), which is typically used to inhibit methanogenesis but is not volatile. For example, acetylene might be used in bench scale microcosm studies and single well push-pull field tests. As an inhibitor it can be added to the subsurface to inhibit transformations. This might result in the build-up in parent compounds and a decrease in daughter compound concentrations at a location.

1,1-CFE was found to be an excellent reactive tracer to quantify the anaerobic VC transformation. The advantage of using a transformation analogue is that a unique

analytical signature for in-situ biotransformation is possible when background concentrations of the CAHs of interest are present. It is often difficult to demonstrate transformation is occurring when other complicated physical processes, such as sorption and desorption of the contaminant and nonaqueous phase liquid (NAPL) dissolution are occurring.

Kinetic studies evaluated kinetic parameters (K_s and $k_{max}X$) for TCE, VC, and 1,1-CFE transformation. Acetylene inhibition constants (K_I) for models of acetylene inhibition on TCE and VC were determined. Inhibition between VC and 1,1-CFE was also evaluated. The competitive inhibition model best fit these experimental data using independently determined K_s values and fitted inhibition constants.

This study developed methods for use in evaluating the potential for groundwater remediation via anaerobic treatment. If acetylene and 1,1-CFE are allowed to be added to contaminated groundwater, tests can be designed to determine if intrinsic remediation is occurring and the rate of intrinsic and enhance remediation.

CONCLUSIONS

The inhibition, kinetic, and modeling studies presented in this dissertation used acetylene as an inhibitor to evaluate reductive dechlorination of TCE and VC. Acetylene was found to be a reversible inhibitor of reductive dechlorination of TCE and VC, and the inhibition was proportional to the acetylene concentration. Acetylene also inhibited methanogenesis, but not acetogenesis. Acetylene being a reversible

inhibitor allows the biological activities being investigated to be switched on and off, which is helpful when studying transformation mechanisms.

Kinetic and modeling studies were also used to evaluate acetylene inhibition of TCE and VC transformation. A competitive inhibition model better fit the transient experimental data than the uncompetitive inhibition model. Non-linear regression methods were used to determine kinetic parameters (K_s and $k_{max}X$) for TCE and VC, and the acetylene inhibition constants (K_{IC} and K_{IU}). Since the inhibition appears to be competitive in nature the result suggest acetylene might inhibit the active site of the dehalogenation reaction.

1,1-CFE was also shown to be an excellent reactive tracer for VC transformation. Kinetic studies quantified the kinetic parameters for VC and 1,1-CFE transformation. The K_s values were 63 and 87 μM for VC and 1,1-CFE, respectively, while similar maximum utilization rates ($k_{max}X$), of 334 and 350 μM/day were obtained. Acetylene inhibition studies also indicated that VC and 1,1-CFE were likely transformed by the same enzyme. A competitive inhibition model with the independently measured K_s values used as the inhibition constants predicted rates of transformation of both VC and 1,1-CFE reasonable well when both compounds were present in batch kinetic tests.

The following conclusions can be drawn from this work:

1. Acetylene was found to be a reversible inhibitor of VC, *c*-DCE and TCE transformations.
2. Acetylene inhibited H_2 consumption associated with reductive dechlorination and methanogenesis, but not acetogenesis.
3. VC transformation was more susceptible to acetylene inhibition than the methane production: 12 μM of acetylene significantly inhibited VC transformation, while 48 μM of acetylene was required to inhibit methane production.
4. 192 μM of acetylene achieved 90% inhibition of TCE; the required concentration was 16 time higher than that for VC.
5. The acetylene (K_{IC}) inhibition constants for TCE and VC based on the independently measured K_s values and a competitive inhibition model were essentially same value (0.4 μM).
6. Uncompetitive modeling of acetylene inhibition yielded constants for transformation steps of, TCE to *c*-DCE, TCE to 1,1-DCE, and VC to ethene of 13.3, 14.1 and 2.2, respectively.
7. Competitive and uncompetitive models fit equally well inhibition experiments conducted at high TCE and VC concentrations near 400 μM.

8. The competitive inhibition model fit transient experimental data better than the uncompetitive inhibition model, conducted at TCE and VC concentrations near their respective K_s values

9. TCFE was transformed to three DCFE isomers (DCFEs), with *c*-DCFE being the main isomer formed. *c*-DCFE was transformed mainly to 1,1-CFE, with FE as an end-product.

10. Ratios of Ethene and FE production rates were proportional to the aqueous concentration of VC and 1,1-CFE, and the transformation rates could be predicted through competitive inhibition modeling using K_s values as inhibition constants.

11. Acetylene inhibited both VC and 1,1-CFE transformation indicating that the transformations were likely carried out by the same reductase enzyme

12. The K_s and $k_{max}X$ values for VC, and 1,1-CFE were 63 and 87 μM and 334 and 350 μM/day, respectively, indicating the same reductase enzyme was responsible for their transformation.

13. The multiple equilibration method provides a simple and precise means of determining $k_{max}X$ and K_s values, and was less labor intensive than the traditional method.

14. With three different cultures 1,1-CFE transformation tracked well VC transformation, indicating 1,1-CFE is an excellent surrogate for estimating rates and extents of VC transformation.

FUTURE WORK

Future studies should investigate the mechanism of acetylene inhibition using a pure culture or a purified enzyme. Our inhibition studies showed that acetylene competitively inhibited TCE and VC transformation. More detailed kinetic studies are needed to determine the type of acetylene inhibition on these transformations. The inhibition of other CAH transformations, including PCE and *c*-DCE, is also of interest.

Studies should also investigate why the fluoride substitution acts similar to a hydrogen substitution in the sequential transformations. We observed that 1,1-CFE transformation rates were similar to those of VC. It is also of interest to perform detailed kinetic studies to determine whether TCFE is transformed at rates similar to PCE or TCE. Additional kinetic studies of acetylene inhibition on fluorinated analogues, such as TCFE and 1,1-CFE, are necessary to evaluate the in-situ TCE and VC transformation.

Since acetylene and 1,1-CFE are gaseous chemicals, they can be easily removed through purging. On the other hand, 2-bromoethane-sulfonate (BES) is not volatile and thus can not be easily removed. Since acetylene is a natural gas, regulatory approval might be obtained for its use in field studies to evaluate intrinsic or enhanced anaerobic dehalogenation, such as the push-pull tests described by Hageman *et al.* (1). Since it is an inhibitor, it can be added to the subsurface to inhibit

1,1-CFE or TCFE transformations. It might also enable the evaluation of in-situ VC or TCE transformation, by inhibiting their transformations, and upon removal from the system permitting the reactions to proceed.

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APPENDIX: Equation Coefficient Nomenclature

Symbols	Definitions	Units
H_{CI}	Henny's constant of inhibitor	unitless
H_{CS}	Henny's constant of inhibitor	unitless
I_L	inhibitor liquid concentration	μM
K_I	inhibition constant	μM
K_{IC}	competitive inhibition constant	μM
K_{IU}	uncompetitive inhibition constant	μM
K_s	half-velocity coefficient	μM
kmax	maximum utilization rate	μmoles/mg/day
M_I	mass of inhibitor	μmoles
M_S	mass of substrate	μmoles
v	substrate degradation rate	μmoles/hr
S_L	substrate liquid concentration	μM
t	time	sec., min., day
V_G	volume of gas	L
V_L	volume of liquid	L
X	biomass of cell	mg/L
α_I	conversion factor of inhibitor mass to liquid concentration	L