Kinetics of Reductive Dechlorination of Trichloroethane (TCA) by Anaerobic Biofilms

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by

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and

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U.S. Environmental Protection Agency

January 1995
Printed on recycled/recyclable paper
Kinetics of Reductive Dechlorination of Trichloroethane (TCA) by Anaerobic Biofilms

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Prepared for
Hazardous Waste Research and Information Center
One East Hazelwood Drive
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HWRIC Project Number HWR 90-079

Printed by Authority of the State of Illinois
95/250
This report is part of HWRIC's Research Report Series. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

All research for this project was conducted at the University of Illinois at Urbana-Champaign.
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ABSTRACT

Reductive dechlorination, in which cleavage of carbon-halogen bonds is accompanied by electron transfer to the carbon molecule, is a critical mechanism for the anaerobic biodegradation of highly chlorinated aliphatic hydrocarbons. The concentrations of primary electron-donor and -acceptor substrates, which control the intracellular availability of electrons, can affect the rate of dechlorination reactions. A mechanism-based model that describes the effects of primary electron donors and acceptors on the kinetics of reductive dechlorination was developed and tested with 1,1,1-trichloroethane (TCA) and anaerobic biofilm reactors. The model is based upon the hypothesis that the rate of reductive dechlorination is controlled by the intracellular concentration of a reduced metalloenzyme, whose concentration is controlled by the external concentrations of electron donors and acceptors.

Experimental results with methanogenic and sulfate-reducing biofilms confirmed the predictions of the mechanistic model. First, Monod kinetics represented the rate of TCA degradation when concentrations of the primary donor and acceptor were constant. Second and most importantly, the Monod kinetic parameters depended on the concentrations of the primary substrates. The apparent maximum specific rate of TCA biodegradation, \( q_{\text{in,ap}} \), and the half-maximum-rate concentration, \( K_{\text{ap}} \), increased as the concentration of the primary electron donor increased. The primary electron-acceptor substrate increased \( K_{\text{ap}} \), but had no effect on \( q_{\text{ap,ap}} \). These results provide a quantitative tool for controlling the rate of reductive dechlorination in biological treatment of reactors and \textit{in situ} bioremediation.
CHAPTER 1 INTRODUCTION

In order to assess and control the risk of organic contaminants to the health of humans and the environment, the physical, chemical, and biological processes that affect their fate and transport must be quantified. This report addresses key factors that affect the kinetics of anaerobic biotransformation of halogenated aliphatic hydrocarbons. Specifically, this research focuses on the interactions that occur among primary electron-donor, primary electron-acceptor, and halogenated-aliphatic substrates during their anaerobic biodegradation. Primary electron-donor and acceptor substrates are the compounds whose utilization supports microbial growth (Namkung and Rittmann, 1987). Halogenated aliphatic hydrocarbons are among the most ubiquitous xenobiotic contaminants of groundwaters, surface waters, and wastewaters.

The importance of halogenated aliphatic hydrocarbons as pollutants is due, in large part, to their importance in industry and agriculture. Halogenated aliphatics, such as carbon tetrachloride (CCl₄), chloroform (CHCl₃), 1,1,1-trichloroethane (TCA), and trichloroethene (TCE), are used industrially in large volumes for many purposes. They are used as solvents, degreasers, refrigerants and propellants, and in dry cleaning and chemical and plastics manufacturing (Windholtz et al., 1976, OTA, 1984). Agriculturally, halogenated aliphatics have been used as insecticides, nematocides, and fungicides (OTA, 1984). Compounds that have been used in agriculture include hexachlorocyclohexane (Lindane), 1,2-dibromoethane (EDB), and dibromochloropropane (DBCP).

This large industrial and agricultural demand results in the production of huge quantities of halogenated aliphatic hydrocarbons. Eight of the top fifty organic chemicals produced in the United States are halogenated aliphatics. Over the past decade, the production of five of these compounds has increased at an annual rate of at least 2% per year, but the production of the other three (CCl₄, dichloromethane, and tetrachloroethene) has decreased dramatically.

Halogenated aliphatic hydrocarbons often enter the environment as a result of accidental spills or improper disposal (Zoeteman, 1985). Many of these are stable, non-reactive compounds that persist when released into the environment. The half-lives for abiotic disappearance of the most common halogenated aliphatics range from 0.5 to 7,000 years (Vogel et al., 1987). Because abiotic reactions of these compounds are slow, biodegradation can be an important mechanism through which they are removed from the environment.

Their large annual production rates and their persistence in the environment place halogenated aliphatic hydrocarbons among the most common organic pollutants in natural waters (Zoeteman, 1985; Schwarzenbach and Giger, 1985; Macalady et al., 1986). In fact, of the thirty-three organic compounds that were found most commonly in drinking water wells, eighteen were halogenated aliphatics (CEQ, 1981). The eighteen halogenated aliphatics that contaminate drinking water wells most commonly and the concentration ranges that have been reported for them in groundwater and drinking water supplies are listed in Table 111.
Table 1.1 Halogenated Aliphatic Compounds that are Detected Most Commonly in Drinking Water Wells (CEQ, 1981, OTA, 1984)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>Concentration Range (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>trichloroethene</td>
<td>TCE</td>
<td>210 - 37,000</td>
</tr>
<tr>
<td>1,1,1-trichloroethane</td>
<td>1,1,1-TCA</td>
<td>0.2 - 26,000</td>
</tr>
<tr>
<td>methylene chloride</td>
<td>CH₂Cl₂</td>
<td>4 - 8,400</td>
</tr>
<tr>
<td>tetrachloroethene</td>
<td>PCE</td>
<td>717 - 2,405</td>
</tr>
<tr>
<td>chloroform</td>
<td>CHCl₃</td>
<td>1.4 - 1,890</td>
</tr>
<tr>
<td>carbon tetrachloride</td>
<td>CCl₄</td>
<td>0.3 - 18,700</td>
</tr>
<tr>
<td>1,2-dichloroethene</td>
<td>1,2-DCE</td>
<td>0.2 - 323</td>
</tr>
<tr>
<td>1,2-dibromoethane</td>
<td>1,2-DBA (EDB)</td>
<td>35 - 300</td>
</tr>
<tr>
<td>1,1-dichloroethene</td>
<td>1,1-DCE</td>
<td>1.2 - 4,000</td>
</tr>
<tr>
<td>1,2-dichloroethane</td>
<td>1,2-DCA</td>
<td>250 - 847</td>
</tr>
<tr>
<td>dibromochloropropane</td>
<td>DBCP</td>
<td>1 - 37</td>
</tr>
<tr>
<td>trifluorotrichloroethane</td>
<td>F₃C-CCl₃</td>
<td>35 - 135</td>
</tr>
<tr>
<td>dibromochloromethane</td>
<td>CHBr₂Cl</td>
<td>2.1 - 55</td>
</tr>
<tr>
<td>vinyl chloride</td>
<td>VC</td>
<td>50 - 740</td>
</tr>
<tr>
<td>chloromethane</td>
<td>CH₃Cl</td>
<td>44</td>
</tr>
<tr>
<td>1,1,2-trichloroethane</td>
<td>1,1,2-TCA</td>
<td>0.2 - 26,000</td>
</tr>
<tr>
<td>bromoform</td>
<td>CHBr₃</td>
<td>2.4 - 110</td>
</tr>
<tr>
<td>1,1-dichloroethane</td>
<td>1,1-DCA</td>
<td>0.5 - 1,330</td>
</tr>
</tbody>
</table>

This research focuses on anaerobic biodegradation of the halogenated aliphatic hydrocarbons for two reasons. First, anaerobic conditions are common in many natural and engineered systems, including certain wastewater treatment processes, many sediments, and some aquifers (Macalady et al., 1986). More importantly, however, some
highly halogenated aliphatics, such as tetrachloroethane and CCl₄, are not biodegradable under aerobic conditions. For these compounds, anaerobic biotransformation must initiate reactions that lead to complete dechlorination and mineralization.

In order to study microbial consortia that have characteristics similar to natural populations, the experimental systems used in this research are designed to maintain low growth rates and low steady-state concentrations of the primary substrates. In most aquifers, organic compounds that can support growth of indigenous microbial populations are present at very low concentrations (Ghiors and Wilson, 1988). Many sediments and waste treatment processes also are characterized by low substrate concentrations. Environments of this type select for oligotrophic bacteria, microorganisms that are capable of growing in the presence of very low concentrations of nutrients (Alexander, 1985). Typically, these bacteria can grow on a wide variety of substrates, which they may be able to use simultaneously. Oligotrophic bacteria often have very high affinities for these substrates, but have low maximum specific growth and substrate utilization rates (Alexander, 1985; Rittmann et al., 1986). In contrast, traditional techniques used to enrich bacteria from environmental samples tend to select organisms with high maximum growth rates (Parkes, 1982). Bacteria isolated from enrichment cultures often have relatively low affinities for substrates and a more restricted substrate range. These differences suggest that natural microbial populations may metabolize xenobiotic substrates very differently than pure or mixed cultures obtained using traditional methods.

The anaerobic biodegradation of halogenated aliphatic hydrocarbons is critically reviewed in Chapter 2. The mechanisms of reductive dehalogenation reactions are explained, and the expected effects of substrate structure on reaction kinetics are described. Microbial coenzymes that are likely to catalyze reductive dehalogenation reactions are identified, and the implications of the relative effectiveness of these catalysts for the microbial ecology of reductive dehalogenation are considered.

A structured model for the kinetics of reductive dehalogenation reactions is developed in Chapter 3. This model is based on the reaction mechanism that is presented in Chapter 2, and it describes the interactions that occur among primary electron-donor and -acceptor substrates and halogenated aliphatics during reductive dehalogenation reactions.

The experimental methods that were used to measure TCA biodegradation kinetics are presented in Chapter 4. Also in this chapter, the use of once-through biofilm reactors in the kinetics experiments is justified. The procedures that were used to analyze the kinetic data are described, and the accuracy of the approximation that was used to simplify the data analysis is demonstrated.

The effects of primary electron-donor and electron-acceptor substrate concentrations on the apparent Monod kinetic parameters for TCA biodegradation in the methanogenic and sulfate-reducing biofilm reactors are presented in Chapter 5. The experimental data are compared to the predictions of the model. For all primary-substrate concentrations tested, the results demonstrate that the Monod equation provides an adequate description of the kinetics of TCA biodegradation. However, the Monod kinetic parameters are
functions of the primary-substrate concentrations, which is consistent with the predictions of the model developed in Chapter 3.

This research demonstrates that the kinetics of anaerobic biotransformation of TCA are affected by the concentrations of the primary substrates. Primary electron-donor substrates stimulate the rate of TCA biodegradation, and primary electron acceptors inhibit it. Thus, the concentrations of TCA and biomass are insufficient descriptors of TCA biodegradation kinetics, but a Monod-like kinetic expression can be used if the kinetic parameters are allowed to be functions of the primary substrate concentrations.
CHAPTER 2 REDUCTIVE DEHALOGENATION

Anaerobic conditions are common in natural and engineered systems (Macalady et al., 1986). In order to understand the fate of halogenated aliphatic hydrocarbons in these systems, it is essential to understand the processes by which they are removed. One such process is microbial transformation, and reductive dehalogenation is an important mechanism by which anaerobic biotransformation of halogenated aliphatics can occur. Many highly halogenated aliphatics, such as tetrachloroethene (PCE) and carbon tetrachloride (CCl₄), react slowly or not at all by the common non-reductive pathways for biotransformation of halogenated organic compounds (e.g., oxidative or hydrolytic dehalogenation). Thus, biodegradation of such compounds begins with reductive dehalogenation.

This chapter begins by describing the most common types of reductive dehalogenation reactions that are catalyzed by anaerobic bacteria. The known effects of primary electron-donor and -acceptor substrates on reductive dehalogenation reactions are reviewed next. Then, the biochemical mechanisms through which reductive dehalogenation can occur are described, and this information is used to predict the effects of substrate structure on reaction rates. Finally, the microbial coenzymes that are likely catalysts of reductive dehalogenation are identified.

2.1 Anaerobic Biotransformation of Halogenated Aliphatic Hydrocarbons

Anaerobic biotransformation of halogenated aliphatic hydrocarbons has been studied in systems ranging in complexity from anaerobic microcosms, which are of poorly defined composition, to pure cultures growing in defined media, where the reaction conditions can be precisely defined and biotic and abiotic reactions can be readily distinguished. Anaerobic biotransformations of halogenated aliphatics usually are slow—the time scales of these experiments range from days to months—and recovery (and/or identification) of products is often very poor.

Replacement of a carbon-halogen bond by a carbon-hydrogen bond—or "hydrogenolysis" (Vogel et al., 1987)—is the most common type of reductive dehalogenation reaction. Two examples of reactions that proceed by this mechanism are shown in Figure 2.1. These reactions involve transfer of two electrons to the halogenated substrate per halogen atom that is removed.

If the substrate is polyhalogenated, it may be possible to remove all of the halogen substituents through a series of reductive reactions similar to those shown in Figure 2.1. Biodegradation of tetrachloroethene (PCE) in anaerobic microcosms (Parsons et al., 1984, Parsons and Lage, 1985, Lage et al., 1986) and in methanogenic (Vogel and McCarty, 1985, Freedman and Gossett, 1989) and acetogenic (DiStefano et al., 1991) laboratory reactors is believed to have occurred via sequential dehalogenation reactions, such as those illustrated in Figure 2.2. Complete dechlorination of PCE to ethene is sometimes observed (Freedman and Gossett, 1989, DiStefano et al., 1991), but incompletely dechlorinated intermediates, such as vinyl chloride and cis or trans-1,2-DCE, frequently accumulated (Parsons et al., 1984, Parsons and Lage, 1985, Vogel and McCarty, 1985;
Lage et al., 1986, Lage et al., 1987) Incompletely dechlorinated compounds accumulate in some systems, because the reactivity of halogenated aliphatics to reductive dehalogenation decreases as halogen atoms are removed.

Although hydrogenolysis is the only reductive mechanism by which halomethanes can react, multi-carbon halogenated aliphatics with halogen substituents on adjacent ("vicinal") carbon atoms can reductively eliminate two vicinal halogen atoms. The products of this type of reductive dehalogenation reaction are unsaturated Dihaloelimination, which is illustrated in Figure 2.3, involves transfer of one electron to the halogenated substrate per halogen atom that is removed.

Reductive eliminations of 1,2-dihaloethanes were catalyzed by several pure cultures of H₂-utilizing methanogens (Belay and Daniels, 1987, Elgi et al., 1987) A similar reaction appeared to be the initial step in anaerobic biodegradation of lindane (γ-hexachlorocyclohexane) by pure cultures of several species of common anaerobic heterotrophs (Jagnow et al., 1977). Hexachloroethane was rapidly dechlorinated to PCE under aerobic conditions (Cridle et al., 1986) In addition, Belay and Daniels (1987) observed a small amount of dibromoelimination from 1,2-dibromoethene, resulting in the formation of acetylene. Dihaloelimination of haloethenes, however, is very slow relative to the rates of dihaloelimination from haloethanes.

Oxidized and oxygenated products cannot be formed through the reactions that have been described in this section. The ultimate products should be volatile alkanes or alkenes, such as methane, ethane, and ethene, which are not readily oxidized under anaerobic conditions. Nevertheless, many products observed in anaerobic mixed cultures
were oxidized, oxygenated, or non-volatile and water soluble. Some important examples of transformations that cannot have resulted from hydrogenolytic or dihaloelimination reactions are compiled in Table 2.1. For example, carbon dioxide was a frequently observed product of the anaerobic biodegradation of halogenated aliphatics. The production of volatile fatty acids and other polar products from metabolism of 1,1,1-TCA by a pure culture of a *Clostridium* sp (Galli and Mccarty, 1989) and the conversion of CCl₄ to a polar, non-volatile product by a denitrifying mixed culture obtained from sewage (Bouwer and Mccarty, 1983b) are further examples of observations that cannot be explained by the reductive pathways that have been presented.

Pathways that are initiated by one or more reductive dehalogenation steps, but lead to oxygenated and oxidized products have been proposed. These pathways involve reaction of incompletely dehalogenated products with water, either through hydrolytic dehalogenation or by addition of water to a carbon-carbon double bond (Vogel and Mccarty, 1985, Vogel and Mccarty, 1987). An alternative reductive mechanism that leads directly to many of the oxygenated products that are commonly observed has been proposed (Cridle and Mccarty, 1991) and is discussed in more detail in Section 2.3.
2.2 Primary Substrate Effects on Reductive Dehalogenation

Electrons are transferred to halogenated substrates during reductive dehalogenation reactions. Therefore, it is not surprising that many investigators have found that anaerobic biotransformation of halogenated aliphatic and aromatic hydrocarbons is affected by electron-donor and -acceptor substrates. The effects that have been observed include stimulation of reductive dehalogenation by electron donors, inhibition by electron acceptors, alterations of the acclimation times that precede the onset of biotransformation by enrichments from environmental samples, and shifts in the distribution of products. A mechanism by which primary substrates can directly affect reductive dehalogenation reactions is through control of the intracellular concentrations of electron carriers. This direct mechanism is described in Chapter 3, where it is used as the basis for a kinetic model for reductive dehalogenation reactions. Indirect effects can result from changes in the biomass density or composition and from genetic effects, such as induction or repression of enzyme synthesis. In many cases reported in the literature, the direct and indirect effects of primary substrates cannot easily be distinguished.

The effects of electron-donor and -acceptor substrates on reductive dechlorination of chlorinated aromatic substrates was studied in microcosms that contained material from methanogenic aquifers. Short-chain organic acids (e.g., formate, propionate, and butyrate) and alcohols (e.g., ethanol) decreased the time required for the microcosms to begin dechlorinating 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and increased the extent of dechlorination observed after four months (Gibson and Suffita, 1990). Addition of butyrate to similar microcosms caused the products formed by biotransformation of 2,3,4,5-tetrachloroaniline to change. In the absence of butyrate, a major pathway involved sequential removal of chlorine from the para position (to form 2,3,5-trichloroaniline) and the ortho position (giving 3,5-dichloroaniline). In the presence of butyrate, the initial dechlorination could occur at the meta position, as well as the para position (Kuhn et al., 1990). The 2,4,5-trichloroaniline that was produced by meta dechlorination was not
Table 2.1 Production of Oxidized and Oxygen-Containing Products During Anaerobic Biodegradation of Halogenated Aliphatic Hydrocarbons

<table>
<thead>
<tr>
<th>System</th>
<th>Substrates</th>
<th>Products</th>
<th>Product Recovery</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>xylan- and cellulose-fed</td>
<td>CCl₄</td>
<td>CO₂</td>
<td>99%</td>
<td>Bouwer and McCarty (1983a)</td>
</tr>
<tr>
<td>waste activated sludge</td>
<td>CHCl₃</td>
<td>CO₂</td>
<td>88%</td>
<td></td>
</tr>
<tr>
<td>acetate-fed methanogenic</td>
<td>1,1,1-TCA</td>
<td>CO₂</td>
<td>9 - 13%</td>
<td>Vogel and McCarty (1987)</td>
</tr>
<tr>
<td>biofilm reactor (Methanothrix)</td>
<td>1,1-DCA</td>
<td>CO₂</td>
<td>34%</td>
<td></td>
</tr>
<tr>
<td>(vinyl chloride)</td>
<td>1,1-DCE</td>
<td>CO₂</td>
<td>18%</td>
<td></td>
</tr>
<tr>
<td>Methanosarcina sp. strain DCM</td>
<td>CHCl₃</td>
<td>CH₂Cl₂</td>
<td>65%</td>
<td>Mikesell and Boyd (1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH₃Cl</td>
<td>trace</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>7%</td>
<td></td>
</tr>
<tr>
<td>Acetobacterium woodii</td>
<td>CCl₄</td>
<td>CH₂Cl₂</td>
<td>10%</td>
<td>Elgi et al. (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>13%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>acetate</td>
<td>38%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pyruvate</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>biomass</td>
<td>6%</td>
<td></td>
</tr>
<tr>
<td>Clostridium sp.</td>
<td>1,1,1-TCA</td>
<td>1,1-DCA</td>
<td>30 - 40%</td>
<td>Galli and McCarty (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acetate</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>0.2%</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp. strain KC</td>
<td>CCl₄</td>
<td>CHCl₃</td>
<td>1%</td>
<td>Criddle et al. (1990a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>47%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>UI,NV*</td>
<td>42%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>biomass</td>
<td>5%</td>
<td></td>
</tr>
</tbody>
</table>

*UI,NV* = unidentified, non-volatile product

Transformed further. Addition of sulfate to both sets of microcosms usually inhibited reductive dechlorination (Gibson and Suffit, 1990; Kuhn et al., 1990), but conversion of tetrachloroaniline to trichloroaniline was unaffected by sulfate (Kuhn et al., 1990)
Sulfate always increased the time required for the microcosms to begin dechlorinating 2,4,5-T (Gibson and Suflita, 1990)

Because of the long acclimation times that were required prior to the onset of reductive dechlorination of 2,4,5-T and tetrachloroaniline, major changes in the size or composition of the microbial populations in the microcosms probably occurred. Thus, the effects of electron donors and acceptors in these systems might have been due to their effects on the composition of the microbial populations. Direct effects and other indirect effects, such as genetic changes, may have occurred as well, but it is impossible to distinguish among these possibilities based on the available data. Effects of the primary substrates on the compositions of the microbial populations are consistent with all of the data. Addition of sulfate to the methanogenic aquifer slurries probably stimulated the growth of sulfate-reducing bacteria at the expense of the dechlorinating bacteria, resulting in increased acclimation times and decreased dechlorinating activity. The acclimation times that preceded dechlorination of 2,4,5-T decreased when short-chain organic acids and alcohols were added, because these electron donors probably stimulated growth of the dechlorinating populations. The stimulation of PCE degradation that was observed upon addition of toluene to anaerobic aquifer microcosms might be another example of the primary electron donor's effect on the size of the population of dechlorinating bacteria (Sewell and Gibson, 1991). On the other hand, a mechanism through which a direct effect of the primary electron donor on the availability of electrons could change the position at which 2,3,4,5-tetrachloroaniline was dechlorinated is difficult to envision.

The effects of primary electron-acceptor substrates on reductive dechlorination of CCl₄ by E. coli K-12 (Cridle et al., 1990b) and Pseudomonas sp strain KC (Cridle et al., 1990a) were probably caused by changes in gene expression (i.e., induction or repression of dehalogenase activity) controlled by the primary substrate. Both of these organisms are facultative aerobes that can transform carbon tetrachloride completely. Pseudomonas sp KC rapidly degrades CCl₄ to CO₂ and an unidentified, non-volatile, aqueous product in the presence of nitrate, but not in the presence of O₂ or fumarate (Cridle et al., 1990a). E. coli K-12, on the other hand, transforms CCl₄ completely under fermentation and fumarate-respiring conditions, but not under aerobic or nitrate-respiring conditions (Cridle et al., 1990b). The products formed from CCl₄ by E. coli K-12 also were dramatically affected by the electron acceptor that was supplied. The major products formed in the presence of fumarate were biomass and unidentified non-volatile compounds, whereas biomass and CHCl₃ dominated under fermentation conditions (Cridle et al., 1990b). Well documented differences exist in the respiratory electron transport chains of E. coli when it is grown in the presence of different electron acceptors (Jones, 1982), and these differences might be responsible for the observed metabolic changes. Genetic effects such as these may represent an important level of control for reductive dehalogenation activity, but they are not considered further, because there is insufficient information regarding the nature of microbial dehalogenases and the mechanisms by which their expression is controlled.

Several examples illustrate the likely direct effects of electron-donor and acceptor substrates. For example, reductive dechlorination of CHCl₃ to CH₂Cl₂ by two strains of Methanosarcina sp did not occur in the absence of electron-donor substrates, but proceeded readily in the presence of methanol and mono, di, and trimethylamines.
One of these strains—*Methanosarcina* sp. strain DCM—also dechlorinated PCE to TCE (Fathepure and Boyd, 1988a). Acetate, methanol, and mono- and trimethylamine supported reductive dechlorination of PCE by this organism. A small amount of PCE dechlorination occurred in the absence of electron-donor substrates, but only while methane production also occurred. Presumably, *Methanosarcina* sp. DCM has an endogenous source of electrons that can sustain methanogenesis and reductive dechlorination in the absence of exogenous electron donors.

*Methanosarcina barkeri* reductively dechlorinated CCl₄ and CHCl₃ in the absence of an exogenous electron donor, but the rates of these reactions were twice as fast in the presence of 6% carbon monoxide than when no electron donors were added (Krone et al., 1989b). When *M. barkeri* cultures were killed by boiling, carbon monoxide-supported reductive dechlorination ceased. Titanium(III) citrate, however, was an effective electron donor for the dechlorination of CCl₄ and CHCl₃ that was catalyzed by heat-killed cells. Thus, living cells were not required to catalyze the dehalogenation reaction, but the dehalogenase could not be reduced by physiological electron donors unless the cells were alive.

*Desulfomonile tiedjei* is a sulfate-reducing bacterium that is capable of using 3-chloro-benzoate as its primary electron acceptor. *D. tiedjei*, formerly known as DCB-1, was isolated from a 3-chlorobenzoate-degrading, methanogenic consortium. Although this organism can dehalogenate 3-chlorobenzoate in the absence of an electron donor, the reaction was greatly stimulated by H₂, formate, and pyruvate (DeWeerd et al., 1991). H₂, formate, and carbon monoxide stimulated reductive dechlorination in cell-free extracts of *D. tiedjei*, but pyruvate had no effect (DeWeerd and Sulfita, 1990). Sulfate, which is preferred over 3-chlorobenzoate as the primary electron acceptor, did not inhibit reductive dechlorination of the aromatic substrate in either intact cells or in cell-free extracts (DeWeerd and Sulfita, 1990, DeWeerd et al., 1991). Other sulfur oxygions that can be used as primary electron acceptors by this organisms, such as sulfite ad thiosulfate, did inhibit the reaction in both systems.

Electron donors were required to sustain reductive dechlorination of PCE and TCE to ethylene by mixed methanogenic cultures (Freedman and Gossett, 1989). Methanol, H₂, formate, acetate, and glucose could support reductive dechlorination by these cultures. Methanol was the most effective. If primary substrates were not added to the enrichments, the rates of PCE dechlorination gradually decreased. Reductive dechlorination of PCE by these mixed cultures continued after methane production had ceased (Freedman and Gossett, 1989), indicating that endogenous electron donors were available to the dechlorinating organisms.

The work most immediately relevant to this research was carried out by Wrenn (1991), who demonstrated that formate stimulated the reductive dechlorination of TCA by methanogenic and sulfate-reducing consortia present as biofilms. Acetate, methanol, and propionate had no effects. Glucose provided a degree of stimulation, but that stimulation probably was caused directly by formate and H₂ produced by fermentation of the glucose. The inhibitor molybdate stimulated TCA dechlorination in the sulfate-reducing system, presumably because it prevented SO₄²⁻ from being an electron sink. The
inhibitor 2-bromoethanesulfonate (BES) had no effect on TCA reduction, although it greatly reduced methane generation in the methanogenic system. The BES result suggests that the methyl reductase enzyme has greater affinity for TCA reduction than for methane formation. All of these results demonstrate that TCA dechlorination was carried out by \( \text{H}_2 \)-oxidizing anaerobes, which use formate as an alternate electron donor.

2.3 Mechanism of Reductive Dehalogenation

Reductive dehalogenation reactions of halogenated aliphatic compounds often are catalyzed by coordination complexes of reduced transition metals (Wade and Castro, 1973a, Kochi, 1978, Bakac and Espenson, 1986). The mechanism of reductive dehalogenation is illustrated in Figure 2.4. A bridged activated complex, in which the halogen atom serves as the bridging ligand, probably forms as an intermediate during electron transfer from the metal ion to the alkyl halide (Kochi, 1978). The halogen atom forms a coordinate covalent bond with the metal ion by interaction of a filled halogen \( p \) orbital with an unoccupied \( d \) orbital of the metal atom. A single electron is transferred.

![Mechanism of Reductive Dehalogenation](image)

Figure 2.4 Mechanism for the reductive dehalogenation of halogenated aliphatic hydrocarbons
from the reduced metal to the organic substrate, resulting in homolytic cleavage of the carbon-halogen bond. When a covalent bond is broken by homolysis, one electron from the molecular orbital remains with each of the two previously bonded atoms (Morrison and Boyd, 1973). Therefore, the products of homolytic cleavage of a carbon-halogen bond are an alkyl-free radical and an oxidized metal-halide complex (Wade and Castro, 1973a; Kochi, 1978; Bakac and Espenson, 1986). The metal-halide bond can dissociate rapidly (Wade and Castro, 1973a), providing some of the energy to drive the bond cleavage reaction (Kochi, 1978). This type of reaction is called a ligand transfer reaction and is said to occur through an "inner sphere" mechanism, because it requires intermediate formation of a coordination complex between the halogenated substrate and the reduced metal (Kochi, 1978).

In most cases, the subsequent reactions of the alkyl radical began by interaction of its unpaired electron with another reduced metal complex (Wade and Castro, 1973a). Covalent bond formation between these reactants results in (effective) oxidation of the metal ion and reduction of the carbon atom. Evidence for the formation of alkylated metal complexes during reductive dehalogenation reactions has been obtained in several different systems (Mansuy, 1980; Bakac and Espenson, 1986; Krone et al., 1989a). The products of the reaction are dictated by the mechanism through which this carbon-metal bond breaks (Figure 2.4). In hydrogenolytic reactions, the oxidized metal ion, $M^{n+}$, is displaced by a $H^+$ (Wade and Castro, 1973a, Bakac and Espenson, 1986). When one of the "R" groups of the organic substrate is a halogen-substituted carbon atom, dihaloelimination can occur (Wade and Castro, 1973a). Both of these pathways are shown in Figure 2.4.

The third pathway that is available for the further reaction of the alkyl radical is dimer formation (Wade and Castro, 1973a; Vogel et al., 1987). Because none of the products listed in Table 2.1 are a result of reaction by this pathway, it appears to be of limited importance in reactions of the halogenated aliphatics that are of environmental concern. Wade and Castro (1973a) found that only benzylic, allylic, and propargylic halides reacted by this pathway. These compounds have the following structure:

![Structure](image)

The C-X bonds in these compounds are very reactive to reductive dehalogenations (Wade and Castro, 1973a), and the organic free radicals that are produced by homolysis of the C-X bond are very stable (Morrison and Boyd, 1973). These characteristics may facilitate dimer-forming reactions by enabling the organic free radicals to persist long enough to react with each other. As very few important pollutants have these structures, dimer formation is not considered further, except to note that reductive dechlorination of $CCl_4$ by aquocobalamin (Krone et al., 1989a) and coenzyme $F_{430}$ (Krone et al., 1989b) resulted.
in the production of trace amounts (less than 0.05%) of ethane. Ethane could have formed in these systems only by dimerization of methyl radicals. Thus, detection of ethane supports the hypothesis that these reactions occurred by the mechanism outlined in this section, but indicates that radical coupling was a minor pathway for further reaction of the alkyl radical intermediates.

Many of the products listed in Table 2.1 were not formed by only the reductive reactions that have been described in this section. Pathways through which these products can be formed from the incompletely dehalogenated intermediates by reaction with water were proposed by Vogel and McCarty (1985 and 1987). These pathways appear to be reasonable, because lightly halogenated alkanes react readily with nucleophiles, such as H$_2$O or OH$^-$ (Morrison and Boyd, 1973), and addition of water to carbon-carbon double bonds is a common reaction in many microbial metabolic pathways (Gottschalk, 1986). Many examples of microbially catalyzed hydrolyses of mono- and dihalomethanes and ethanes are known (Keuning et al., 1985; Janssen et al., 1985; Strotmann and Roschenthaler, 1987; Scholtz et al., 1988). The effect of the extent of halogen substitution on the rates of reductive dehalogenation and nucleophilic substitution, however, suggests that halogenated intermediates should accumulate, because the rates of reductive dehalogenation decrease as halogens are removed, and the rates of hydrolysis decrease as halogen substitution increases. Therefore, intermediates with about two halogens per carbon react slowly by both mechanisms. Accumulation of these intermediates, however, is often not observed. Furthermore, highly halogenated aliphatic substrates (e.g., CCl$_4$) are frequently converted to oxygen-containing products (e.g., CO$_2$) by bacteria that cannot metabolize less halogenated analogs (e.g., CHCl$_3$ and CH$_2$Cl$_2$). Thus, pathways that involve sequential reductive dehalogenation and hydrolytic dehalogenation reactions cannot be the only way to form oxygenated products.

A reductive reaction mechanism that leads directly to non-volatile, dehalogenated products has recently been proposed (Criddel and McCarty, 1991). For many halogenated aliphatic substrates, the products of this reaction mechanism can be readily oxidized by many anaerobic bacteria. This mechanism results in complete dechlorination of polyhalogenated aliphatics by transfer of only two electrons to the halogenated substrate, rather than one or two electrons per halogen, as is required by the mechanism illustrated in Figure 2.4. The initial steps of this mechanism are identical to those illustrated in Figure 2.4, but the alkyl-free radical that is produced by homolysis of the carbon-halogen bond reacts differently. The unpaired electron does not react with a reduced metal complex, instead, a second dehalogenation reaction occurs. Presumably, this can occur in the same manner as does the first dehalogenation. The result of these two dehalogenation reactions is an alkyl diradical (i.e., a carbene). The carbene is expected to react further, by an unspecified mechanism, to produce completely dechlorinated, oxygenated products. This pathway is outlined in Figure 2.5.

Criddel and McCarty (1991) tested this hypothesis in an electrolytic system in which reductive dechlorination of CCl$_4$ and 1,1,1-TCA occurred at a silver electrode. Their results were consistent with a carbene mechanism. They observed complete dechlorination of these substrates with input of only two electrons per molecule of substrate that was transformed and production of carbon monoxide and formate from CCl$_4$. Only about 12% of the CCl$_4$ was converted to CHCl$_3$. The non-volatile dechlorinated
products of 1,1,1-TCA were not identified, but very little (approximately 5%) was converted to the hydrogenolysis product 1,1-dichloroethane. Although these results do not prove that these reactions proceed through carbenes, they do demonstrate that it is possible to produce oxygenated, completely dehalogenated products from highly halogenated aliphatics through a purely reductive process.
2.4 Effects of Substrate Structure on the Kinetics of Reductive Dehalogenation Reactions

The pathway by which the alkyl radical reacts determines the structure of the products that are formed by reductive dehalogenation. However, it does not significantly affect the rate at which the overall reaction proceeds. Cleavage of the carbon-halogen bond is believed to be the rate-limiting step in reductive dehalogenation reactions (Wade and Castro, 1973a, Kochi, 1978, Bakac and Espenson, 1986). The mechanisms that were described in Section 2.3 suggest that three characteristics of the halogenated substrate will influence the reaction rate. First, halogenated substrates must form stable coordination complexes with reduced metal ions. "Stable" means that the complexes must persist long enough for electron transfer to occur. Therefore, the strengths of the metal-halogen coordinate bonds should affect the rates of reaction. Second, carbon-halogen bond cleavage must occur. Therefore, the strengths of the C-X bonds, which measure the ease with which bond cleavage occurs, will influence the rates of reaction. Finally, the allyl radicals that form by homolytic cleavage of the C-X bonds must be stable enough that they do not recombine with the coordinated halogen atoms immediately. That is, the reverse reaction—carbon-halogen bond formation—must be slow relative to the rates at which the products (R* and X-) diffuse away from the catalytic metal centers.

The ability of halogens to form coordinate bonds and the strength of the covalent bonds that they form with carbon follow opposite trends. As halogen size increases, the ability of the atom to form coordinate bonds increases (Modena and Scorrano, 1973), and the strength of the carbon-halogen bond decreases (Morrison and Boyd, 1973, Wagniere, 1973). Both of these trends result in greater reactivity of C-X bonds to reductive cleavage as the size of the halogen substituents increases from F to Cl to Br to I. This predicted trend in reactivity is in agreement with most of the experimental observations. In general, brominated compounds react more readily than do chlorinated compounds (Wade and Castro, 1973a, Bouwer and McCarty, 1983a,b, Bouwer and Wright, 1987, Beley and Daniels, 1987). However, because biodegradation reactions are catalyzed by enzymes in cells, not by reduced metal complexes that exist free in solution, steric effects may limit the reactivity of some substrates, especially those that possess bulky substituents at the reactive carbon atom.

The extent of halogen substitution at the carbon atom bearing the C-X bond of interest (i.e., the α carbon) also affects bond strength. In general, carbon-halogen bond strength decreases as the halogen substitution of the α carbon increases. Thus, highly halogenated aliphatics should be reductively dechlorinated more quickly than aliphatics that are only mono- or di-substituted. Again, this predicted trend in reactivity is consistent with many of the observations that have been made in microbial systems (Vogel and McCarty, 1985, Elgi et al., 1987, Elgi et al., 1988, Galli and McCarty, 1989, Freedman and Gossett, 1989).

The third characteristic of substrate structure that influences the rates of reductive dehalogenation reactions is the stability of the organic free radicals that are formed by homolytic cleavage of the C-X bonds. The standard enthalpies of formation of several
Table 2.2  Standard Enthalpies of Formation for Free Radicals
(Dean, 1985, Weast, 1987)

<table>
<thead>
<tr>
<th>Free Radical</th>
<th>$\Delta H_{f_{25}}$ (kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃·</td>
<td>34.8</td>
</tr>
<tr>
<td>CH₃CH₂·</td>
<td>26.0</td>
</tr>
<tr>
<td>H₂C=CH·</td>
<td>63</td>
</tr>
<tr>
<td>CH₂Cl·</td>
<td>29.2</td>
</tr>
<tr>
<td>CHCl₂·</td>
<td>24.1</td>
</tr>
<tr>
<td>CCl₃·</td>
<td>19.1</td>
</tr>
<tr>
<td>CH₂F·</td>
<td>-7.8</td>
</tr>
<tr>
<td>CH₂Cl·</td>
<td>29.2</td>
</tr>
<tr>
<td>CH₂Br·</td>
<td>40.9</td>
</tr>
<tr>
<td>CF₃·</td>
<td>55.0</td>
</tr>
</tbody>
</table>

Alkyl and haloalkyl-free radicals are compiled in Table 2.2. Chloromethyl-free radicals become more stable (i.e., have a lower enthalpy of formation) as the degree of chlorine substitution increases. This trend parallels the decrease in C-Cl bond strength that occurs as the degree of chlorine substitution increases. In fact, within a homologous series, bond-strength and free-radical stability are not entirely independent, as the free-radical enthalpy contributes to the enthalpy of carbon-halogen bond dissociation (Dean, 1985). It is not clear why the stabilities of alkyl-free radicals increase as the degree of chlorine substitution increases, but it may be related to the ability of halogen atoms to form $\pi$ bonds with the radical carbon atom (i.e., they can form carbon-halogen double bonds). This allows the unpaired electron to become delocalized (Modena and Scorrano, 1973), as the following resonance structures suggest:

\[
\begin{align*}
\text{Cl} & \longrightarrow \text{C} \\
\rightarrow & \quad \rightarrow \\
\text{H} & \longrightarrow \text{Cl}
\end{align*}
\]

The ability of halogens to stabilize free radicals decreases as the size of the halogen increases (Table 2.2). Stabilization of free radicals, like the stabilization of carbon-carbon double bonds, is probably related to the strength of the $\pi$ bond that forms between the halogen and carbon atoms. Bond strength is related to the amount of overlap that occurs between the atomic orbitals that combine to form the bonding molecular orbital. The extent of overlap is determined by the relative sizes of the atomic orbitals (Wagniere, 1973). The $p$ orbitals of carbon and fluorine are approximately the same size. Thus, the
maximum overlap occurs for \( \pi \) bonds formed between these two atoms. As halogen size increases, so does the size of the halogen \( p \) orbital that forms the \( \pi \) bond with carbon. Thus, the strength of the carbon-halogen \( \pi \) bond decreases as the size of the halogen increases. The amount of resonance stabilization that is achieved through conjugation (i.e., \( \pi \) bond formation) is proportional to the contribution that each possible resonance structure makes to the true electron distribution (Morrison and Boyd, 1973), and the magnitude of the contribution of each resonance structure is determined by the strength of the \( \pi \) bond that forms between carbon and the halogens. Thus, fluorine may stabilize alkyl-free radicals most efficiently, because it forms the strongest \( \pi \) bonds with carbon.

Whereas the stability of chloromethyl free radicals parallels the trends in dissociation energy of the carbon-chlorine bonds, the stability of the alkyl radicals in the series methyl, ethyl, vinyl is not related to the dissociation energies of the carbon-halogen bonds for the corresponding halomethane, haloethane, and vinyl halide. For the monochlorinated compounds—chloromethane, chloroethane, and chloroethene (i.e., vinyl chloride)—the strengths of the C-Cl bonds are similar, but there are very large differences in the stabilities of the alkyl radical that are formed by homolytic cleavage of these bonds (Table 2). Thus, the differences in reactivity among these three series of halogenated aliphatics might be due to the relative stabilities of the free radicals that are formed. In general, for the same degree of halogenation, the rates of reductive dehalogenation of haloethanes are greater than those of halomethanes, and both are much greater than those of haloethenones (Parsons and Lage, 1985, Bouwer and McCarty, 1983a, Belay and Daniels, 1987).

2.5 Catalysis of Reductive Dehalogenation by Microbial Coenzymes

In general, the mechanisms of reductive dehalogenation reactions have been studied using coordination complexes that are not found in microorganisms. Complexes of metals that do not have normal biological functions, such as chromium, tin, and platinum, have been used in much of this research (Kochi, 1978). Metals that are physiologically important, such as iron, cobalt, and nickel, also have been used, but the organic ligands were usually nonphysiological (Kochi, 1978; Bakac and Espenson, 1986). In those few instances in which physiologically relevant catalysts were used to study the mechanisms of reductive dehalogenation, the halogenated substrates were usually not important pollutants (Wade and Castro, 1973a,b; Castro et al., 1985). Thus, there are no examples in which the mechanisms of reductive dehalogenation of environmentally important halogenated aliphatics were studied using physiologically important transition metal coordination complexes. Recently, however, several groups have investigated the ability of metal-containing microbial coenzymes to catalyze reductive dehalogenation of halogenated aliphatic substrates that are important pollutants. The results of these investigations are consistent with the reaction mechanisms that have been established using more exotic catalysts and substrates.

Three types of microbial coenzymes have been used: iron porphyrins (e.g., hematin), cobalt corrinoids (e.g., vitamin B\(_{12}\)), and the nickel-containing coenzyme F\(_{430}\) (see Gantzer and Wackett (1991) for the structures of these coenzymes). All of these coenzymes have tetrapyrrroles as the organic ligands. The metal ions are coordinated by
the four nitrogen atoms of the tetrapyrrrole rings in an approximately square planar fashion. Since all of these metals can coordinate up to six ligands (Hughes, 1981), two axial positions are potentially available to coordinate halogenated aliphatic substrates. In one class of the corrinoids—the cobalmines—one of the two axial positions is occupied by a ligand that is covalently attached to the tetrapyrrrole ring. Thus, compounds like vitamin B_{12} probably can coordinate halogenated aliphatic substrates at one position only, but this is sufficient for the mechanism that was described in Section 2.3.

The ability of hematin, an iron porphyrin, to catalyze the reductive dechlorination of several chlorinated methanes and ethanes was studied by Klecka and Gonsior (1984). They used either sulfide or cysteine as the electron donor for these reactions. Carbon tetrachloride, chloroform, and 1,1,1-trichloroethane (1,1,1-TCA) were dechlorinated by hematin in the presence of both electron donors at neutral pH, but methylene chloride, 1,1,2-TCA, and 1,1-dichloroethane (1,1-DCA) were not. Apparently, hematin required the presence of at least three chlorine substituents on a single carbon atom in order to catalyze reductive dechlorination. Tetrachloroethene (PCE) and trichloroethene (TCE) also were unreactive under these conditions.

Several corrinoids (Krone et al., 1989a) and coenzyme F_{430} (Krone et al., 1989b) catalyzed the reductive dechlorination of CCl_{4} and its less highly chlorinated analogs to methane. As expected, the rates of dechlorination catalyzed by corrinoids and coenzyme F_{430} decreased dramatically as the extent of chlorine substitution decreased. Titanium(III) citrate could serve as the electron donor for reactions catalyzed by both coenzymes, but dithiothreitol could supply electrons only to reactions catalyzed by the corrinoids. Although the rates of conversion of CCl_{4} to CHCl_{3} by aquocobalamin and coenzyme F_{430} were approximately equal, the rate of methane production from CHCl_{3} was fifty times faster when the reaction was catalyzed by F_{430} than when it was catalyzed by aquocobalamin. The authors suggested that this difference in reactivity was due to the greater lability of the C-Ni bonds of the alkylated metal intermediates (Krone et al., 1989b). In support of this hypothesis, they were able to detect alkylated corrinoid intermediates in corrinoid-catalyzed reactions, but were unable to find analogous compounds during the F_{430^-} catalyzed reactions.

Gantzer and Wackett (1991), in an interesting extension of the previous research, compared the abilities of hematin, vitamin B_{12} (i.e., cyanocobalamin), and coenzyme F_{430} to catalyze reductive dechlorination of CCl_{4} and the homologous series of chlorinated ethenes (i.e., PCE, TCE, the DCEs and vinyl chloride). The electron donor for these reactions was Ti(III) citrate. Their results were consistent with the predictions made in the previous section. CCl_{4} was dechlorinated much faster (approximately 10 to 30 times faster) than was PCE, and the rates of dechlorination of the chloroethenes decreased as the extent of chlorine substitution decreased. In general, vitamin B_{12} and coenzyme F_{430} catalyzed reductive dechlorination at similar rates. Both of these cofactors were much more efficient catalysts of reductive dechlorination than was hematin.

Although the hematin-catalyzed reductive dechlorination of PCE and TCE was slow relative to the B_{12^-} and F_{430^-} catalyzed reactions, it did occur (Gantzer and Wackett, 1991) Klecka and Gonsior (1984), on the other hand, reported that these substrates were
unreactive in their system. The reason for the differences between these two systems is unclear. The efficiencies of the two systems were not markedly different. In fact, the mixed second-order rate coefficients for dechlorination of CCl₄ were similar in both systems. One possibility is that the reaction pHs affected the rates of chloroethene transformation, because Gantz and Wackett (1991) conducted their reactions at a higher pH than did Klecka and Gonsior (1984). The mechanism through which pH affects reductive dehalogenation reactions is not clear, but Krone et al. (1989a) found that high pH stimulated the formation of methane from CCl₄ in (cyanoaquo)cobinamide-catalyzed reactions. Also, the coenzyme F₄₃₀-catalyzed formation of methane from methyl sulfonium salts was stimulated by the addition of base to the reaction mixtures (Juan and Pfaltz, 1988). Perhaps the hematin-catalyzed reductive dechlorinations of PCE and TCE, too, were stimulated by high pH.

Product recovery, as dechlorinated volatile compounds, from the chlorinated methanes and ethanes was incomplete (Klecka and Gonsior, 1984, Krone et al., 1989a,b). Recovery typically ranged between 30 and 60%. The remaining products were unidentified, but probably were dechlorinated, non-volatile compounds, such as those observed in the electrolytic system of Cridlle and McCarty (1991). Notably, formation of carbenes during transition-metal catalyzed reductive dehalogenation of tetrahalomethanes has been observed in other systems (Mansuy, 1980, Dolbier and Burkholder, 1988).

The recovery of dechlorinated, volatile products from PCE and TCE was essentially complete, but reductive dechlorination of cis-1,2-DCE by vitamin B₁₂ and coenzyme F₄₃₀ produced only about 10 to 20% of the vinyl chloride that was expected (Gantz and Wackett, 1991). The differences between the products of reductive dehalogenation of halomethanes and haloethanes and those produced from haloethenes, like PCE and TCE, might be due to differences in the stabilities of the free-radical intermediates that are formed. The instability of vinyl radical might preclude the formation of carbenes by strongly favoring reaction of the unpaired electron of the radical with the reduced metal complex (i.e., forcing the reaction to proceed as illustrated in Figure 2.4). Alternatively, the distribution of products formed from PCE and TCE may indicate that reductive dechlorination of these substrates proceeds through a mechanism that does not involve intermediate formation of free radicals, such as oxidative addition (Kochi, 1978), rather than through the ligand-transfer mechanism that was described in this chapter.

The relative rates of reductive dehalogenation that were catalyzed by hemes, corrinoids, and coenzyme F₄₃₀ have important ecological implications. Heme proteins are widely distributed among aerobic and anaerobic bacteria (Wackett et al., 1989). Heme proteins in which the reduced iron atom is accessible to substrate, such as hemoglobin, myoglobin, and cytochrome P-450, catalyze reductive dehalogenation reactions (Wade and Castro, 1973b, Castro et al., 1985). Because the rates of heme-catalyzed reductive dehalogention reactions are much closer than the rates of corrinoid- and coenzyme F₄₃₀-catalyzed reactions, however, hemes are probably not important catalysts of these reactions in anaerobic bacteria.
The distribution of corrinoids and coenzyme F₄₃₀ among bacteria is much more restricted than is that of hemes. Coenzyme F₄₃₀, for example, has been found only in methanogens (Daniels et al., 1984). Corrinoids are more widely distributed than coenzyme F₄₃₀, but the highest concentrations are found in bacteria that use the acetyl-CoA pathway for acetate metabolism (Zeikus et al., 1985, Dangel et al., 1987).

Coenzyme F₄₃₀ is a component of methyl reductase, the enzyme that catalyzes the formation of methane from methyl-CoM (Daniels et al., 1984, Vogels et al., 1988). Thus, it is present in all methanogens. Methyl reductase can constitute a very large fraction (up to 12%) of the total protein in methanogens (Ellefson and Wolfe, 1981). Furthermore, methyl reductase is known to be inhibited by alkyl halides (Gunsalus and Wolfe, 1978). Thus, the active site of methyl reductase is likely to be accessible to and reactive with halogenated aliphatics. These characteristics, coupled with its high concentration in methanogens, makes methyl reductase a likely catalyst of reductive dehalogenation reactions in methanogenic ecosystems.

Corrinoid proteins are involved in methyl transfer reactions (Wackett et al., 1989) and are particularly important during carbon monoxide dehydrogenase-catalyzed synthesis of acetyl-CoA from single-carbon substrates and during methanogenesis from acetate and methanol (Wood et al., 1986, Zeikus et al., 1985). These methyl transferase are inactivated by alkyl halides, and alkylated cobamides have been detected (Ghambeer et al., 1971). Thus, the reduced cobalt ion is accessible to soluble substrates and reactive with halogenated aliphatics. Acetoclastic methanogens (Wood et al., 1986, Zeikus et al., 1985) and some acetate-oxidizing sulfidogens (Widdel, 1988) metabolize acetate using carbon monoxide dehydrogenase. Homoacetogens use carbon monoxide dehydrogenase to produce acetate from single-carbon substrates, such as CO₂, formate, and methanol (Wood et al., 1986; Zeikus et al., 1985). Many autotrophic anaerobes, including methanogens, sulfidogens, and homoacetogens, use the acetyl-CoA pathway to fix CO₂ into acetate for biosynthesis (Wood et al., 1986; Zeikus et al., 1985). In a survey of the reductive dehalogenating activity of a variety of anaerobes, only those bacteria that possessed the acetyl-CoA pathway (either anabolic or catabolic pathways) were able to dechlorinate carbon tetrachloride (Egli et al., 1988).

The distribution and some of the important functions of the metal-containing coenzymes that were discussed in this section are summarized in Table 2.3. This table also lists the mixed second-order rate coefficients for the reductive dechlorination of several important halogenated aliphatics by hematin, vitamin B₁₂, and coenzyme F₄₃₀. It shows that reductive dechlorination reactions are catalyzed most rapidly by corrinoids and coenzyme F₄₃₀, which are present in high concentrations only in strictly anaerobic bacteria.

2.6 Conclusions

In anaerobic environments, the most important mechanism for biotransformation of highly halogenated aliphatic hydrocarbons is reductive dehalogenation. In these reactions, carbon-halogen bond cleavage is accompanied by transfer of electrons to the halogenated substrate. Reductive dehalogenation reactions are catalyzed by reduced metal complexes, which probably occur at the active sites of redox-active metalloenzymes in
### Table 2.3 Summary of the Reductive Dechlorination Activity and Distribution of Several Important Metal-Containing Coenzymes

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>$l_e$ $X$ $^{(1)}$ (L/μmole coenzyme-hr)</th>
<th>Distribution: Examples of Functions</th>
</tr>
</thead>
</table>
| Hemes (e.g., hematin) | $k_{CCl_4} = 0.052$  
                 | $k_{PCE} = 0.0035$  
                 | $k_{TCE} = 0.00052$ | many aerobic and anaerobic bacteria$^{(2)}$:  
                           | important functions:  
                           | (a) cytochromes  
                           | (b) mono- and dioxygenases  
                           | (c) sulfite reductase |
| Corrinoids (e.g., vitamin B$_{12}$) | $k_{CCl_4} = 1.6$  
                 | $k_{PCE} = 0.16$  
                 | $k_{TCE} = 0.0061$ | widely distributed, but especially high concentrations in$^{(3,4,5,6)}$:  
                           | (1) acetoclastic methanogens and sulfidogens;  
                           | (2) methanol-consuming methanogens; and  
                           | (3) homoacetogens:  
                           | important function: methyl transferases |
| Coenzyme $F_{430}$ | $k_{CCl_4} = 2.2$  
                 | $k_{PCE} = 0.080$  
                 | $k_{TCE} = 0.0043$ | found only in methanogens$^{(3)}$:  
                           | important function: methyl reductase |

$^{(1)}$ Gantzer and Wackett (1991)  
$^{(2)}$ Wackett et al. (1989)  
$^{(3)}$ Daniels et al. (1984)  
$^{(4)}$ Dangel et al. (1987)  
$^{(5)}$ Zeikus et al. (1985)  
$^{(6)}$ Wood et al. (1986)

Bacterial cells Corrinoids and coenzyme $F_{430}$ are the most likely microbial catalysts of reductive dehalogenation reactions.

Reductive dehalogenation reactions are believed to proceed through alkyl-free radical intermediates. The products that have been observed frequently result from replacement of a carbon-halogen bond by a carbon-hydrogen bond or from elimination of vicinal halogen atoms with formation of a carbon-carbon double bond between the dehalogenated carbon atoms. Oxygenated or dehalogenated non-volatile products are also common. Recently, a reductive mechanism that involves the intermediate formation of carbenes (alkyl diradicals) was proposed to account for these products.
Electron-donor and electron-acceptor substrates affect the rates of reductive dehalogenation reactions in a variety of systems, ranging from microcosms to cell-free extracts. The mechanism through which these substrates affect reductive dehalogenation is not always clear. Because they control the availability of electrons in bacterial metabolic pathways, primary electron donors and acceptors may have a direct effect on the rate of reductive dehalogenation reactions by controlling the concentration of one of the reactants. Observations presented in the literature point to the operation of this direct effect. In the following chapter, a kinetic model for the direct effects of primary substrates is developed. The experimental section of this report tests the kinetic model and provides quantitative demonstration of the effects of primary substrates on the rates of reductive dehalogenation reactions.
CHAPTER 3 A MODEL FOR THE RATE OF REDUCTIVE DEHALOGENATION

Although biodegradation kinetics are commonly analyzed by assuming that the reaction rate is controlled only by the concentrations of the substrate and biomass, the mechanism of reductive dehalogenation suggests that the concentrations of the primary substrates, which represent the major source and sink for electrons in bacterial metabolism, also may be important. In this chapter, a kinetic model, which is called the Primary Substrates Model, is developed to describe the effects of electron-donor and acceptor substrates on the availability of intracellular electrons, and, therefore, on the rate of reductive dehalogenation.

3.1 Conceptual Model

The mechanisms of reductive dehalogenation reactions are described in detail in Chapter 2. Briefly, reductive dehalogenation occurs when a halogenated aliphatic, RX, reacts with a reduced metalloenzyme, E-M^\(n^-\). The reaction involves intermediate formation of a coordination complex between a halogen atom of the substrate and a reduced metal ion at the active site of the enzyme. During the course of the reaction, two electrons are transferred from the enzyme to the halogenated substrate, resulting in formation of a reduced, dehalogenated aliphatic product and an oxidized metalloenzyme. The stoichiometry of the reductive dehalogenation reaction is

\[ RX + E-M^n + H_2O \rightarrow RH + E-M^{n+2} + X^- + OH^- \] (R3 1)

If the metalloenzyme that catalyzes reductive dehalogenation reactions is a component of the normal metabolic pathways for energy generation, then a second pathway through which the reduced enzyme can become oxidized exists. Here, oxidation of the reduced metalloenzyme is coupled to reduction of the primary electron-acceptor substrate. Although oxidation of the metalloenzyme and reduction of the primary electron acceptor can be linked via a complicated pathway that involves intermediate electron carriers, only the net reaction must be considered in this model. The stoichiometry of this reaction is

\[ A + \delta E-M^n + 2\delta H^+ \rightarrow AH_{2\delta} + \delta E-M^{n+2} \] (R3 2)

in which A is the primary electron acceptor, AH_{2\delta} is its reduced product, and \( \delta (M_{\text{Av}}M_A^{-1}) \) is a stoichiometric coefficient for this reaction.

The reductive dehalogenation reaction, R 3 1, involves the reduced metalloenzyme as a reactant. The oxidized enzyme that is a product of the reaction is incapable of reacting with another halogenated substrate. The enzyme can function as a true catalyst only when reactions R 3 1 and R 3 2 are coupled to a second reaction in which the oxidized metalloenzyme becomes reduced. This can occur if another pathway allows electrons derived from oxidation of the primary electron donor, DH_{2\delta}, to be transferred.
to the oxidized enzyme. Such a pathway may involve one or many intermediate electron carriers (e.g., NADH and reduced ferredoxin). Oxidation of the electron donor and reduction of the metalloenzyme need not be directly coupled, and only the net reaction must be considered in this model. The stoichiometry of the overall reaction that links oxidation of the electron donor to reduction of the oxidized metalloenzyme is:

\[
DH_{2\beta} + \beta E-M^n \cdot 2 \rightarrow D + \beta E-M^n + 2\beta H^+
\]  \hspace{1cm} (R 3.3)

where \( \beta \) \((M_{enc}^{-1}DH_{2\beta})\) is a stoichiometric coefficient for the net reaction, and D represents the products of electron donor oxidation.

The oxidized metalloenzyme also can be regenerated to its reduced form when the source of electrons is the oxidation of biomass, a process that always is possible in intact cells. In this case, the electron donor is an endogenous electron-donor substrate. The reaction is exactly parallel to R 3.3, except that D is replaced by biomass, given the symbol X.

The four processes—reductive dehalogenation, reduction of the primary electron acceptor, oxidation of the primary electron donor, and oxidation of biomass—all involve the metalloenzyme that functions as the reductive dehalogenase. The relationships among these processes form the conceptual basis for this model and are shown in Figure 3.1. In this model, the effects of primary substrates on the rate of reductive dehalogenation are mediated through their effects on the steady-state concentration of the reduced form of the enzyme (i.e., E-M^n) that catalyzes this reaction. The central role of the metalloenzyme in these four processes provides a means by which the effects of primary substrates can be considered explicitly in the reductive dehalogenation rate equation. The method by which this is accomplished is described in the following section.

3.2 Mathematical Model

Figure 3.1 shows the interactions that occur among the reactions that produce and consume the reduced form of the reductive dehalogenase. If the concentrations of the electron-donors, electron-acceptor, and halogenated aliphatic substrates remain relatively constant then the concentrations of the reduced and oxidized forms of the dehalogenase reaches a steady state. The reduced enzyme is a reactant in the reductive dehalogenation reaction, and a rate equation that is a function of the concentration of this form of the enzyme can be written. The steady-state concentration of the reduced dehalogenase can be obtained by solving the appropriate mass-balance equation, and this concentration can be substituted into the rate equation for reductive dehalogenation. The result is an expression that gives the rate of reductive dehalogenation as a function of the concentrations of the primary substrates biomass, the halogenated substrate, and the metalloenzyme.

25
Figure 3.1 Conceptual model. Interactions occur among exogenous and endogenous electron-donor substrates, the primary electron-acceptor substrate, and the halogenated-aliphatic substrate during reductive dehalogenation.
The mass balance on the reduced dehalogenase has the following general form.

\[
\frac{d[E-M^n]}{dt} = \text{rate of production of } E-M^n - \text{rate of consumption of } E-M^n \quad (3.1)
\]

The details of the mass balance are omitted for now, because the specific reactions that are considered in the production and consumption of the reduced enzyme are described in the next section. At this point, the connection of \([E-M^n]\) to the rate of reductive dehalogenation is developed.

The oxidized enzyme is a reactant in the reactions that produce the reduced enzyme. Therefore, to solve Equation 3.1 for the steady-state concentration of the reduced enzyme, an equation that relates the concentrations of the oxidized and reduced forms of the dehalogenase is required. This relationship can be obtained from a mass balance on the total dehalogenase. In this model, we assume that only two forms of the enzyme are important. The resulting mass balance is

\[
[E-M]_{\text{total}} = [E-M^n] + [E-M^n + 2] \quad (3.2)
\]

Since the total concentration of the dehalogenase cannot be measured, it must be related to some measurable quantity, such as biomass. This can be done by assuming that the dehalogenase represents some constant fraction, \(f \left( M_{\text{enz}} M_X^{-1} \right) \), of the total biomass.

\[
[E-M]_{\text{total}} = fX \quad (3.3)
\]

where \(X \left( M_X L^{-3} \right) \) is the biomass concentration. The experimental conditions employed in this research preclude short-term (i.e., during the course of an experiment) changes in \(f\), and no evidence exists for long-term changes (i.e., over the course of several years). Factors that affect \(f\), however, can have an important influence on the success or failure of reductive dehalogenation as a tool for bioremediation. The fraction of biomass that comprises the reductive dehalogenase can be affected by events at a molecular level (i.e., enzyme induction and inactivation) and at a community level (i.e., changes in the population structure of the microbial community). Therefore, understanding what controls \(f\) may be a very fruitful area for future research.

When all of the manipulations that have been described above are performed, the resulting equation describes the dependence of the steady-state rate of reductive dehalogenation on the concentrations of the primary substrates, the halogenated substrate, and the total biomass. In general, this kinetic expression can be rearranged and written in the following form:

\[
r_{RX} = \frac{q_{m,ap} X[RX]}{K_{ap} + [RX]} \quad (3.4)
\]
in which $r_{RX} (M_{RX}L^{-3}T^{-1})$ is the steady-state rate of reductive dehalogenation, $q_{m,ap} (M_{RX}M_{X}^{-1}L^{-1}T^{-1})$ is the apparent maximum specific rate of reductive dehalogenation, and $K_{ap} (M_{RX}L^{-3})$ is the apparent half-saturation concentration. Equation 3.4 is formally similar to the Monod equation, which is frequently used to describe biodegradation kinetics, except that $q_{m,ap}$ and $K_{ap}$ are not constants. Instead, they are functions of the concentrations of the primary substrates.

Regardless of the specific functional relationship between primary-substrate concentrations and the values of the apparent Monod parameters, Equation 3.4 states that, if reductive dehalogenation reactions are studied in the presence of constant concentrations of the primary electron-donor and -acceptor substrates, Monod kinetics provide an adequate description of the observations. Therefore, it should be possible to test this aspect of the model by determining the apparent Monod kinetic parameters for reductive dehalogenation in the presence of different, but constant concentrations of the primary substrates.

In the remainder of this chapter, a solution to the Primary Substrates Model is derived. Particular emphasis is placed on the functional relationships that are predicted for the apparent Monod kinetic parameters, $q_{m,ap}$ and $K_{ap}$, and for the apparent pseudo-first-order kinetic coefficient for reductive dehalogenation, $k_{ap} (L^{-3}M_{X}^{-1}T^{-1})$, which is defined to be

$$k_{ap} = \frac{q_{m,ap}}{K_{ap}} \quad (3.5)$$

3.3 Solution to the Primary Substrates Model

When biomass and the primary electron donor contribute electrons to reductive dehalogenation, the steady-state mass balance on the reduced form of the dehalogenase is

$$\frac{d[E-M^n]}{dt} = \beta r_{di} + \gamma r_{d2} - \delta r_{a1} - \alpha r_{RX} = 0 \quad (3.6)$$

where $\beta$ is the stoichiometric coefficient ($M_{enz}M_{D}^{-1}$) that relates the amount of primary donor oxidized to the amount of enzyme reduced, $r_{di}$ is the rate at which primary donor is oxidized ($M_{D}L^{-3}T^{-1}$), $\gamma$ is a stoichiometric coefficient ($M_{enz}M_{X}^{-1}$) that relates the amount of biomass that is oxidized to the amount of oxidized enzyme that is reduced, $r_{d2} (M_{X}L^{-3}T^{-1})$ is the rate at which biomass is oxidized, $\delta$ is the stoichiometric coefficient ($M_{enz}M_{A}^{-1}$) that relates the amount of enzyme that is oxidized to the amount of acceptor that is reduced, $r_{a1}$ is the rate at which acceptor is reduced ($M_{A}L^{-3}T^{-1}$), $\alpha$ is the stoichiometric coefficient ($M_{enz}M_{RX}^{-1}$) that relates the amount of enzyme oxidized to the amount of halogenated compound reduced, and $r_{RX}$ is the rate at which the halogenated compound
is reduced \((M_{RX}L^{-3}T^{-1})\). The rates of the four reactions are assumed to follow mixed second-order kinetics:

\[
    r_{d1} = k_{d1} [E-M^n]^2 [DH_{2B}]
\]

\[
    r_{d2} = k_{d2} [E-M^n]^2 [X] = bX
\]

\[
    r_{a1} = k_{a1} [E-M^n] [A]
\]

\[
    r_{RX} = k_{RX} [E-M^n] [RX]
\]

where \(k_{d1}, k_{d2}, k_{a1},\) and \(k_{RX}\) are the mixed second-order rate coefficients \((L^3T^{-1}M^{-1})\) for donor oxidation, biomass oxidation, acceptor reduction, and halogenated compound reduction, respectively. The coefficient \(b\) is the traditional biomass-decay coefficient \([T^{-1}]\).

Substituting Equations 3.7(a-d), 3.2, and 3.3 into Equation 3.6 and letting \(\frac{d [E-M^n]}{dt} = 0\) gives the steady-state concentration of reduced metalloenzyme:

\[
    [E-M^n]_{ss} = \frac{(\beta k_{d1}[DH_{2B}] + \gamma k_{d2}X) fX}{\beta k_{d1}[DH_{2B}] + \gamma k_{d2}X + \delta k_{a1}[A] + \alpha k_{RX}[RX]}
\]

When Equation 3.8 is substituted in Equation 3.7d, a Monod-like equation is obtained. The apparent Monod kinetic parameters are shown in Table 3.1.

The apparent maximum specific rate of reductive dehalogenation \((q_{im,ap})\) is a linear function of the exogenous electron-donor concentration, but it has a positive intercept (Equation 3.9a), \(f \frac{\gamma}{\alpha} k_{d2}X\). The model does not require the rate of reductive dehalogenation to be zero in the absence of added electron donor, because biomass serves as an endogenous electron donor. Increasing the donor increases \(q_{im,ap}\), but \([A]\) has no effect. The apparent half-saturation concentration, \(K_{ap}\) (Equation 3.9b) depends on \([DH_{2B}]\), \(X\), and \([A]\). It has a positive intercept value \(\frac{\gamma k_{d2}X}{\alpha k_{RX}}\) when the primary substrates are absent, but the presence of either primary substrate increases \(K_{ap}\).
Table 3.1 Apparent Monod Kinetic Parameters for the Primary Substrates Model

\[
q_{in,ap} = \beta k_{d1}\frac{[D]}{\alpha k_{RX}} + \gamma k_{d2}\frac{X}{\alpha k_{RX}} + \delta k_{a1}\frac{[A]}{\alpha k_{RX}} \tag{3.9a}
\]

\[
K_{ap} = \frac{\beta k_{d1}[DH_2\beta]}{\alpha k_{RX}} + \frac{\gamma k_{d2}X}{\alpha k_{RX}} + \frac{\delta k_{a1}}{\alpha k_{RX}}[A] \tag{3.9b}
\]

\[
k_{ap} = \frac{fk_{RX}(\beta k_{d1}[DH_2\beta] + \gamma k_{d2}X)}{\beta k_{d1}[DH_2\beta] + \gamma k_{d2}X + \delta k_{a1}[A]} \tag{3.9c}
\]

\[
k_{ap}' = \frac{fk_{RX}(\beta k_{d1}[DH_2\beta] + \gamma k_{d2}X)}{\beta k_{d1} + \delta k_{a1}[1]} + \frac{\gamma k_{d2}X}{\delta k_{a1}} \tag{3.9d}
\]

**NOTES**

- \( k_{ap} \) is the apparent first-order rate constant and equals \( q_{in,ap}/K_{ap} \)
- \( k_{ap}' \) is the apparent first-order rate coefficient for the special case when the concentrations of the electron donor and electron acceptor covary in a fixed ratio g moles\(_a\)/mroles\(_p\)

When the concentrations of the exogenous primary substrates vary independently (e.g., for sulfate reduction or when \( H_2 \) is the electron donor for autotrophic methanogens), the apparent first-order rate coefficient (Equation 3.9c) increases as the concentration of the electron donor increases. Although \( k_{ap} \) is a hyperbolic function of the electron-donor concentration, its intercept is not at the origin, because biomass oxidation provides a source of electrons when an exogenous electron donor is absent.

The effects of electron donor concentration on the kinetics of reductive dehalogenation, in the presence of a constant concentration of the primary electron acceptor and a constant biomass concentration, are shown in Figure 3.2. The kinetic and stoichiometric parameters used to generate these predictions are presented in Table 3.2. These parameters were chosen to correspond to a hypothetical situation in which CCl\(_4\) is reductively dechlorinated to CHCl\(_3\) by a pure culture of formate-oxidizing, sulfate-reducing bacteria. The dehalogenase enzyme is assumed to be corrinoid containing. If a parameter could not be found for a sulfate reducer, it was assumed to be equal to those reported for \( H_2 \) or formate-oxidizing methanogens.

Because \( q_{in,ap} \) and \( K_{ap} \) increase as the concentration of the exogenous donor increases, the rate of reductive dehalogenation increases as the donor and halogenated-
compound [RX] concentrations increase. The effect of [RX] is hyperbolic, while the effect of the donor concentrations depends on [RX]. Only for relatively large [RX] does an increase in donor concentration bring about a significant increase in the rate of reductive dehalogenation. Figure 3.2 illustrates that the rate of reductive dehalogenation does not go to zero as the concentration of the exogenous electron donor approaches zero, because the endogenous electron supply is sufficient when [RX] is relatively small.

Figure 3.3 illustrates the competitive effect of the primary electron acceptor. The trend is very clear. Increasing the acceptor concentration greatly slows the rate of reductive dehalogenation, especially when [RX] is small. (The results in Figure 3.3 do not match exactly those in Figure 3.2, because $b = k_{a2} = 0$ was imposed for Figure 3.3.)

An important special case occurs when the concentrations of the exogenous electron donor and acceptor covary with a constant ratio $g$ (e.g., when formate is the electron donor and acceptor for H$_2$-oxidizing methanogens). Then, the apparent first-order rate coefficients, $k_{ap}$, is given by Equation 3.9d (Table 3.1). Equation 3.9d is similar to Equation 3.9c in that $k_{ap}$ is a hyperbolic function of the concentration of the exogenous primary donor substrate. However, an important difference is that $k_{ap}$ decreases as the concentration of the exogenous primary substrate increases. Thus,
### Table 3.2 Kinetic and Stoichiometric Parameters Used in Primary Substrates Model Predictions

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{RX} = 450$ L/mole enz-s</td>
<td>Gantzer and Wackett (1991)</td>
</tr>
<tr>
<td>$k_{d1} = 1.4 \times 10^5$ L/mole enz-s</td>
<td>Schauer, <em>et al.</em> (1982)</td>
</tr>
<tr>
<td>$k_{d2} = \frac{b}{\sqrt{X}}$; $b = 1.04 \times 10^{-6}$ s$^{-1}$</td>
<td>Costello, <em>et al.</em> (1991)</td>
</tr>
<tr>
<td>$q_{m,a1} = 6.45$ mole SO$_4^{2-}$/mole enz-s</td>
<td>Ingvorsen, <em>et al.</em> (1984)</td>
</tr>
<tr>
<td>$K_{a1} = 5.5 \times 10^{-5}$ mole/L</td>
<td>Ingvorsen and Jorgensen (1984)</td>
</tr>
<tr>
<td>$k_{a1} = \frac{q_{m,a1}}{K_{a1}} = 1.2 \times 10^5$ L/mole enz-s</td>
<td>Nethe-Jaenchen and Thauer (1984)</td>
</tr>
<tr>
<td>$k_{a2} = 1.4 \times 10^5$ L/mole enz-s*</td>
<td>assumed</td>
</tr>
</tbody>
</table>

* $[A2] = 9 \times 10^{-5}$ mole/L ($[A2] = [H_2CO_3]$, pH = 7.3, $C_{T,CO_3} = 10^{-3}$ M)

<table>
<thead>
<tr>
<th>Stoichiometric Parameters</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$f = 2 \times 10^{-10}$ mole enz/mg cells</td>
<td>Dangel, <em>et al.</em> (1987)*</td>
</tr>
<tr>
<td>$\beta = 1$ mole enz/mole formate</td>
<td>HCOOH + $E-M^{n+2}$ ----&gt; CO$_2$ + $E-M^n$ + 2H$^+$</td>
</tr>
<tr>
<td>$\delta = 3$ moles enz/mole SO$_4^{2-}$</td>
<td>HSO$_3^{-}$ + 3$E-M^n$ + 6H$^+$ ----&gt; HS$^-$ + 3H$_2$O + 3$E-M^{n+2}$</td>
</tr>
<tr>
<td>$\gamma = 8.8 \times 10^{-5}$ moles enz/mg biomass</td>
<td>C$_5$H$_7$O$_2$N + 13H$_2$O + 10$E-M^{n+2}$ ----&gt; 5HCO$_3^{-}$ + NH$_4^+$ + 24H$^+$ + 10$E-M^n$</td>
</tr>
<tr>
<td>$\kappa = 1$ mole enz/mole acceptor</td>
<td>assumed</td>
</tr>
<tr>
<td>$g = \frac{1}{3}$ mole acceptor/mole donor</td>
<td>assumed</td>
</tr>
</tbody>
</table>

* assuming 1 mole enzyme/mole contaminant

\[
k_{op,o} = f k_{RX} \tag{5.10}
\]

and reductive dehalogenation is controlled by the endogenous decay rate The opposite
limiting case is when the rate of reduction of the dehalogenase by electrons from the exogenous donor is much larger than the rate at which the enzyme is reduced by electrons from the endogenous donor (i.e., \( \beta k_{dl}[\text{DH}_{2p}] \gg \gamma k_{d2}X \)). Then the apparent first-order rate coefficient is

\[
k'_{ap,\infty} = \frac{\beta f_{RX} k_{dl}}{\beta k_{dl} + \delta g k_{al}}
\] (311)

By definition, all of the stoichiometric and kinetic coefficients are positive. Therefore, \( \frac{\beta k_{dl}}{\beta k_{dl} + \delta g k_{al}} < 1 \) and \( k_{ap,0} > k_{ap,\infty} \). That is, this special case of the Primary Substrates Model predicts that the apparent first-order rate coefficient decreases in the presence of exogenous primary substrates if the concentrations of the electron donor and acceptor covary.

The decrease in \( k'_{ap} \) that accompanies an increase in the concentrations of the exogenous primary substrates is a result of one of the assumptions that was made in this formulation of the model. In the absence of exogenous primary substrates, the
halogenated aliphatic substrate, RX, is the only electron acceptor that is considered. Under these conditions, all of the electrons derived from biomass oxidation must go to reductive dehalogenation. Therefore, the rate is limited only by the intrinsic kinetics of the reaction (i.e., enzyme turnover). When primary substrates are added, the supply of electrons increases, but competition for those electrons also increases, because the concentrations of the electron donor and acceptor increase simultaneously. The net result is a decrease in the rate of reductive dehalogenation upon addition of the primary substrates, because the primary acceptor increasingly competes with the halogenated substrate.
CHAPTER 4 EXPERIMENTAL METHODS

The methods that were used to study the kinetics of TCA biodegradation in anaerobic biofilm reactors are described in this chapter. It includes descriptions of the experimental methods that were used, as well as a discussion of the method that was used to analyze the kinetic data.

4.1 Cultivation of Anaerobic Consortia

4.1.1 Physical Characteristics of Biofilm Reactors

Sulfate-reducing and methanogenic consortia were grown in completely mixed, packed-bed biofilm reactors. A schematic diagram of these reactors is shown in Figure 4.1, and the physical characteristics are given in Table 4.1.

Completely mixed conditions, promoted by using a large recycle ratio, were used to provide a uniform distribution of biomass throughout the reactors. When the fractional substrate removal is large, however, concentration gradients can develop across the reactor. The effects of these gradients on the distribution of biomass were minimized by reversing the direction of flow through the reactors every week.

Methanogens and sulfate reducers are strict anaerobes and are very sensitive to inhibition by traces of $O_2$. Oxygen contamination of the systems by diffusion of $O_2$ through the reactor tubing was minimized by using the most oxygen-impermeable tubing that was available. PVC tubing (1.52 mm I.D., $D_0 = 1.26 \times 10^{-7}$ molle $O_2$/cm$^2$/day-atm, Gilson Medical Electronics) was used in the feed pump. Norprene (Size 15, $D_{O2} = 9.42 \times 10^{-8}$ mole $O_2$/cm$^2$/day-atm, Cole-Parmer) was used in the recycle pump, and the transfer tubing was 1/8" (O.D.) Teflon (Cole-Parmer) encased within 1/4" (O.D.) x 1/8" (I.D.) Norprene (Cole-Parmer). The feed pump was a Gilson Minipuls 2 peristaltic pump (Gilson Medical Electronics), and the recycle pump was a Masterflex variable speed peristaltic pump (Model # 7520-10, Cole-Parmer).

4.1.2 Media Preparation

The composition of the anaerobic consortium that was cultivated in each reactor was controlled by the electron acceptor that was supplied in the feed medium. One of the reactors (R1) received no exogenous electron acceptor, and a methanogenic consortium developed. The other reactor (R2) was fed sulfate, leading to development of a sulfidogenic consortium. Both systems received glucose as the primary electron-donor substrate. The glucose concentration in the feed media was low ([glucose]$_{inf} = 10$ mg/L) to encourage the growth of oligotrophs, which are probably the most important bacteria in subsurface environments (Gihiorse and Wilson, 1988; Balkwill et al., 1989) and in some biological treatment reactors. The compositions of the feed media are presented in Table 4.2.

Because methanogens and sulfidogens are inactivated by traces of oxygen, precautions were taken to remove $O_2$ from the media during preparation and to prevent...
recontamination during storage. Dissolved O₂ was removed from autoclaved media by bubbling the hot solutions extensively with O₂-free N₂. The deoxygenated media were reduced by addition of Na₂S (0.1 mM). The media were stored under a positive pressure of N₂ at all times to prevent contamination by atmospheric O₂.

4.2 Quantitative Analysis of Substrate and Product Concentrations

The analytical methods that were used to quantify the concentrations of important substrates and products are described in this section. The substrate that is of most interest is 1,1,1-trichloroethane (TCA), because the kinetics of TCA biodegradation were the main topic of this research. The concentrations of other substrates (e.g., formate and sulfate) and products (e.g., methane) were also monitored, because the removal (or production) of these compounds reflects the metabolic activity of specific groups of bacteria within the reactors.
Table 4.1 Physical Characteristics of Biofilm Reactors

<table>
<thead>
<tr>
<th>Dimension</th>
<th>Symbol (units)</th>
<th>Methanogenic Reactor (R1)</th>
<th>Sulfate-Reducing Reactor (R2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>reactor length</td>
<td>r (cm)</td>
<td>7.4</td>
<td>7.1</td>
</tr>
<tr>
<td>diameter</td>
<td>d (cm)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>cross-sectional area</td>
<td>A_{cs} (cm²)</td>
<td>4.9</td>
<td>4.9</td>
</tr>
<tr>
<td>total volume</td>
<td>V_T (cm³)</td>
<td>36.3</td>
<td>34.9</td>
</tr>
<tr>
<td>diameter of glass beads</td>
<td>d_p (cm)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>surface area of glass beads</td>
<td>A (cm²)</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td>specific surface area of reactors</td>
<td>a (cm⁻¹)</td>
<td>12.6</td>
<td>12.8</td>
</tr>
<tr>
<td>flow rate</td>
<td>Q (cm³/hr)</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>recycle flow rate</td>
<td>Q_R (cm³/hr)</td>
<td>3,000</td>
<td>3,000</td>
</tr>
<tr>
<td>recycle ratio</td>
<td>Q_{R} / Q</td>
<td>75</td>
<td>75</td>
</tr>
</tbody>
</table>

4.2.1 1,1,1-Trichloroethane (TCA)

The concentration of TCA in reactor influent and effluent samples was measured using the methods of Henderson et al (1976). In this method, TCA was extracted from aqueous samples into an organic solvent (octane was used in this research), and the concentration of TCA in the organic phase was determined by gas chromatography. This method differs from traditional liquid-liquid extraction procedures in that the extraction was performed in the absence of a gas phase, because TCA (like all low molecular weight halogenated aliphatics) is highly volatile.
Table 4.2 Composition of Feed Media for Completely-Mixed Anaerobic Biofilm Reactors

<table>
<thead>
<tr>
<th>Component</th>
<th>Methanogenic Reactor (mg/L)</th>
<th>Sulfate-Reducing Reactor (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>resazurin</td>
<td>0.375</td>
<td>0.375</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>58.2</td>
<td>58.2</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>82.1</td>
<td>82.1</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>69.1</td>
<td>69.1</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>8.4</td>
<td>8.4</td>
</tr>
<tr>
<td>CaCl₂ 2H₂O</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>MnCl₂ 4H₂O</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Na₂MoO₄ 2H₂O</td>
<td>0.1</td>
<td>---</td>
</tr>
<tr>
<td>MgCl₂ 6H₂O</td>
<td>16.9</td>
<td>---</td>
</tr>
<tr>
<td>MgSO₄ 7H₂O</td>
<td>---</td>
<td>25.0</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>---</td>
<td>11.4</td>
</tr>
<tr>
<td>FeCl₂ 4H₂O</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>glucose</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>vitamins*</td>
<td>1.25 mL/L</td>
<td>1.25 mL/L</td>
</tr>
</tbody>
</table>

*Composition of vitamins solution:

<table>
<thead>
<tr>
<th>vitamin</th>
<th>Stock Solution Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>biotin</td>
<td>2</td>
</tr>
<tr>
<td>folic acid</td>
<td>2</td>
</tr>
<tr>
<td>pyridoxine-HCl</td>
<td>10</td>
</tr>
<tr>
<td>riboflavin</td>
<td>5</td>
</tr>
<tr>
<td>thiamine</td>
<td>5</td>
</tr>
<tr>
<td>nicotinic acid</td>
<td>5</td>
</tr>
<tr>
<td>pantothenic acid</td>
<td>5</td>
</tr>
<tr>
<td>vitamin B₁₂</td>
<td>0.1</td>
</tr>
<tr>
<td>p-aminobenzoic acid</td>
<td>5</td>
</tr>
<tr>
<td>thiamic acid</td>
<td>2</td>
</tr>
</tbody>
</table>

The extractions were performed in 14 8-mL serum bottles, which were completely filled with a mixture containing a known volume of aqueous sample (usually 10 to 30 mL) and acidified distilled water. The serum bottles were sealed with Teflon-faced rubber stoppers (Supleco, Inc) and aluminum crimp seals (Wheaton). Octane (Aldrich, 99+%) was
injected into the serum bottles through the septa using Hamilton Gas-Tight syringes (Supelco, Inc.), displacing an equal volume of diluted sample. TCA was extracted into octane by vigorously shaking the serum bottles by hand for approximately five minutes.

The extraction efficiency of TCA into octane was essentially 100%, but its concentration in the organic phase was very sensitive to variations in the volume of octane that was added and to the actual volume of the serum bottles in which the extractions were performed. These sources of variation were minimized by adding a precise volume of an internal standard (usually 5.0 μL of a 100 mg/L solution of TCE in methanol) to the diluted aqueous sample before adding octane. The internal standard, like the octane, was added to the samples by injection through the septa.

When an internal standard is used, the aqueous concentration of TCA can be calculated using measurements that are known accurately (e.g., the volume of internal standard, \( V_{\text{int \, std}} \), and the volume of sample, \( V_{\text{sample}} \)) or which possess uncertainties that can be quantified (e.g., the concentrations of TCA and TCE in octane after extraction—[TCA]_{oct} and [TCE]_{oct}). The following relationship was used:

\[
[TCA]_{aq} = \frac{[TCA]_{oct} \times V_{\text{int \, std}}}{[TCE]_{oct} \times V_{\text{sample}}} \tag{4.1}
\]

The concentrations of TCA and TCE in octane following extraction were measured by gas chromatography using a Hewlett-Packard HP-5890 gas chromatograph. TCA and TCE were separated on a 6' x 1/4" glass column packed with 80/100 Carbopak B coated with 1% SP-1000 (Supelco, Inc.) They were detected and quantified using an electron capture detector (ECD). The column was operated isothermally at 190°C. The injector port and detector temperatures were 230°C. The carrier gas was 5% methane in argon, and the flow rate was 30 mL/minute. Under these conditions, the retention times were 18 minutes for TCA and 25 minutes for TCE.

Quantification of TCA and TCE in octane was performed by comparison to standards that were prepared in octane by dilution of neat compounds (Supelco, Inc.). These standards were stored at -20°C in 15 mL screw-cap vials sealed with Teflon-faced silicone septa. The concentrations of standards stored in this way were stable indefinitely. The detector response was slightly nonlinear over the range of standard concentrations that were routinely used (approximately 20 to 600 μg/L), but the standard curves could be adequately represented using two linear regions (0 to 200 μg/L and 200 to 600 μg/L).

4.2.2 Methane

Methane is the product of the electron-acceptor reactions that occur in methanogenic bacteria, and its production is an important indicator of the activity of these organisms.

The concentration of methane in reactor effluent was determined by extracting dissolved methane into \( \text{N}_2 \) and measuring its gas phase concentration by gas
Nitrogen gas was added directly to the 30-mL ground-glass syringes that were used to collect the effluent samples. Dissolved methane was extracted into N₂ by vigorously mixing the two phases for approximately 45 seconds and allowing them to separate for 30 seconds. The gas phase was then transferred to 15-mL serum bottles, displacing the acidified distilled water with which they had been filled. This procedure was performed twice on each effluent sample to insure that all methane was stripped from solution. Additional methane could not be recovered from the aqueous samples by a third extraction.

The methane concentration in N₂ was measured using an HP-5830A gas chromatograph equipped with a 6’x1/8” stainless-steel column packed with 80/100 Porapak Q (Supelco, Inc). The column temperature was 100°C, and the injector port and detector temperatures were 150°C. The carrier gas was N₂, and the flow rate was 30 mL/minute. Under these conditions, methane had a retention time of 0.4 minutes.

Methane was detected and quantified using a flame ionization detector (FID). Standards were prepared by dilution of 100% methane (Supelco, Inc) in 120- to 160-mL serum bottles containing N₂. The standard curve was linear over the entire range of concentrations that were used (from approximately 160 pmoles CH₄/injection to 5 nmoles CH₄/injection). These standards could be used many times, but damage to the rubber stoppers that were used to seal the serum bottles eventually caused them to become unreliable, at which time they were replaced.

4.2.3 Sulfate

Sulfate is the primary electron acceptor for sulfate-reducing bacteria, and its consumption is an important indicator of the activity of these organisms.

Sulfate was analyzed by ion chromatography (analyses were conducted by Jens Sandberger of the Illinois Natural History Survey). A Dionex 2020i ion chromatograph equipped with a conductance detector and an anion micro-membrane suppressor (AMMS-1) was used in this analysis. Separation of sulfate from other anions in solutions was accomplished using an HPIC AS4A column with a HPIC AG4A guard column. The eluant consisted of a solution of 0.75 mM NAHCO₃ and 2.2 mM Na₂CO₃ (isocratic). The detection limit for this method was approximately 10⁻⁷ mole sulfate/L.

Effluent samples were prepared for storage and chromatography by removing sulfide, which can be oxidized to sulfate during storage. Sulfide was precipitated as ZnS by adding an excess of zinc acetate (0.01 M) in the presence of 0.015 M NaOH. This concentration of NaOH was sufficient to increase the pH of the samples to between 11 and 12. The serum bottles in which the samples were stored was vigorously sparged with N₂ before, during, and for several minutes after transferring the aqueous samples. This procedure helped to prevent oxidation of sulfide to sulfate during precipitation of ZnS. The serum bottles were sealed with rubber stoppers and stored (under N₂) at 4°C. The precipitate was removed (usually within 24 hours) by centrifugation at 1,000 x g for 15 minutes. The supernatant was then carefully transferred to a fresh serum bottle using a Pasteur pipette. A Sorvall GLC-2 centrifuge and an Omni-Carrier swinging bucket rotor.
were used to separate the precipitate from the supernatant solution. A centrifugal force of 1,000 x g is obtained at an angular velocity of 2,400 rpm with this system.

4.2.4 Organic Acids

Two organic acids were of particular interest. Formate, which was used as the primary electron-donor substrate in many of the experiments, and acetate, which can be produced from formate by many homoacetogenic bacteria (which may compete with formate-oxidizing methanogens and sulfidogens) and by metabolism of fatty acids and proteins by fermentative bacteria. Consumption of formate and production of acetate are indicators of metabolic activity in the reactors, and this activity can be used to determine which groups of bacteria catalyzed TCA biodegradation in the anaerobic biofilm reactors.

Organic acids were analyzed by high-performance liquid chromatography using the method described by Ehrlich et al (1981). Formate, acetate, and other volatile fatty acids can be separated using a 9.5 mm x 300 mm stainless-steel column packed with HPX-87 cation exchange resin (Bio-Rad Laboratories). The column was operated at 50°C, and compounds were eluted isocratically using 0.013 N H$_2$SO$_4$ at a flow rate of 9.7 mL/minute. The instrument was a Waters HPLC, and detection was by absorbance at 190 nm using a Waters 486 Tunable Absorbance Detector.

Standards were prepared in distilled water from formic acid or the sodium salt of acetate. The standard curve for these three compounds was linear over the range from 0.01 mM to 4.5 mM. Under the conditions described above, the retention times for the organic acids were 12.1 minutes for formate and 13.2 minutes for acetate.

Influent and effluent samples were prepared for chromatography by filtration through 0.45-μm Gelman Acrodisc filters. The samples were acidified by addition of 10 μL of H$_2$SO$_4$ per 1.5 mL of sample and were stored in 1.5-mL screw-cap vials sealed with Teflon-faced silicone septa. The samples were stored at 4°C until analysis. Samples stored in this manner were stable for several months.

4.3 Biofilm Kinetics: Measurements of Transport Parameters and Biofilm Characteristics

The kinetics of TCA biodegradation were measured in a series of short-term experiments in which the pseudo-steady-state effluent concentration of TCA was measured over a wide range of influent TCA concentrations. The effluent substrate concentration is called pseudo-steady state to distinguish it from true steady state, because the biofilm thickness and density did not change significantly during the course of the experiments. Thus, the effluent concentration reached steady state with respect to the amount of biomass that was initially present in the system, but the thickness, density, and composition of the biofilms were determined by the long-term, completely mixed operating conditions. The method that is described here was used to estimate biodegradation kinetic parameters from short-term experiments of this type in completely mixed biofilm reactors (Rittmann et al., 1986).
In completely mixed reactors at pseudo-steady state, the substrate concentration is uniform throughout and is equal to the effluent substrate concentration. If the biofilm thickness and density are also uniform throughout the reactor, then the flux of substrate into the biofilm, \( J (\text{M} \cdot \text{L}^{-2} \cdot \text{T}^{-1}) \), is the same everywhere. In these reactors, the local flux of substrate into the biofilm at any point in the reactor is assumed to be equal to the mass-balance flux, \( J_{mb} (\text{M} \cdot \text{L}^{-2} \cdot \text{T}^{-1}) \), that is given by the mass balance on the reactor (assuming that reactions catalyzed by suspended bacteria are insignificant):

\[
J = J_{mb} = \frac{S_{inf} - S_{eff}}{a \theta} \quad (4.2)
\]

where \( S_{inf} \) and \( S_{eff} \) (\( \text{M} \cdot \text{L}^{-3} \)) are the influent and effluent substrate concentrations, respectively, \( a \) (\( \text{L}^{-1} \)) is the specific surface area of the reactor, and \( \theta \) (\( \text{T} \)) is the empty-bed detention time.

A pseudo-analytical solution that predicts the local flux into a biofilm of constant thickness as a function of the substrate concentration at the biofilm-liquid interface, \( S_s \) (\( \text{M} \cdot \text{L}^{-3} \)), and kinetic parameters has been developed (Atkinson and Davies, 1974, Rittmann and McCarty, 1981)

\[
J = \eta q_{m,p} X_f L_f \frac{S_s}{K_{ap} + S_s} \quad (4.3)
\]

where \( X_f \) (\( \text{M} \cdot \text{L}^{-3} \)) is the biomass density within the biofilm, \( L_f \) (\( \text{L} \)) is the biofilm thickness, and \( \eta \) is the effectiveness factor, which expresses the decrease in flux caused by mass-transport resistance inside the biofilm. Analyses of the kinetics obtained for TCA in this study (Wrenn, 1991) revealed that \( \eta = 1 \) and \( S_s \) approximately equalled the bulk-liquid concentration, \( S \). Furthermore, Wrenn (1991) showed that concentration changes across the reactor did not alter the kinetic analysis when the bulk liquid concentration was represented by the log-mean concentration

\[
S = S_{inf} - S_{eff} \ln \left( \frac{S_{inf}}{S_{eff}} \right) \quad (4.4)
\]

Geometry and simple experimental measurements can be used to estimate the empty-bed detention times, \( \theta \), and the specific surface areas, \( a \), of the biofilm reactors. The empty-bed detention time can be computed from the total reactor volume, \( V_T \) (Table 4.1), and the flow rate, \( Q \) (\( \text{L} \cdot \text{T}^{-1} \))

\[
\theta = \frac{V_T}{Q} \quad (4.5)
\]
The flow rates were measured during each experiment. The specific surface areas were computed using the following relationship (Rittmann et al., 1986)

\[ a = \frac{6}{d_p} (1 - \epsilon) \]  

(4.6)

where \(d_p(L)\) is the diameter of the glass beads that were used to pack the reactors and \(\epsilon\) is the porosity. The porosities of the reactors were estimated from conservative tracer experiments (Wrenn, 1991). The specific surface areas of the methanogenic and sulfate-reducing biofilm reactors are given in Table 4.1.

In order to evaluate the results of biodegradation kinetics experiments in biofilm reactors, the biofilm thickness, \(L_f\), and density, \(X_f\), must be known. Exact measurement of these parameters may be impossible, but it is relatively simple to obtain reasonable estimates.

The biofilm thickness was estimated by determining the mass of water that was contained within the biofilm (Rittmann et al., 1986). This was accomplished by measuring the change in mass that occurred when beads coated with biofilm were dried overnight at 105°C. The thickness of the biofilm is given by:

\[ L_f = \frac{W}{\rho \ n \ A \ (0.96)} \]  

(4.7)

where \(W\) (M) is the mass of water that was lost during drying, \(n\) is the number of glass beads that were dried, and \(A\) (L²) is the surface area of a single bead (Table 4.1). The biofilm was assumed to be 96% water by mass.

The biomass density was estimated by using the Hach method for chemical oxygen demand (Jirka and Carter, 1975) to measure the "biomass" attached to \(n\) beads. The biofilm density is given by:

\[ X_f = \frac{\text{biomass}}{nAL_f} \]  

(4.8)

Estimates of the biofilm thickness and density are presented in Table 4.3.

4.4 Experiments with Once-Through Biofilm Reactors

When the biodegradation of chlorinated aliphatic hydrocarbons was studied in the completely mixed anaerobic biofilm reactors, substantial abiotic losses were observed (Wrenn, 1991). These losses were due to interaction between the chlorinated substrates and the reactor tubing, especially the Tygon (i.e., PVC) tubing that was used in the peristaltic pumps. The extent of removal of chlorinated aliphatic hydrocarbons by interaction with Tygon tubing was dependent on the surface area of the tubing, the contact
Table 4.3 Estimates of the Biofilm Thickness ($L_f$) and Density ($X_f$) for Anaerobic Biofilm Reactors

<table>
<thead>
<tr>
<th>Reactor</th>
<th>$L_f$ (cm)</th>
<th>$X_f$ (mg COD/cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanogenic (R1)</td>
<td>0.017 ± 0.007</td>
<td>74 ± 13</td>
</tr>
<tr>
<td>sulfate-reducing (R2)</td>
<td>0.020 ± 0.007</td>
<td>58 ± 20</td>
</tr>
</tbody>
</table>

time, and the concentration of the chlorinated substrate. Sorption of chlorinated aliphatics to Teflon was also observed, but these losses saturated relatively quickly and became minor after a short contact period. Saturation of Tygon tubing, on the other hand, was never observed. Losses remained undiminished even after one week of exposure. Sorption of chlorinated aliphatic and aromatic compounds by polymers that are commonly used in laboratory equipment has also been observed by other researchers (Cseh et al., 1989, Stanley et al., 1989).

All types of flexible tubings that could be used in the recycle peristaltic pump adsorbed chlorinated aliphatic substrates to an unacceptable degree. Therefore, the biodegradation experiments were conducted using a once-through configuration, shown schematically in Figure 4.2. Influent media were fed to the reactors using a syringe pump (Model 355, Sage Instruments) and 50-mL Hamilton Gas-Tight syringes (Supelco, Inc.). Between experiments, the biofilm reactors were operated as completely mixed reactors (i.e., with the effluent recycle, as in Figure 4.1) in order to preserve the uniform distribution of biomass.

4.4.1 Tracer Studies

Accurate estimate of the rate of reaction in continuous-flow reactors requires that effluent concentrations be measured after they have reached steady state. In plug-flow reactors, steady state is reached after a single detention time. When dispersion is important and when reversible reactions—such as adsorption—occur, the time required to reach steady state increases. Tracer experiments were performed with two goals (1) to determine when the effluent TCA concentration reached steady state, and (2) to provide insight into processes, such as dispersion and adsorption, that affected solute transport within the biofilm reactors. Wrenn (1991) reported details of the tracer studies, a summary of which is given here.
Figure 4.2 Schematic diagram of the once-through biofilm reactors that were used to study the biodegradation kinetics of TCA. This configuration was chosen to minimize the abiotic losses of TCA.

4.4.1.1 Conservative Tracer Studies

A conservative tracer (chloride) was used to estimate dispersion in the reactors. The anaerobic biofilm reactors and two control reactors, which were physically identical except that they lacked biomass, were subjected to step increases in the influent chloride concentration. The effluent chloride concentration was measured until steady state was achieved. The porosities and dispersion coefficients were estimated by fitting a model for the one-dimensional transport of a non-reactive solute to the experimental results (van Genuchten and Alves, 1982).

Table 4.4 summarizes the results for porosity ($\varepsilon$) and dispersion coefficient ($D_{th}$). The presence of biomass reduced the porosity and increased the dispersivity. Both effects are direct consequences of biofilm accumulation and are consistent with observations of other researchers (Taylor et al., 1990, Taylor and Jaffe, 1990).

4.4.1.2 Reactive Tracer Studies

TCA was used as a reactive tracer in the biofilm and control reactors. The reactors were subjected to step increases in the influent TCA concentration, and the effluent TCA
concentration was measured as a function of time. The model that was fit to these data considers solute transport by advection and dispersion, adsorption of the solute to the solid phase, and first-order decay of the tracer. This model was simplified by assuming that, at any point in the reactor, the dissolved and sorbed substrate concentrations were in equilibrium (local equilibrium assumption) and that the adsorption isotherm was linear.

The analytical solution for this model was given by van Genuchten (1982). This solution was solved for the best-fit values of the retardation coefficient (R) and first-order decay rate (kᵢ). The porosity and dispersion coefficients were those presented in Table 4.4. The best-fit values of R and kᵢ are presented in Table 4.5.

The breakthrough of TCA was only slightly retarded in the control reactors (R = 1.1), which lacked biomass, but it was more substantially slowed in both biofilm reactors (R ≈ 2). (A retardation factor of 1.0 indicates that the solute moves at the same rate as would a conservative tracer.) Therefore, biomass retarded the movement of TCA through the reactors, probably by adsorption. Because of the increased adsorption and dispersion that resulted from biomass accumulation, the effluent TCA concentration required approximately three times as long to reach steady state in the biofilm reactors as was required in the control reactors.

At steady state, the effluent TCA concentration in the control reactors never reached the influent concentration. This abiotic removal was traced to the perforated aluminum disks that were used to distribute the influent evenly at the reactor inlets. When these were replaced by Teflon disks, the abiotic removal dropped to approximately 4%. The residual abiotic removal appeared to be due to adsorption of TCA to the Teflon components of the systems (e.g., tubing, end plugs, and flow distribution disks). This conclusion is supported by observations of the reactor effluents after TCA was removed from the influent media. The effluent TCA concentrations initially decreased rapidly when TCA was removed from the influent, but then decreased steadily and more slowly for a prolonged period. Sufficient time was allowed between experiments to reduce the

<table>
<thead>
<tr>
<th>Reactor</th>
<th>System</th>
<th>ε (V voids/V bulk)</th>
<th>D_H (cm²/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1</td>
<td>control</td>
<td>0.41</td>
<td>0.15</td>
</tr>
<tr>
<td>CR2</td>
<td>control</td>
<td>0.42</td>
<td>0.15</td>
</tr>
<tr>
<td>R1</td>
<td>methanogenic</td>
<td>0.37</td>
<td>1.27</td>
</tr>
<tr>
<td>R2</td>
<td>sulfate-reducing</td>
<td>0.36</td>
<td>1.28</td>
</tr>
</tbody>
</table>

Table 4.4 Estimates of Porosity (ε) and Hydrodynamic Dispersion Coefficient (D_H) from Conservative Tracer Experiments
Table 4.5 Estimates of the Retardation Factor (R) and the First-Order Decay Rate (k₁) for TCA in Control and Biofilm Reactors

<table>
<thead>
<tr>
<th>Reactor</th>
<th>System</th>
<th>R</th>
<th>k₁ (hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1</td>
<td>control</td>
<td>1.1</td>
<td>0.0218</td>
</tr>
<tr>
<td>CR2</td>
<td>control</td>
<td>1.1</td>
<td>0.0206</td>
</tr>
<tr>
<td>R1</td>
<td>methanogenic</td>
<td>1.9</td>
<td>0.0244</td>
</tr>
<tr>
<td>R2</td>
<td>sulfate-reducing</td>
<td>2.0</td>
<td>0.0753</td>
</tr>
</tbody>
</table>

...effluent TCA concentrations to the point at which they did not interfere with the next experiment

...Retardation of TCA by adsorption to biomass had important implications for experimental design. The effluent TCA concentration required approximately 6 detention times (approximately 20 hours at a flow rate of 4 mL/hr) to reach steady state, whereas the concentration of the conservative tracer, chloride, had reached steady state after only 3 detention times. This delay was significant and had to be accounted for to achieve steady-state conditions in experiments conducted for 24 to 72 hours. The systems were typically operated for 24 to 72 hours at a constant flow rate and influent TCA concentration prior to initiation of sample collection. The steady-state effluent TCA concentrations were monitored for up to three days with no apparent change, indicating that the steady states were stable throughout the duration of typical experiments.

4.4.2 Temperature

During a series of experiments designed to test the effect of formate concentration on the first-order rate of TCA biodegradation (Wrenn, 1991), variations that could not be attributed to formate were observed. Eventually, this variability was traced to the ambient temperature. At the time that these observations were made, experiments were conducted at room temperature, and this temperature varied considerably on time scales ranging from hours to months.

Soon after the discovery that the reaction rate was a strong function of temperature, the biofilm reactors were transferred to a temperature control room, and all subsequent experiments were conducted at 20°C. Although data that had been collected at room temperature could be corrected for the effects of temperature (Wrenn, 1991), the results presented here are for 20°C.
CHAPTER 5 RESULTS AND DISCUSSION

In this chapter, the effects of formate concentration on the rate of TCA biodegradation are presented for an approximately 1000-fold range of TCA concentrations. These results demonstrate that formate stimulates TCA biodegradation over the entire concentration range and that the Monod equation is an adequate description of TCA biodegradation kinetics if the Monod kinetic parameters, \( q_m \) and \( K_s \), are allowed to be a function of the influent formate concentration. The functional dependence of \( q_m \) and \( K_s \) on the formate concentration, however, is not simple.

5.1 Experimental Methods and Data Analysis

The effect of formate concentration on the kinetics of TCA biodegradation was determined by measuring the flux, \( J \) (\( \text{M} \cdot \text{L}^{-2} \cdot \text{T}^{-1} \)), of TCA in the methanogenic and sulfate-reducing biofilm reactors for influent TCA concentrations ranging from approximately 50 \( \mu \text{g/L} \) to 100 mg/L. The influent formate concentrations in these experiments ranged from 0 to 4 mM. The TCA flux was computed using the reactor mass balance

\[
J = \frac{[\text{TCA}]_{\text{inf}} - [\text{TCA}]_{\text{eff}}}{a\theta}
\]  

(5.1)

where \( a \) (L\(^{-1}\)) is the specific surface area of the reactor and \( \theta \) (T) is the empty-bed detention time.

The intrinsic kinetic parameters were estimated from the data at each formate concentration by minimizing the following function:

\[
SS_{rel} = \sum \left( \frac{J - J_{\text{mod}}}{J_{\text{mod}}} \right)^2
\]  

(5.2)

The model flux, \( J_{\text{mod}} \), is the average local flux in a once-through biofilm reactor, which is given by:

\[
J_{\text{mod}} = \eta X_f L_f q_{m,ap} \frac{[\text{TCA}]_{1 - m}}{K_{ap} + [\text{TCA}]_{1 - m}}
\]  

(5.3)

where \([\text{TCA}]_{1-m}\) is the log-mean TCA concentration in the reactor.

\[
[\text{TCA}]_{1 - m} = \frac{[\text{TCA}]_{\text{inf}} - [\text{TCA}]_{\text{eff}}}{\ln \left( \frac{[\text{TCA}]_{\text{inf}}}{[\text{TCA}]_{\text{eff}}} \right)}
\]  

(5.4)
and \( q_{m,ap} \) and \( K_{ap} \) are "apparent" Monod kinetic parameters. These parameters were assumed to be constants when the influent formate concentration was constant. For the purposes of this analysis, the effectiveness factor, \( \eta \), was assumed to be equal to one, because the biofilm always was fully penetrated for TCA (Wrenn, 1991).

The kinetic parameters, \( q_{m,ap} \) and \( K_{ap} \), were estimated by finding the values that minimized \( SS_{res} \). The best estimates of these parameters are given by the following equations (Cornish-Bowden, 1979):

\[
q_{m,ap} = \frac{1}{\sum J_i J_f} \frac{\sum \left( \frac{J_i}{[TCA]_{1-m,i}} \right)^2 \sum J_i^2 - \left( \sum \frac{J_i^2}{[TCA]_{1-m,i}} \right)^2}{\sum \left( \frac{J_i}{[TCA]_{1-m,i}} \right)^2 \sum J_i - \sum \frac{J_i^2}{[TCA]_{1-m,i}} \sum \frac{J_i}{[TCA]_{1-m,i}}} \tag{5.5}
\]

\[
K_{ap} = \frac{\sum J_i \sum \frac{J_i}{[TCA]_{1-m,i}} \sum \frac{J_i^2}{[TCA]_{1-m,i}} \sum J_i}{\sum \left( \frac{J_i}{[TCA]_{1-m,i}} \right)^2 \sum J_i - \sum \frac{J_i^2}{[TCA]_{1-m,i}} \sum \frac{J_i}{[TCA]_{1-m,i}}} \tag{5.6}
\]

in which \( J_i \) (Equation 5.1) is the flux that was measured at a specific \([TCA]_{1-m,i}\) (Equation 5.4).

5.2 TCA Biodegradation Kinetics and Parameter Estimates

The flux of TCA in the anaerobic biofilm reactors is plotted as a function of a log-mean TCA concentration in Figures 5.1 (methanogenic reactor) and 5.2 (sulfate-reducing reactor) for each influent formate concentration. The solid lines are plots of Equation 5.3 using the best estimates of \( q_{m,ap} \) and \( K_{ap} \) that were given by Equations 5.5 and 5.6. The formate concentration had a clear and dramatic effect on the TCA flux, and this effect was similar in both reactors. Formate between 0.5 and 2 mM stimulated TCA biodegradation over the entire range of TCA concentrations that were tested, but the observed fluxes were approximately independent of the formate concentration above and below this range.
Figure 5.1 Effect of formate concentration on the flux of TCA in the methanogenic biofilm reactor. The lines plotted in this figure are the best fits of Equation 5.3 to the flux data for each influent formate concentration.
Equation 5.5 and 5.6 were used to find the best estimates of \( q_{\text{m,ap}} \) and \( K_{\text{ap}} \) for each data set.

Figure 5.2 Effect of formate concentration on the flux of TCA in the sulfate-reducing biofilm reactor. The lines plotted in this figure are the best fits of Equation 5.3 to the flux data for each influent formate concentration.
Equation 5.5 and 5.6 were used to find the best estimates of \( q_{\text{m,ap}} \) and \( K_{\text{ap}} \) for each data set.
The model that was developed in Chapter 3 to describe the effects of primary substrate concentrations on the kinetics of reductive dehalogenation showed that Monod kinetics can be used to describe the relationship between the biodegradation rate and the substrate concentration if the Monod kinetic parameters were defined to be functions of the concentrations of the primary substrates. At all formate concentrations and in both reactors, a Monod-type function fit the data reasonably well, demonstrating that the kinetic parameters could be treated as constants when the influent formate concentration was constant.

When formate and biomass serve as sources of electrons for reductive dehalogenation, the apparent Monod kinetic parameters for both reactors are linear functions of the formate concentration (recall Figures 3.9a and 3.9b and Table 3.1). When the concentrations of biomass and sulfate are the same for all concentrations of formate, Equations 3.9a and 3.9b can be written in the form

\[ q_{m,ap} = C_1 \left[\text{formate}\right] + C_2 \]  \hspace{1cm} (5.7)

\[ K_{ap} = C_3 \left[\text{formate}\right] + C_4 \]  \hspace{1cm} (5.8)

The best estimates of the apparent Monod kinetic parameters are presented in Tables 5.1 (methanogenic reactor) and 5.2 (sulfate-reducing reactor) and are plotted as a function of the influent formate concentration in Figures 5.3 ($q_{m,ap}$) and 5.4 ($K_{ap}$). Least-squares linear regression was used to fit Equations 5.7 and 5.8 to the data, and the results are plotted along with the data in these figures. Examination of these plots reveals that the general increasing trends are as expected, but the data deviate from linearity in similar ways for both systems. At low concentrations (below 0.5 mM for $q_{m,ap}$ and 1 mM for $K_{ap}$), the formate concentration did not affect either parameter. At intermediate concentrations, both parameters increased as the formate concentration increased. Finally, at high concentrations (greater than 2 mM), the parameters became independent of the formate concentration again.

The behavior of these parameters at low formate concentrations suggests a threshold effect. The results presented in Figures 5.1 and 5.2 suggest a switch in electron donor from biomass to formate for formate concentrations between 0.5 and 1 mM. The apparent maximum specific rates of TCA degradation increased when the influent formate concentration increased from 0.5 to 1 mM, but the half-saturation concentrations remained constant. This caused the apparent first-order rate coefficients, $K_{ap}$, to increase abruptly over this concentration range (see $K_{ap}$ values in Tables 5.1 and 5.2). The results presented here for a TCA concentration range of 50 to 20,000 μg/l are confirmed by the behavior reported by Wrenn (1991) for TCA concentration less than 100 μg/l. Both results suggest the conclusion of a threshold in the formate response.
### Table 5.1  Best-Fit Parameters for Equation 5.3 to TCA Flux Data for the Methanogenic Reactor

<table>
<thead>
<tr>
<th>[formate] (mM)</th>
<th>( q_{m,ap} ) (( \mu\text{g} ) TCA/mg COD-h)</th>
<th>( K_{ap} ) (( \mu\text{g}/L ))</th>
<th>( k_{ap} ) (L/mg COD-hr)</th>
<th>( s^2_{rel} ) (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.31 x 10^{-3}</td>
<td>6,360</td>
<td>1.15 x 10^{-6}</td>
<td>0.0316</td>
</tr>
<tr>
<td>0.5</td>
<td>8.66 x 10^{-3}</td>
<td>8,130</td>
<td>1.07 x 10^{-6}</td>
<td>0.0928</td>
</tr>
<tr>
<td>1.0</td>
<td>1.46 x 10^{-2}</td>
<td>7,870</td>
<td>1.86 x 10^{-6}</td>
<td>0.0145</td>
</tr>
<tr>
<td>2.0</td>
<td>4.59 x 10^{-2}</td>
<td>23,500</td>
<td>1.95 x 10^{-6}</td>
<td>0.0295</td>
</tr>
<tr>
<td>4.0</td>
<td>4.71 x 10^{-2}</td>
<td>24,600</td>
<td>1.91 x 10^{-6}</td>
<td>0.0242</td>
</tr>
</tbody>
</table>

(1) \( s^2_{rel} = \frac{SS_{rel}}{n-2} \),

where \( SS_{rel} \) is given by Equation 5.2

---

### Table 5.2  Best-Fit Parameters for Equation 5.3 to TCA Flux Data for the Sulfate-Reducing Reactor

<table>
<thead>
<tr>
<th>[formate] (mM)</th>
<th>( q_{m,ap} ) (( \mu\text{g} ) TCA/mg COD-h)</th>
<th>( K_{ap} ) (( \mu\text{g}/L ))</th>
<th>( k_{ap} ) (L/mg COD-hr)</th>
<th>( s^2_{rel} ) (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.92 x 10^{-3}</td>
<td>2,790</td>
<td>2.12 x 10^{-6}</td>
<td>0.0219</td>
</tr>
<tr>
<td>0.5</td>
<td>5.83 x 10^{-3}</td>
<td>3,230</td>
<td>1.80 x 10^{-6}</td>
<td>0.0817</td>
</tr>
<tr>
<td>1.0</td>
<td>1.06 x 10^{-2}</td>
<td>2,850</td>
<td>3.72 x 10^{-6}</td>
<td>0.0083</td>
</tr>
<tr>
<td>2.0</td>
<td>5.86 x 10^{-2}</td>
<td>14,800</td>
<td>3.96 x 10^{-6}</td>
<td>0.0763</td>
</tr>
<tr>
<td>4.0</td>
<td>5.28 x 10^{-2}</td>
<td>11,500</td>
<td>4.59 x 10^{-6}</td>
<td>0.0420</td>
</tr>
</tbody>
</table>

(1) \( s^2_{rel} = \frac{SS_{rel}}{n-2} \),

where \( SS_{rel} \) is given by Equation 5.2
Figure 5.3  Effect of the influent concentration on the maximum substrate utilization rate for methanogenic and sulfate-reducing biofilm reactors. The best-fit line for the methanogenic reactor is

\[ q_{map} = 0.00765 + 0.0114 \cdot [\text{formate}]_{inf}, \quad r^2 = 0.803 \]

and for the sulfate-reducing reactor, the best fit line is

\[ q_{map} = 0.00556 + 0.0141 \cdot [\text{formate}]_{inf}, \quad r^2 = 0.707 \]
Figure 5.4  Effect of the influent formate concentration on the apparent half-saturation coefficients. The best-fit line for the methanogenic reactor is

\[ K_{ap} = 6,340 + 5,146 \cdot [\text{formate}]_{\text{infl}}, r^2 = 0.801 \]

and for the sulfate-reducing reactor, the best-fit line is

\[ K_{ap} = 2,930 + 2,740 \cdot [\text{formate}]_{\text{infl}}, r^2 = 0.576 \]
The apparent Monod parameters increased dramatically when the influent formate concentration increased from 1 to 2 mM. In the methanogenic reactor, $q_{m,ap}$ and $K_{ap}$ increased approximately three-fold, and they increased by a factor of more than five in the sulfate-reducing reactor. Because $q_{m,ap}$ and $K_{ap}$ increased simultaneously, the apparent first-order rate coefficients increased much less dramatically. The changes in $K_{ap}$ that were calculated from these data were of similar magnitude to those that were observed in $K_{ap}$ for only low TCA concentrations (Wrenn, 1991).

Above 2 mM formate, the electron-donor effect appeared to saturate, and no further increases in $q_{m,ap}$ and $K_{ap}$ were observed. Saturation of the electron-donor effect could have been caused by saturation of the electron-donor pathway (i.e., the rate at which electrons were transferred to the reductive dehalogenase) or saturation of the reductive dehalogenation reaction itself. The former seems unlikely, because methane production in the methanogenic reactor was a linear function of the influent formate concentration up to 8 mM formate (data not shown). Therefore, saturation of the dechlorination reaction is the most probably cause of the observed behavior.

The apparent maximum specific TCA biodegradation rates were slightly higher in the methanogenic reactor than in the sulfate-reducing reactor when the formate concentration was less than 1 mM. At formate concentrations greater than or equal to 2 mM, however, $q_{m,ap}$ was larger in the sulfate-reducing reactor. The apparent half-saturation concentrations, on the other hand, were always higher in the methanogenic reactor, indicating that the sulfate-reducing consortium had a higher affinity for TCA. Thus, the larger first-order rate coefficients that were observed for the sulfate-reducing system were primarily due to its higher affinity for TCA. Because of this higher affinity, the sulfate-reducing consortium transformed TCA much more rapidly under most conditions that were studied.

TCA dechlorination kinetics will be first-order when $[TCA]$ is low compared to $K_{ap}$. In particular, the TCA concentration must be sufficiently low relative to $K_{ap}$ so that

$$\frac{q_{m,ap} \ X_f \ L_f \ [TCA]_{1-m}}{K_{ap} + [TCA]_{1-m}} = \frac{q_{m,ap} \ X_f \ L_f}{K_{ap}} \ [TCA]_{1-m}$$

(5.9)

The values that are reported for $K_{ap}$ in Tables 5.1 and 5.2 show that the first-order approximation is justified for TCA concentrations less than about 200 µg/ℓ in the most stringent case, no exogenous electron donor.

5.3 Electron Flow to TCA

The rates of electron transfer to TCA ($r_{e,TCA}$) and from the donors ($r_{e,x}$ and $r_{e,form}$) indicate the relative importance of reductive dechlorination to the total electron flow in the cells. The rate of electron transfer to TCA was computed by assuming that two electrons were transferred per molecule of TCA reduced (to DCA)

$$r_{e,TCA} = 2Q \ ( [TCA]_{in} - [TCA]_{eff} )$$

(5.10)
When formate was not present in the influent, electrons were assumed to be released through biomass oxidation. The biomass decay coefficient, \( b \), was assumed to be 0.01 day\(^{-1}\), and only electrons derived from \( \text{H}_2 \)-oxidizing methanogens and sulfidogens were considered. These trophic groups were assumed to constitute 16% of the total biomass (Wrenn, 1991). Thus, the rate of liberation of electrons from biomass is given by

\[
\frac{r_{ex}}{Y_{e,COD}} = 0.16 \times \frac{X_f}{b} = \frac{Y_{e,COD}}{(0.16)} \times X_f \times L_f \times V_f \times b
\]

where \( Y_{e,COD} \) is the electron yield per unit mass of COD:

\[
Y_{e,COD} = 1.25 \times 10^{-4} \text{ moles } e^-/\text{mg COD}
\]

When the formate was present in the influent, it was assumed to be the sole source of electrons in the reactors (i.e., biomass oxidation was not considered when an exogenous electron acceptor was present). Formate oxidation yields two electrons per mole. Because the effluent formate concentration was not measured in these experiments, the maximum rate at which electrons could become available through formate oxidation was calculated

\[
\frac{r_{e,\text{form}}}{2} = \frac{[\text{formate}]_{\text{inf}} \cdot Q}{2}
\]

The fraction of available electrons that were transferred to TCA when TCA-degradation kinetics were first order was small, because the influent TCA concentrations and the rates of TCA removal were low. Table 5.3 summarizes these results and shows that less than 0.05% of the available electrons were transferred to TCA when TCA concentrations were low. This is true regardless of whether the electrons came mainly from biomass or formate. Thus, TCA was not an important electron sink in either reactor when TCA concentrations were in the first-order region.

As the influent TCA concentration increased, the fraction of available electrons that were transferred to TCA also increased. Nevertheless, the electrons transferred to TCA always represent a low fraction of those available. For the highest rate of TCA removal that was observed, less than 3% of the available electrons were transferred to TCA. The results of the calculations for high TCA concentrations are presented in Table 5.4.
Table 5.3 Electron Flow to TCA when TCA Concentrations Were Less than 100 μg/l

<table>
<thead>
<tr>
<th>Reactor</th>
<th>$[\text{formate}]_{\text{inf}}$ (mM)</th>
<th>$r_{e,TCA}$ (moles e^-/day)</th>
<th>$r_{e,X}$ (moles e^-/day)</th>
<th>$r_{e,\text{form}}$ (moles e^-/day)</th>
<th>$r_{e,TCA}$/$r_{e,d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>0</td>
<td>$2.39 \times 10^{-8}$</td>
<td>$1.15 \times 10^{-4}$</td>
<td>0</td>
<td>$2.07 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>$3.57 \times 10^{-8}$</td>
<td>0</td>
<td>$1.88 \times 10^{-4}$</td>
<td>$1.90 \times 10^{-4}$</td>
</tr>
<tr>
<td>R2</td>
<td>0</td>
<td>$3.78 \times 10^{-8}$</td>
<td>$1.21 \times 10^{-4}$</td>
<td>0</td>
<td>$3.12 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>$5.48 \times 10^{-8}$</td>
<td>0</td>
<td>$1.90 \times 10^{-4}$</td>
<td>$2.88 \times 10^{-6}$</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>$1.11 \times 10^{-7}$</td>
<td>0</td>
<td>$1.55 \times 10^{-3}$</td>
<td>$7.16 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

Table 5.4 Electron Flow to TCA when the Influent TCA Concentration Was High

<table>
<thead>
<tr>
<th>Reactor</th>
<th>$[\text{formate}]_{\text{inf}}$ (mM)</th>
<th>$[\text{TCA}]_{\text{inf}}$ (mg/L)</th>
<th>$r_{TCA}$ (moles e^-/day)</th>
<th>$r_{X}$ (moles e^-/day)</th>
<th>$r_{\text{form}}$ (moles e^-/day)</th>
<th>$r_{e,TCA}$/$r_{e,d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>0</td>
<td>24.7</td>
<td>$1.76 \times 10^{-6}$</td>
<td>$1.15 \times 10^{-4}$</td>
<td>0</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>68.8</td>
<td>$7.21 \times 10^{-6}$</td>
<td>0</td>
<td>$3.89 \times 10^{-4}$</td>
<td>0.019</td>
</tr>
<tr>
<td>R2</td>
<td>0</td>
<td>30.0</td>
<td>$1.22 \times 10^{-6}$</td>
<td>$1.21 \times 10^{-4}$</td>
<td>0</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>69.6</td>
<td>$1.14 \times 10^{-5}$</td>
<td>0</td>
<td>$3.90 \times 10^{-4}$</td>
<td>0.023</td>
</tr>
</tbody>
</table>
5.4 Inhibition of Methane Production by TCA

The models that were developed to describe the effects of primary substrates on the kinetics of reductive dehalogenation assume that the primary electron-donor and acceptor substrates are metabolized at rates that are independent of the concentration of the halogenated aliphatic substrate. However, the effects of formate on the rate of TCA biodegradation were examined over a wide range of TCA concentrations, and the rate of methane production was inhibited by concentrations of TCA greater than 100 μg/L. Figure 5.5 illustrates the decrease in the first-order rate of methane formation (k_{m,dp}) with increasing [TCA]. The rate of sulfate reduction was not similarly affected.

Although methane production was almost completely inhibited in the presence of less than 10 mg/L TCA, formate still stimulated the rate of TCA biodegradation, even when the influent TCA concentration was nearly 100 mg/L (recall Figure 5.1). A better understanding of the mechanisms of TCA biodegradation and inhibition of methanogenesis by TCA are required to incorporate these interactions quantitatively into the Primary Substrates Model. Nonetheless, a possible mechanism for the interaction of methanogenesis and reductive dehalogenation can be postulated.

Halogenated aliphatics probably inhibit methanogenesis by reacting directly at the active site of methyl reductase. Several halogenated aliphatics, including CHCl₃ and chloral hydrate (2,2,2-trichloro-1,1-ethanediol), inhibited methanogenesis from methyl-coenzyme M in cell-free extracts of *Methanobacterium thermoautotrophicum* (Gunsalus and Wolfe, 1978), and so, presumably, they inhibited the methyl reductase enzyme complex directly. Many halogenated methanes are known to form stable covalent complexes with reduced cobamides, such as coenzyme B₁₂ (Wood et al., 1968). Perhaps inhibition of methyl reductase results from a direct interaction with F₄₃₀, a Ni-containing coenzyme that is believed to serve as the terminal methyl carrier in methanogenesis and that has characteristics similar to those of cobamides. Interesting, this complexation mechanism for inhibition by halogenated aliphatics is similar to the complexation mechanism postulated for reductive dehalogenation. In fact, Wood et al. (1968) observed methyl chloride as the major product resulting from photolysis of the haloethylcobamide complexes that they studied, indicating that the halomethanes were reductively dehalogenated.

Methane production measures the activity of the electron-acceptor reactions, but we do not know how high concentrations of TCA affect the electron-donor reaction. Obviously, it must be inhibited as well, but if the inhibition results only from accumulation of reduced electron transfer cofactors and proteins, then one would expect to see enhanced TCA biodegradation. Bauchop (1967), in his original observations of the inhibitory effects of halogenated methanes on methanogenesis in rumen fluid, found that inhibition of methane formation from formate was accompanied by accumulation of H₂, indicating that formate oxidation can proceed to a limited extent, even in the absence of methane production. Formate probably is oxidized through a formate-hydrogen lyase type reaction, in which formate oxidation by formate dehydrogenase is coupled to H₂ production by hydrogenase (Vogels et al., 1988).
Figure 5.5  Inhibition of methane production by TCA. The data were obtained at formate concentrations of 2 mM and 4 mM. The first-order rate coefficient for methane production, $k_{m, sp}$, is

The line plotted through the data is the best-fit of a competitive inhibition model

$$
\frac{k_{m, sp}}{k_m} = 1 + \frac{[\text{TCA}]}{K_{TCA}}
$$

where $k_m$ is the first-order rate coefficient for methane production in the absence of TCA, and $K_{TCA}$ is the concentration of TCA that inhibited methane production by 50%. The best estimates of these parameters are

$$
k_m = 0.290 \text{ hr}^{-1}
$$

$$
K_{TCA} = 790 \mu g/L = 5.92 \times 10^{-4} \text{ mM}
$$
Unfortunately, most of these molecular-level details are speculative. Furthermore, the effects of formate on TCA flux were similar to both reactors, and sulfate reduction was not significantly inhibited by TCA. Biodegradation of TCA in the methanogenic reactor and the effects of primary substrates on the rate of this reaction appear to occur independently of methane production. Thus, methane production can be inhibited without affecting the rate of TCA biodegradation or the ability to formate to stimulate the reaction time.

5.5 Evaluation of the Primary Substrates Model

The Primary Substrates Model makes two important predictions regarding the effects of electron-donor concentration on the kinetics of reductive dehalogenation. First, it predicts that TCA biodegradation kinetics can be described by the Monod equation, if the Monod kinetic parameters are defined to be functions of the concentrations of the primary electron-donor and acceptor substrates. Second, this model predicts that the electron-donor substrate will stimulate the rate of TCA biodegradation. The data provide solid evidence that supports both of these predictions.

The apparent maximum specific rate of TCA degradation, \( q_{m,ap} \), and the apparent half-saturation concentration, \( K_{ap} \), were constant when the data were grouped according to formate concentration, but were strongly affected by the influent formate concentration. Most significantly, the stimulation due to formate occurred through the whole range of TCA concentration tested (50 \( \mu \)g/t to 100 mg/t), but was particularly striking at high TCA concentrations. These data are consistent with the inhibition caused by \( SO_4^{2-} \), the electron acceptor in sulfate-reducing biofilm systems (Wrenn, 1991). The apparent first-order rate coefficients, \( K_{ap} \), were affected less dramatically, but exhibited trends similar to those seen at low TCA concentrations by Wrenn (1991). The first-order biofilm flux coefficients were independent of the formate concentration below 0.5 mM, and they increased abruptly between 0.5 mM and 1 mM formate in both reactors. Above 1 mM formate, the first-order flux coefficients behaved differently in the methanogenic and sulfate-reducing biofilm reactors. In the sulfate-reducing reactor, \( k_{ap} \) continued to increase with the formate concentration increased, but there was no further effect of formate in the methanogenic reactor.

The specific relationships between the apparent Monod parameters and the influent formate concentration did not agree quantitatively with the predictions of the Primary Substrate Model. All of these kinetic parameters—\( q_{m,ap} \), \( K_{ap} \), and \( k_{ap} \)—showed a threshold,
below which they were independent of the formate concentration. In addition, \( q_{\text{m,ap}} \) and \( K_{\text{ap}} \) appeared to saturate (i.e., become independent of the formate concentration) when the formate concentration exceeded 2 mM. Additional research is required to elucidate the basis for this behavior.

The results presented in this chapter show that the major predictions of the Primary Substrates Model are correct. Furthermore, they map the limits over which the electron-donor concentration affects the kinetics of TCA biodegradation.
CHAPTER 6 SUMMARY AND CONCLUSIONS

The rate of reductive dehalogenation, which is the most important mechanism by which highly halogenated aliphatics are biotransformed under anaerobic conditions, ought to be controlled by the intracellular availability of electrons. A kinetic model, based on this hypothesis, was developed to describe the interactions that occur among electron donors, acceptors, and halogenated aliphatic substrates during microbially catalyzed reductive dehalogenation reactors. The concentrations of the primary electron donor, primary electron acceptor, endogenous electron donor (i.e., biomass decay), and the halogenated substrate controlled the intracellular concentration of a reduced metalloenzyme, which ultimately controlled the kinetics of reductive dehalogenation. The primary goal was to quantify the effects of the primary substrates in the reductive dehalogenation of 1,1,1-trichloroethane (TCA) in methanogenic and sulfate reducing consortia.

Previous work identified the H$_2$-oxidizing bacteria as being responsible for reductive dechlorination of TCA (Wrenn, 1991). Therefore, formate an alternate to H$_2$, was employed as the primary electron donor. The primary electron acceptor was SO$_4^{2-}$ in the sulfate-reducing biofilms reactor and formate in the methanogenic biofilm reactor.

The effects of formate (and sulfate) were consistent with the substrate interactions considered in the model. Most importantly, formate stimulated the rate of TCA biodegradation, especially when the TCA concentration was high. Furthermore, the apparent kinetic parameters for TCA dehalogenation were constants, as long as the formate concentration was constant.

The most dramatic effects of the primary electron-donor substrate were observed at high TCA concentrations. In the methanogenic reactor, the apparent maximum specific rate of TCA biodegradation ($q_{m,ap}$) was more than six-fold larger in the presence of 4 mM formate than it was when formate was absent. The apparent half-saturation concentration ($K_{ap}$) increased by a factor of almost four over the same range of formate concentrations. Similar changes in $K_{ap}$ were observed in the sulfate-reducing reactor, but the effect on $q_{m,ap}$ was even more dramatic: The maximum specific rate of TCA biodegradation increased by almost an order of magnitude in this system.

The kinetics of TCA biodegradation were adequately modeled using the Monod equation at all formate concentrations. This provides support for an important prediction of the Primary Substrates Model. The effects of primary substrates can be completely incorporated into terms for the apparent kinetic parameters, $q_{m,ap}$ and $K_{ap}$. The relationships between these apparent kinetic parameters and the concentrations of the primary substrates, however, are not as simple as the model predicts.

Although the responses of the kinetic coefficients for TCA biotransformation in both biofilm reactors were generally consistent with the predictions of the Primary Substrates Model, important discrepancies exist. The most obvious of these is the threshold that occurred in the relationships between the influent formate concentration and the apparent coefficients. Below 0.5 mM formate, TCA biodegradation were independent.
of the format concentration in both reactors. The biodegradation rates increased abruptly between 0.5 and 1 mM formate. At higher influent formate concentrations, the rate coefficients behaved as predicted by the Primary Substrates Model. Further research will be required to resolve whether or not this threshold has a physiological basis.

This research has made several important contributions. First, it has established that the primary electron-donor and -acceptor substrates strongly affect the kinetics of anaerobic biodegradation of halogenated aliphatic hydrocarbons. Clearly, the kinetics of anaerobic biodegradation of halogenated aliphatics cannot be based on the concentration of the halogenated substrate alone. Second, a conceptual and mathematical framework that can be used to describe the interactions that occur among these substrates was developed. Many of the observations that have been made in similar systems by other researchers are consistent with this model. Finally, the model’s limits have been defined. The experimental work identified specific regions in which the model became inaccurate. Further research to determine why the model breaks down in these regions may substantially improve our understanding of reductive dehalogention. Use of the current model and improved models generated in the future can lead to more effective biological treatment process for highly halogenated aliphatics.
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