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Variables Effecting Trichloroethylene Transformation by Methanotrophs

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VARIABLES EFFECTING TRICHLOROETHYLENE TRANSFORMATION BY METHANOTROPHS

By

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ABSTRACT

VARIABLES AFFECTING TRICHLOROETHYLENE TRANSFORMATION BY METHANOTROPHS

By

Linda M Clowater

This research focuses on environmental variables affecting co-metabolism of trichloroethylene (TCE) by methanotroph enrichment MM1, derived from aquifer material sampled at the Moffett Field Naval Air Base, Mountain View, California. Variables evaluated include incubation pH, reducing power availability, and TCE concentration. In order to quantify the effects of these variables, the kinetic models used to describe co-metabolism were reviewed, and inter-relationships between the models were determined.

The effects of pH on transformation capacity, T_C, of the culture were evaluated at pH values of 5.5, 6.8, 8.5 and 10. Transformation capacity decreased at pH 5.5 and 10.

Reducing power was evaluated by addition of formate. At low TCE concentrations, formate addition had no effect on TCE transformation, but at high TCE concentrations, formate addition increased the transformation capacity from 0.023 mg TCE/mg cells to 0.139 mg TCE/mg cells. Formate addition at pH 5.5 increases the T_C from 0.013 mg TCE/mg cells to 0.033 mg TCE/mg cells, but had no effect at pH 10.

The loss of transformation capacity was also evaluated over a five day aeration period in the absence of methane. Transformation capacity decayed at a rate of 0.0381 day^{-1}. Experiments with low levels of TCE gave a transformation capacity of 0.0346 mg TCE/mg cells. This value is close to the values observed at higher concentrations (~ 0.04 mg TCE/mg cells), but does not include the loss of T_C due to decay. If the low level TCE value is adjusted for decay, the initial value for T_C would be 0.192 mg TCE/mg cells. This suggests that T_C may actually increase at low TCE concentrations.

This thesis is dedicated to my family, whom I love.

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b first order decay coefficient (per day)

k maximum specific rate of substrate utilization (mg substrate/mg biomass-day)

K_S half saturation coefficient (mg substrate/L)

k' second order rate coefficient = K/K_S (L/mg-day)

S non-growth substrate concentration (mg/L)

t time

T_C transformation capacity (mg substrate/mg cells)

X active organism concentration (mg/L)

X^o initial active organism concentration (mg/L)

S^o initial non-growth substrate concentration

CHAPTER 1

INTRODUCTION

Background

Contaminated water is a problem facing many communities throughout the world. It is estimated that 16 to 28% of randomly selected U.S. groundwater drinking supplies are contaminated (Westrick *et al.*, 1984). A major cause of groundwater contamination is halogenated aliphatic compounds. These compounds are widely used in industry as solvents for degreasing, paint stripping and dry cleaning. They enter the water supplies mainly through accidental spills and improper disposal and storage. As use of these chemicals has grown, so has the potential for groundwater contamination as these chemicals are mobile and will migrate through soil into groundwater. Once present in a drinking water supply, they pose a threat to public health since many of these chemicals are known or suspected carcinogens.

Methods for remediation of contaminated groundwater include carbon adsorption, air stripping, chemical oxidation and biological methods. The advantage of chemical and biological processes over the others is that these processes provide partial or complete destruction of contaminants. Adsorption and air stripping simply transfer contaminants to a different phase, and may still require further treatment.

One emerging technology for the treatment of contaminated water is bioremediation. During the past decade, aerobic degradation of chlorinated aliphatics, such as trichloroethylene (TCE), has been widely demonstrated using methanotrophic bacteria. Unlike anaerobic degradation which can produce toxic by-products such as vinyl chloride, the products produced aerobically are thought to be relatively harmless. To date, however, there have been relatively few systematic investigations of environmental factors influencing the rate and extent

of methanotrophic transformations. An understanding of these factors is essential in the design and operation of engineered systems and in the comparison of cultures from different sources.

Scope and Objectives

The overall goal of this research is to evaluate the role of environmental factors on the rate and extent of degradation of TCE by methanotrophs. More specifically, this work evaluates the following environmental factors:

1) TCE concentration
2) Reducing power (formate addition)
3) pH
4) Loss of transformation capacity

Biotransformation of chlorinated organics

Many organic compounds found in the environment are mineralized as a result of biological activity. There are, however, some compounds which resist microbial degradation or are only partially transformed. Reasons for this include environmental conditions that prevent competent microbial populations from growing, such as lack of primary substrate or nutrients.

For many years, it was held that many chlorinated aliphatics could only be degraded anaerobically. In 1985, however, Wilson and Wilson demonstrated aerobic degradation of TCE in soil enriched with natural gas. Since then, much research has focused on aerobic biodegradation of chlorinated aliphatics.

Methanotrophic transformation of chlorinated aliphatics can be classified as "co-metabolic". One definition of co-metabolism is "transformation of a non-growth substrate in

the obligate presence of a growth substrate or another transformable compound" (Dalton and Stirling, 1982). In a more general sense, however, co-metabolism can be defined as any transformation of a non-growth substrate that depends upon the concurrent *or previous* metabolism of a growth or energy substrate. This broader definition is more appropriate for methanotrophs inasmuch as methanotrophic transformation of non-growth substrates, such as TCE, can continue, although at decreasing rates, in the absence of a growth substrate (e.g. methane) or an energy substrate (e.g. formate).

Methanotrophs

Methylotrophs are bacteria that can oxidize methane and methanol for energy and growth. Obligate methylotrophs grow on reduced carbon compounds that contain one or more carbon atoms but no carbon-carbon bonds. Facultative methylotrophs can grow on a variety of other organic multi-carbon compounds (Anthony, 1982). Methylotrophic bacteria that are able to grow on methane are called methanotrophs.

Methanotrophs are classified as either Type I or Type II depending upon their internal membrane structure. Type I methanotrophs have a membrane that is arranged in bundles of vesicular discs, use the ribulose monophosphate cycle to assimilate carbon, have an incomplete TCA cycle, and form cysts (*Azotobacter*-like) for resting stages. Type II methanotrophs have their membranes arranged in pairs around the cell periphery, use the serine pathway to assimilate carbon, have a complete TCA cycle, and have exospores or 'lipid' cysts (unique structures) for resting stages (Higgins, 1979).

The metabolism of methane is shown in the Figure 1.1. In the conversion of methane to methanol, several oxidation reactions occur. The initial oxidation is catalyzed by the methane monooxygenase (mmo) enzyme system. Methanol dehydrogenase catalyzes the oxidation of methanol to formaldehyde. Formaldehyde is either incorporated into biomass or oxidized further for energy. Formaldehyde dehydrogenase catalyzes the oxidation of

formaldehyde to formic acid, and formate dehydrogenase catalyzes the final oxidation of formic acid to carbon dioxide and water.

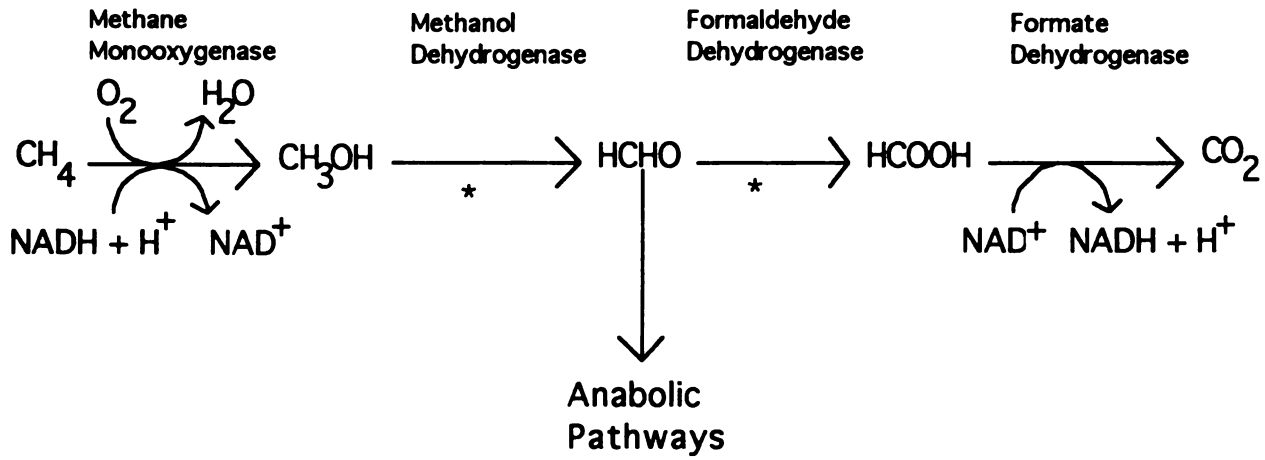

Figure 1.1. The metabolism of methane. (Modified from Higgins, 1979; Anthony, 1982; and Fox, 1990), * co-factors required for these reactions vary.

The mmo system has been identified and characterized in three species of methanotrophs: *Methylococcus capsulatus* (Bath), *Methylosinus trichosporium* OB3b, and *Methylobacterium sp* strain CRL-26. The enzyme is a multicomponent system which catalyzes the oxidation of methane to methanol. The mmo system has two known forms: a particulate form associated with the cell membrane and a soluble or least soluble form (Higgins, 1979). The dominant form of mmo depends upon growth conditions (Patel *et al.*, 1982, Burrows *et al.*, 1984). The actual location of mmo in the cell is dependent upon copper availability and biomass concentration.

For *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b, soluble mmo dominates at low copper concentration; as the copper concentration increases, so does the particulate to soluble ratio until the only form remaining is the particulate form. Dalton *et al.*,

(1984) showed similar variations if the copper concentration was held constant and the biomass concentration varied.

The substrate specificities of the two forms of the enzyme also differ. Particulate mmo efficiently oxidized n-alkanes and n-alkenes, but poorly oxidized alicyclics and aromatic compounds (Stirling *et al..*, 1979, Burrows, 1984). In *in vitro* studies, the soluble fraction of *Methylococcus capsulatus* (Bath) catalyzes the hydroxylation of primary and secondary C-H bonds, the formation of epoxides from internal and terminal alkenes, the hydroxylation of aromatic compounds, the N-oxidation of pyridine, and the oxidation of CO to CO_2. (Colby et al., 1977).

Using crude extracts from *Methylosinus trichosporium* OB3b and *Methylobacterium sp.* CRL-26, Stirling *et al.*, (1979) and Patel *et al.*, (1982) showed that the substrate specificity and oxidation products were the same as those associated with *Methylococcus capsulatus* (Bath). There is a wide but slightly more limited range of transformations that occur with whole-cell suspensions as opposed to the cell-free enzyme. In studies conducted by Stirling and Dalton (1979), resting-cell suspensions of *Methylococcus capsulatus* (Bath) oxidized the following compounds: chloromethane, bromomethane, dimethyl ether, ethylene and propylene. Using formeldehyde to regenerate reducing power, the following additional compounds were oxidized: carbon monoxide, diethyl ether, ethane, propane, 1-butylene, cis-2-butylene, and trans-2 butylene. Higgins *et. al* . (1979) showed that whole cell suspensions of *Methylosinus trichosporium* OB3b had a broader specificity than *Methylococcus capsulatus* (Bath) by oxidizing n-alkanes, n-alkenes, alicyclic and terpenoid hydrocarbons, aromatics, alcohols, phenol, pyridine, and ammonia.

Mixed culture MM1 (Appendix C, Figure C.1) used for this study was originally enriched from aquifer solids from the Moffett Field Naval Base in California (Henry, 1990). MM1 is a stable consortium consisting of one methanotroph and three or four heterotrophs containing predominantly Gram-negative pleomorphic coccobacilli and prosthecates as well as some Gram-negative bacilli and cocci. The methanotroph was a pleomorphic coccobacillus that

contained the internal membrane structures characteristics of Type II methanotrophs (paired membranes inside the periphery of the cell.) Extracts of mixed culture MM1 were tested with an antibody specific to the soluble MMO of *M. trichosporium* OB3b and they cross reacted with it (Tsien *et al.*, 1990). This indicates that the methanotrophs in mixed culture MM1 expressed soluble MMO similar to that of *Methylosinus trichosporium* OB3b under the specified growth conditions (Henry, 1991a).

Aerobic degradation of trichloroethylene

Several investigators have examined the degradation of chlorinated aliphatics. Much of this effort has focused on the chlorinated solvent trichloroethylene (TCE).

For a better understanding of this compound, some of the physical and chemical properties associated with trichloroethylene are summarized in Table 1.1

	Trichloroethylene (TCE)
Molecular Weight	131.39 g (a)
Melting Point	-73°C(a)
Boiling Point	87°C (a)
Density (20°C)	1.464 g/mL (a)
Solubility (25°C)	1100 mg/L(b)
Henry's Constant (dimensionless) 21°C	0.33 (c)

(a) = hand book of Physics and chemistry, 1989
(b) = Horvath (1982)
(c) = Gossett (1987)

Table 1.1 Chemical and physical properties of trichloroethylene.

Fogel *et al.*. (1986) showed that in a mixed culture, ^{14}C-labeled TCE was transformed to CO_2, cell biomass, and nonvolatile or nonchlorinated compounds. Acetylene, a known inhibitor of mmo activity, inhibited degradation indicating that methane-oxidizing bacteria probably initiated TCE oxidation.

Henson *et al.*. (1989) examined the degradation of several chlorinated aliphatics including TCE. Mixed culture experiments were conducted in serum tubes to which a mixture of halogenated hydrocarbons was added. When grown in the presence of methane, the culture transformed TCE.

Using a pure culture, strain 46-1, Little *et al* . (1988) demonstrated degradation of TCE only when grown on methane or methanol. These studies were conducted using liquid cultures in inverted serum bottles. Degradation stopped when methane was depleted and continued if additional methane was added. These researchers concluded that TCE degradation is a cometabolic process that provides little or no benefit to methanotrophs because strain 46-1 initiated the degradation of TCE but was unable to metabolize the intermediates. Preliminary evidence indicated that glyoxylic acid and dichloroacetic acid were the breakdown products. Finally, a mechanism of degradation was proposed. It was hypothesized that TCE is first converted to its epoxide, which breaks down spontaneously, yielding dichloroacetic acid, glyoxylic acid, formate and carbon monoxide.

This proposed pathway seems consistent with the findings of Henschler (1979) who evaluated TCE-epoxide reactivity in aqueous systems. Henschler found that the epoxide decomposes to form formate, carbon monoxide, glyoxylic acid and dichloroacetic acid and that the distribution of products was pH-dependent. As pH decreased, fewer 1-carbon products were observed.

Recently a new pathway was proposed for *Methylosinus trichosporium* OB3b (Newman, 1991). Four different methanotrophs expressing soluble methane monooxygenase produced 2,2,2-trichloroacetaldehyde (chloral hydrate). Chloral hydrate was biologically transformed to trichloroethanol and trichloroacetic acid. Figure 1.2 is a proposed pathway

based on the findings of Henschler *et al.* (1979), Little *et al.* (1988), and Newman, (1991) with some modifications.

Once TCE degradation and its degradation pathway were well established, it became possible to test a number of environmental variables in an attempt to enhance transformation rates and capacity. Most efforts to enhance the transformation have focused on the availability of reducing power. As previously described, the mmo enzyme system requires a source of reducing power to carry out the transformation of TCE. This reducing power is provided by electron carriers in the cell, such as NAD(P)H (Figure 1.1). When a growth substrate, such as methane, or energy substrate such as formate, is metabolized, reducing power is regenerated. Oxidation of any one of the metabolites of methane (methanol, formaldehyde or formate) can regenerate reductant (Figure 1.1). When mmo oxidizes a non-growth substrate, such as TCE, reducing power is not regenerated, and the oxidation will eventually stop. Providing methanotrophs with methanol, formeldehyde or formate will not alleviate the need for methane, but will support continued TCE oxidation. Few methanotrophs grow well on methanol, none grow well on formaldehyde, and formate is not a growth substrate (Anthony, 1982).

Even when methane is provided, reducing power can still be depleted. Using a pure culture, Henry (1991b) showed that carbon monoxide competitively inhibited methane oxidation until formate was added as an exogenous electron donor. She also showed that formate addition to mixed culture MM1 did not enhance degradation rates at low TCE concentrations and assumed this was because MM1 methanotrophs possess lipid storage granules which can serve as an alternate source of electrons. Henry (1991a) also tested the effect of formate addition on the rates of TCE transformation during methane starvation. At a TCE concentration of 30 -60 μg/L, formate addition did not increase rates for mixed culture MM1. However, the rates were enhanced for the the first ten hours of methane starvation when pure culture MM2 was incubated with 2 mM formate. When the culture was incubated without formate and the formate was added simultaneously with the TCE, transformation rates remained significantly enhanced throughout 62 hours of testing.

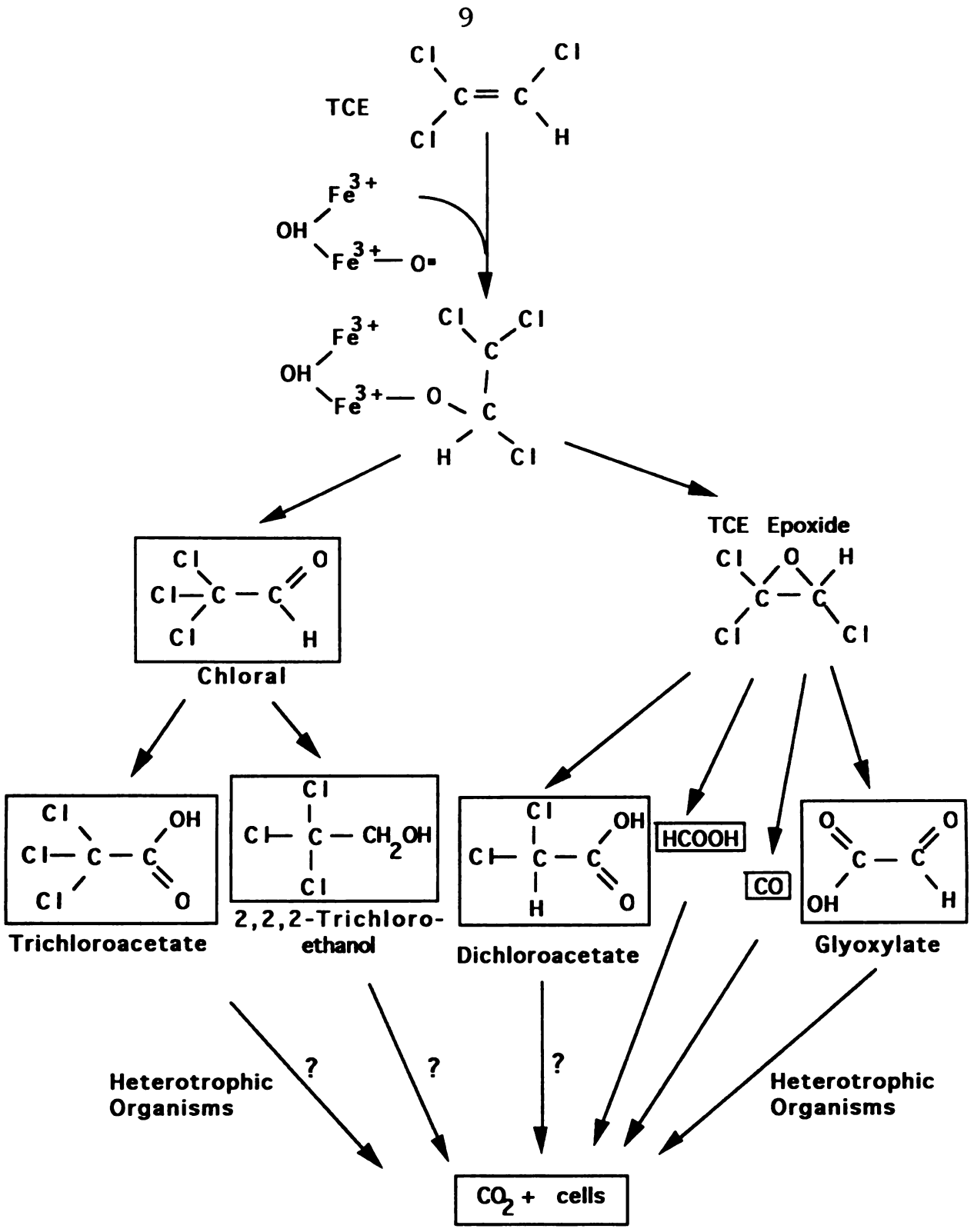

Figure 1.2. Proposed mechanism for aerobic TCE degradation
(After Little *et al.*, 1988; Henschler *et al*, 1979; and Newman, 1990)

Alvarez-Cohen and McCarty (1991a) introduced the concept of transformation capacity, as the amount of non-growth substrate (TCE in this case) degraded per unit biomass. This parameter serves as a useful index making possible the comparison of different co-metabolizing cultures. Alvarez-Cohen (1991b) tested the effect of formate on transformation capacity using a TCE concentration of approximately 20 mg/L. With the addition of 20 mM of formate, transformation capacity of the culture increased from 0.036 to 0.073 mg TCE/mg cells. The transformation rates also increased from 0.6 to 2.1 mg TCE/mg of cells per day. Alvarez-Cohen (1991b) also tested the effect of aeration on a culture's ability to transform TCE. Freshly harvested cells were spiked with TCE and compared to cells that had been aerated for 24 hours before TCE addition. The freshly harvested cells had an initial linearized disappearance rate of 0.5 mg TCE/mg cell-day compared to the aerated cells which had a rate of 0.03 mg TCE/mg cell-day.

The effects of TCE concentration were evaluated by both Henry (1987) and Alvarez-Cohen (1991b). Henry showed that increased TCE concentration resulted in increased transformation rates. When the TCE concentration was increased from 0.1mg/L to 1.0 mg/L, there was approximately an order of magnitude increase in the TCE transformation rate. Alvarez-Cohen showed that fluctuations in TCE concentration had no effect on transformation capacity.

Modeling

Michaelis-Menton or Monod expressions are commonly used to describe cometabolic degradation. One limitation of these models is that they don't account for the loss of transformation activity in the absence of growth substrate or the loss of transformation activity due to other factors such as product toxicity. Consequently, parameters estimated by these models may be in error. Models are needed that account for terms that include loss of biomass or enzyme activity caused by consumption of reducing power, endogenous decay, product

toxicity and suicide inactivation. Recently, a few models that can account for loss of transformation activity have emerged, but to date, no effort has been made to identify the inter-relationships among these models or to critique their basic assumptions. In this section, three models are described together with their underlying assumptions.

All of the models proposed to date can be derived from the following three equations:

$$-\frac{dS}{dt} = \frac{kSX}{K_s + S} \qquad (1.1)$$

$$\frac{dX}{dt} = -bX \qquad (1.2)$$

$$\frac{dS}{dX} = T_c \qquad (1.3)$$

where:

S = non-growth substrate concentration (mg/L)
X = active organism concentration (mg/L))
k = maximum specific rate of substrate utilization
(mg substrate/mg biomass-day)
K_S = half saturation coefficient (mg substrate/l)
k'= second order rate coefficient = k/K_S (L/mg-day)
b = first order decay coefficient (per day)
T_C = transformation capacity (mg substrate/ mg cells)
t = time

Table 1.1 summarizes different ways in which these equations can be combined. Here, S^o= initial substrate concentration (mg/L), and X^o= initial concentration of active organisms (mg/L). Model 1 is obtained by combining equations 1.1 and 1.2. Model 2 is obtained by combining equations 1.1 and 1.3. Model 3 is obtained by combining equations 1.2 and 1.3. Also shown are simplifications for $S^o << K_S$ (equations 1b & 2b) and $S^o >> K_S$ (equations 1c & 2c).

In models 1 and 3, transformation activity is lost with time as a result of the decay of cometabolizing biomass. This is modeled using a simple first-order exponential decay term, as

proposed for enzymes by Bailey and Ollis (1986) and for whole cells by Galli and McMarty (1989).

Table 1-2. Kinetic expressions for cometabolism

model	Differential equations for substrate utilization rate	integrated form	ref.
1a	$-\frac{dS}{dt}=\frac{kSX}{Ks+S}$ and $\frac{dX}{dt}=-bX$ so $-\frac{dS}{dt}=\frac{kSX^{o}}{K_s+S}e^{-bt}$	$K_s\ln\left(\frac{S}{S^{o}}\right)+S-S^{o}=\frac{-kX^{o}}{b}(1-e^{-bt})$	14, 32
1b $S<<K_s$	$-\frac{dS}{dt}=k'SX$ and $\frac{dX}{dt}=-bX$ so $-\frac{dS}{dt}=k'SX^{o}e^{-bt}$	$S=S^{o}e^{\left(\frac{k'X^{o}}{b}(e^{-bt}-1)\right)}$	9
1c $S>>K_s$	$-\frac{dS}{dt}=kX$ and $\frac{dX}{dt}=-bX$ so $-\frac{dS}{dt}=kX^{o}e^{-bt}$	$S=S^{o}-T_cX^{o}(1-e^{-bt})$ where $T_c=\frac{k}{b}$	
2a	$-\frac{dS}{dt}=\frac{kSX}{Ks+S}$ and $\frac{dS}{dX}=T_c$ so $-\frac{dS}{dt}=\frac{kS\left(X^{o}-\frac{1}{T_c}(S^{o}-S)\right)}{K_s+S}$	$t=-\frac{1}{k}\left(\left(\frac{K_s}{S^{o}/T_c-X^{o}}\right)\ln\left\{\frac{SX^{o}}{FS^{o}}\right\}+T_c\ln\left\{\frac{X^{o}}{F}\right\}\right)$ where $F=X^{o}-\frac{1}{T_c}(S^{o}-S)$	1
2b $S<<K_s$	$-\frac{dS}{dt}=k'SX$ and $\frac{dS}{dX}=T_c$ so $-\frac{dS}{dt}=k'S\left(X^{o}-\frac{1}{T_c}(S^{o}-S)\right)$	$S=S^{o}\frac{F'e^{-kF't}}{X^{o}-\frac{S^{o}}{T_c}e^{-kF't}}$ where $F'=X^{o}-S^{o}/T_c$	
2c $S>>K_s$	$-\frac{dS}{dt}=kX$ and $\frac{dS}{dX}=T_c$ so $-\frac{dS}{dt}=\left(kX^{o}-\frac{k}{T_c}(S^{o}-S)\right)$	$S=S^{o}-T_cX^{o}(1-e^{-bt})$	
3	$\frac{dX}{dt}=-bX$ and $\frac{dS}{dX}=T_c$ so $\frac{dS}{dt}=-bT_cX^{o}e^{-bt}$	$S=S^{o}-T_cX^{o}(1-e^{-bt})$	31

In model 2, the loss of transformation activity is directly proportional to the amount of non-growth substrate transformed. A key concept in models 2 and 3 is the idea of a "transformation capacity" T_C for the cometabolizing cells. As stated earlier, Alvarez-Cohen and

McCarty (1991a) defined T_C as the mass of non-growth substrate transformed per unit of biomass, as expressed mathematically by equation 1.3. In both the second and third models, it is assumed that transformation capacity is independent of concentration and is constant. In the first model, however, transformation capacity, while not explicitly defined, can be derived by dividing equation 1.1 by equation 1.2. In this case, T_C is not constant, but depends upon the concentration of the non-growth substrate (equation 1.4).

$$Tc = \frac{kS}{b(K_s + S)} \qquad (1.4)$$

Equation 1.4 has the same form as the Monod expression for substrate utilization, as illustrated in Figure 1.3. For high substrate concentration ($S>>K_S$), transformation capacity T_C becomes constant, $T_C = k/b$, and model 1 converges with models 2 and 3 (compare 1c, 2c, and 3c). When T_C is close to K_S, the transformation capacity is half its maximum value. At low concentrations ($S<<K_S$), model 1a predicts a linear increase in transformation capacity with substrate concentration.

In addition, models 1 and 2 assume a constant maximum substrate utilization rate over all substrate concentrations, and models 1 and 3 assume that the biomass decay rate is independent of substrate concentration and is also constant. A summary of all the assumptions behind the models is shown in Figure 1.4.

Saez and Rittmann (1991) used model 3 and classified their experiments as a "success" or "failure" depending upon the rate and extent of transformation. For a "success" test, the target compound was degraded steadily and completely; for a failure test, only minor amounts of the compound were transformed. The substrate to biomass ratio correlated with the success or failure of an experiment. During failure tests, T_C decreased with increasing time. This was

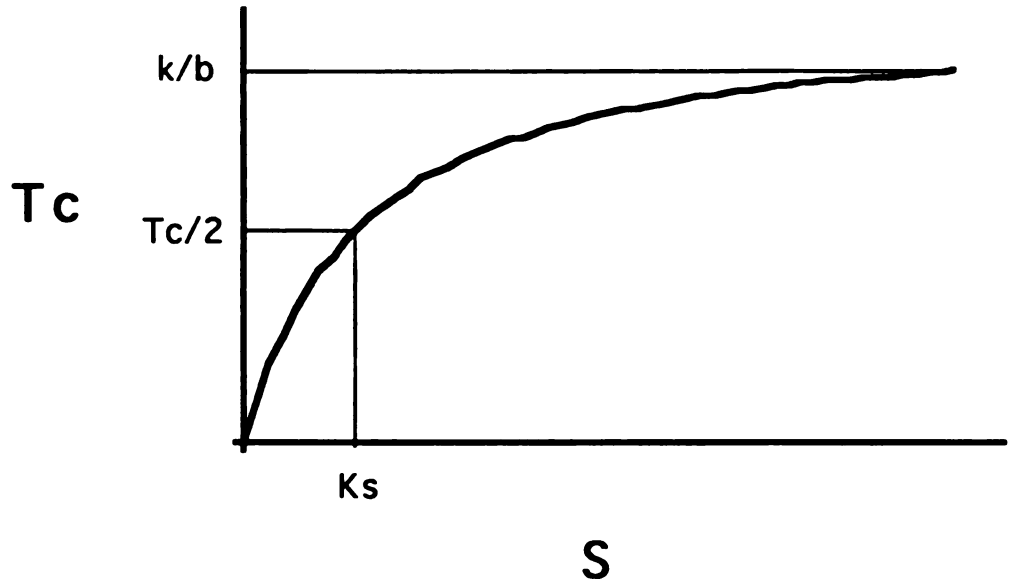

Figure 1.3. Dependence of transformation capacity on substrate concentration for model 1.

MODEL ASSUMPTIONS

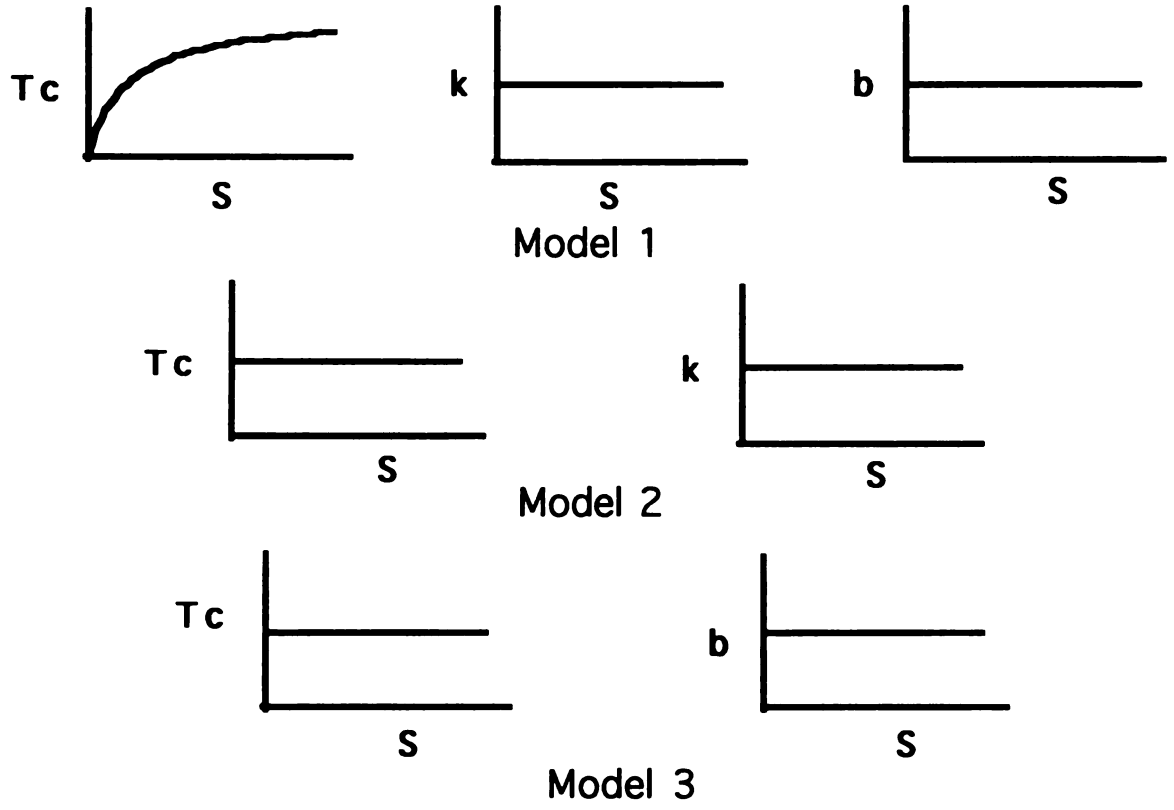

Figure 1.4. Assumptions associated with models 1, 2, and 3.

attributed to increased inhibition as the S:X ratio increased. In other words, the biomass was dying off faster than the compound was transformed. If T_C was greater than the $S^O:X^O$ ratio, the test was classified as a success. If T_C was less than the $S^O:X^O$ ratio, the test failed.

CHAPTER 2

MATERIALS AND METHODS

Mixed culture development.

Mixed culture MM1, a methanotrophic enrichment obtained from aquifer material at the Moffett Field test site, was used for all experiments in this study (courtesy of S. Henry, Stanford University). This culture was grown in Whittenbury Mineral media (Appendix A). A 1-liter culture was grown at room temperature in a continuously stirred 4-liter bottle with approximately 30% methane in air supplied at 68 mL/min. Growth curves were monitored and as stationary phase approached, approximately 10 mL of culture was transferred to 1 liter of Whittenbury Media and incubated as previously described. Cells were harvested in mid-log growth for all testing.

In order to ensure that mid-log growth stage was the optimal stage of growth to utilize, preliminary work was done involving methane utilization rates for different stages of the culture's growth. The materials and methods for this procedure is described in Appendix B, with a summary of the results shown in Table 2.1.

Table 2.1. Methane Utilization Results

Elapsed Time (hr)	Dry Wt. Conc. (mg/L)	$dM/dt/V_L$ (mg/L-day)
24.5	40	-115.4
43	140	-117
48.5	240	-349.2
73.5	490	-574.6
90	760	-975.3
96	820	-810.6
145.5	1590	-308.67

From the data, it is evident that as the culture approaches mid-log phase (dry weight between 500-800 mg/L), the specific rates of methane utilization ($dM/dt/V_L$) increased indicating greater activity within the culture. Based on these results, additional experiments were conducted using cells with high activity (in mid-log growth phase) for the TCE degradation studues.

TCE solutions and analysis.

Water saturated with TCE was used as the spike solution in all experiments. The TCE saturated solution was prepared by adding 3 mL pure TCE (99%+ Aldrich Chemical Company, Milwaukee, MI.) to a 120-mL vial with Teflon®-lined rubber septum containing 117 mL water. The vial was vigorously shaken and stored in a refrigerator until needed. One hour before use, it was shaken again and allowed to settle. A Hamilton microliter syringe was injected through the Teflon® septum and the appropriate amount of TCE saturated water was withdrawn and injected directly into batch bottles containing the culture.

TCE concentrations were determined using headspace analysis. Headspace samples of 0.2 mL were removed from the test bottles with a Precision gas-tight syringe and analyzed on a Perkin Elmer 8500 Gas Chromatograph (GC). For low TCE concentrations (less than 0.5 mg/l), samples were analyzed on a GC equiped with an electron capture detector and a stainless steel packed column with nitrogen as the carrier. For high TCE concentrations (1-10 mg/L), samples were analyzed on a GC equipped with a flame ionization detector and a squalane packed column with helium as the carrier. The oven-temperature program was set at an isothermal temperature of 90°C for both the FID and ECD.

Standards for calibration were prepared by adding approximately 25 mg of TCE to 25 mL of methanol for a concentration of 1 mg TCE/mL MeOH. The vial was sealed with a Teflon®-lined rubber septum and aluminum crimp-top cap. From this stock solution, known

amounts of TCE were transferred to bottles containing mineral media, shaken vigorously on a rotary shaker and sampled three minutes later. A calibration curve was produced for each data set with at least five concentration levels bracketing the concentrations expected in the samples. The TCE concentration in the samples was obtained using the calibration curve, a dimensionless Henry's constant of 0.33 for a temperature of 21°C (Gossett, 1987), and known liquid and gas volumes. Controls without cells were spiked and analyzed in all experiments.

TCE Transformation Studies

TCE transformation studies were performed using 250-mL glass bottles sealed with Teflon Mininert® valves. These bottles were inoculated with 100 mL of a mixture of mineral media and culture. The mass of culture added varied from 10 mg to 16 mg per bottle depending on the desired rate of transformation. TCE saturated water was injected through the Mininert valves into the bottles, shaken vigorously for 20 seconds and placed upside-down on a rotary shaker (250 rpm). The bottles were shaken at 100 rpm and 350 rpm with no increase in TCE utilization, indicating that the system was not mass transfer limited. Doubling the biomass concentration resulted in a doubling of the transformation rates, also indicating that the system was not mass transfer limited under the experimental conditions used. After TCE addition, 0.2 mL gas samples were withdrawn periodically using a 1-mL Precision gas tight syringe and injected on to a GC for analysis.

Culture Density

Cell biomass was determined on a dry weight basis using 0.2 μm filters (Gelman Sciences Inc., Ann Arbor, MI.). The filters were prepared by first soaking them in mineral media for 10 minutes, rinsing on a vacuum filter with deionized water, drying overnight in a 103°C oven, and cooling in a desiccator until needed. The filters were weighed, and once a

known amount of culture was filtered through them, they were rinsed, dried, cooled and reweighed. Optical density was also determined by measuring culture transmittance at 540 nm on a Shimadzu UV-160 spectrophotometer.

CHAPTER 3

EFFECTS OF TCE CONCENTRATION ON TRANSFORMATION CAPACITY

Introduction

As discussed in Chapter 1, it is now clear that the both cell-free mmo enzyme and the whole-cell suspensions of methanotrophs can oxidize many different compounds, including trichloroethylene. From an engineering prospective, the key to the degradation of these compounds is manipulating the environmental conditions of the culture to maximize the rate and capacity of degradation. This chapter focuses on the effects of TCE concentration on transformation capacity.

According to Alvarez-Cohen and McCarty (1991a), transformation capacity (T_C) can be obtained experimentally by determining the maximum amount of TCE degraded by a specific amount of biomass. In this thesis, this method is referred to as the 'measured' method of obtaining T_C. This method can only be applied to data sets in which the TCE concentration does not go to zero because the microorganisms may not have reached their capacity to degrade TCE. A respike may need to be performed until the degradation levels off at a point which is not zero.

Materials and Methods

All materials and methods used for this section are described in Chapter 2. For the batch studies, 30 mL of cells were harvested, giving a mass of cells per bottle of 15 - 17 mg, and placed in 250-mL bottles containing 70 mL mineral media.

Results

The models of Table 1.1 were applied to triplicate samples run at five different concentrations: 0.016 - 0.018 mg/L, 0.22 - 0.26 mg/L, .91 - .97 mg/L, 5.5 - 5.7 mg/L, and 10 - 12.5 mg/L. A graph of this data is shown in Figures 3.1 through 3.3.

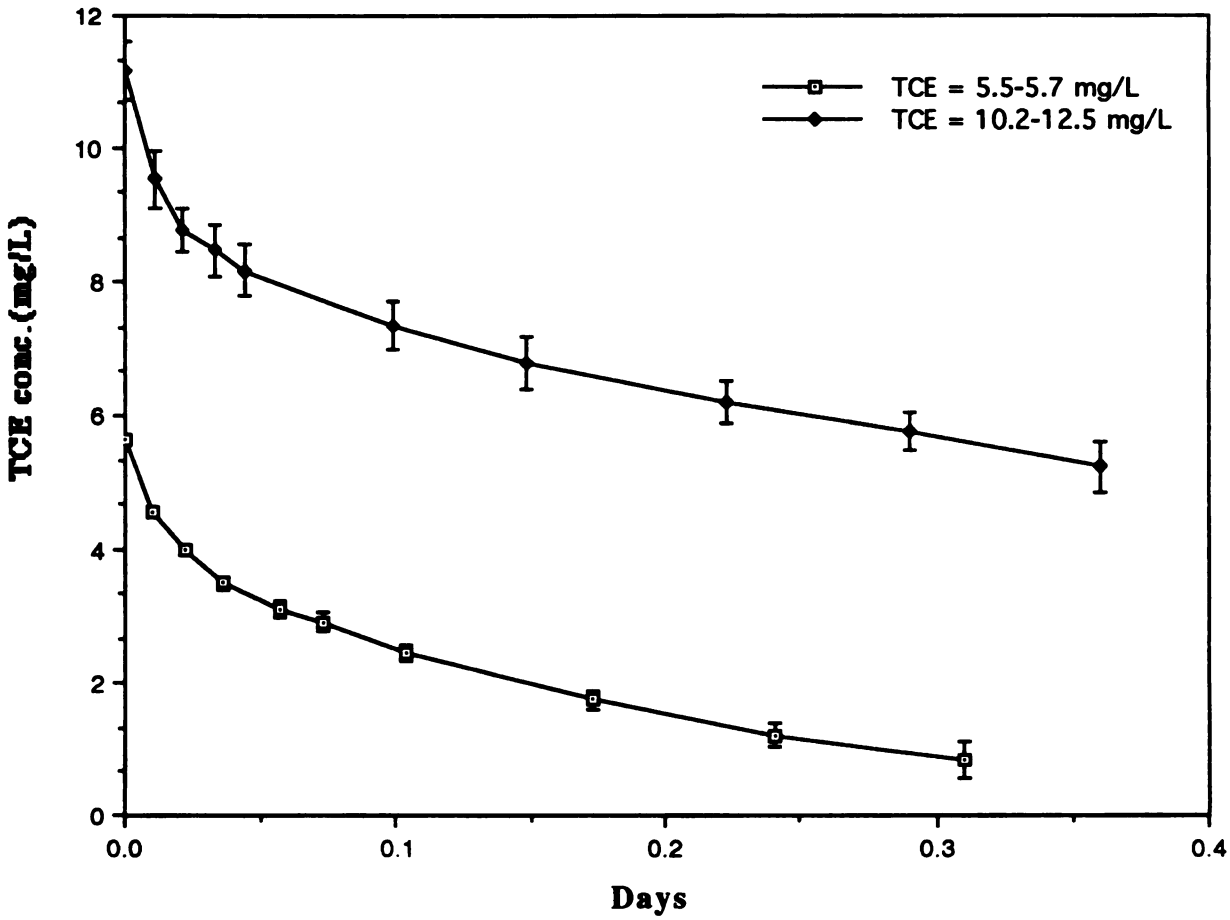

Figure 3.1. TCE degradation at high initial substrate concentrations.

Figure 3.1 shows data from two of the higher initial substrate concentration ranges. The data points are an average of triplicate samples with the error bars representing the respective standard deviation. Figure 3.2 shows data in the middle range of initial substrate concentrations, and Figure 3.3 shows data from the low initial substrate concentration. In addition, a respike experiment was done at the low TCE concentration to determine

transformation capacity. The first six hours of this experiment are shown in Figure 3.4. Cumulative TCE degraded over the five days for the same set of data is shown in Figure 3.5.

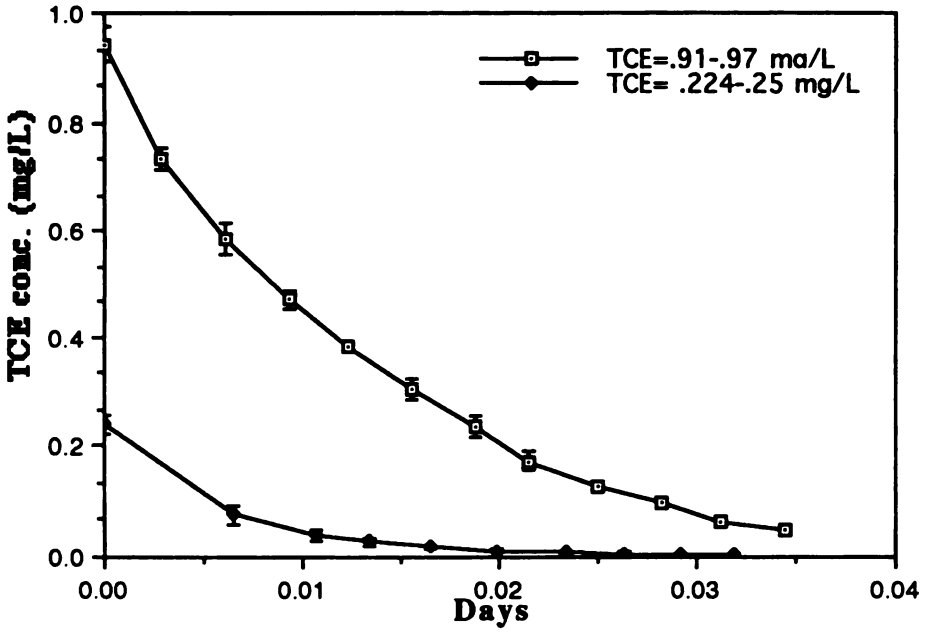

Figure 3.2. TCE degradation at medium initial substrate concentrations.

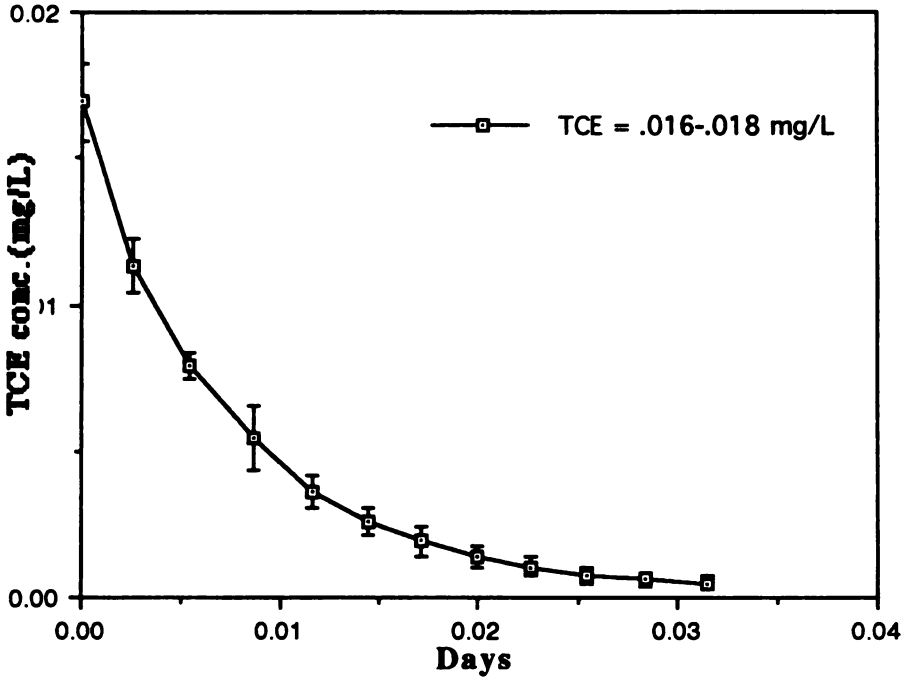

Figure 3.3. TCE degradation at low initial substrate concentrations.

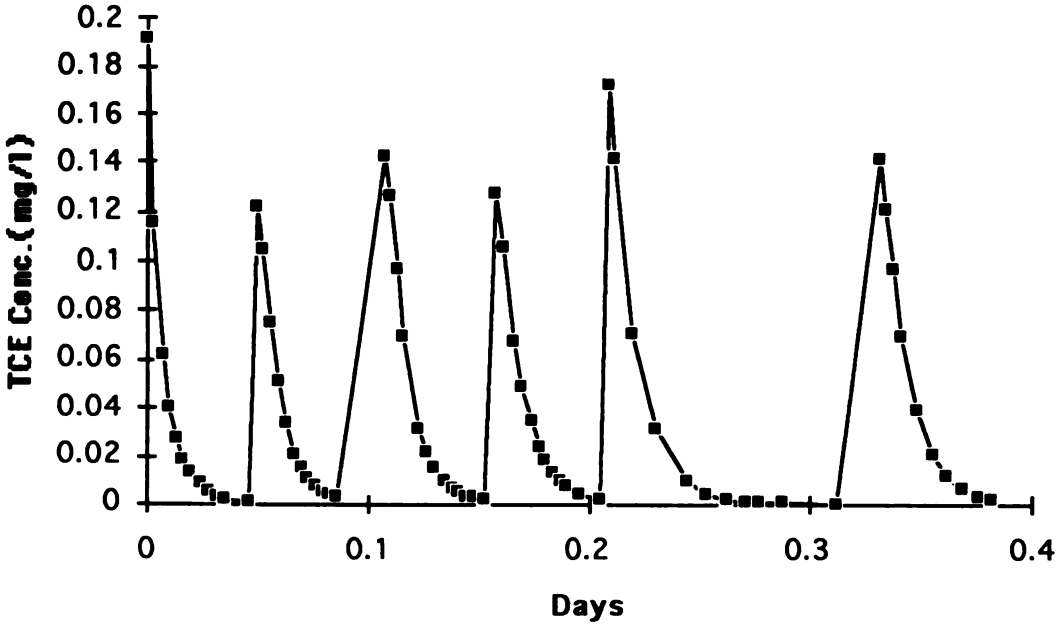

Figure 3.4. Continuous respike experiment for low levels of TCE (experiment continued for five days and required 22 spikes: only the first six spikes are shown here.)

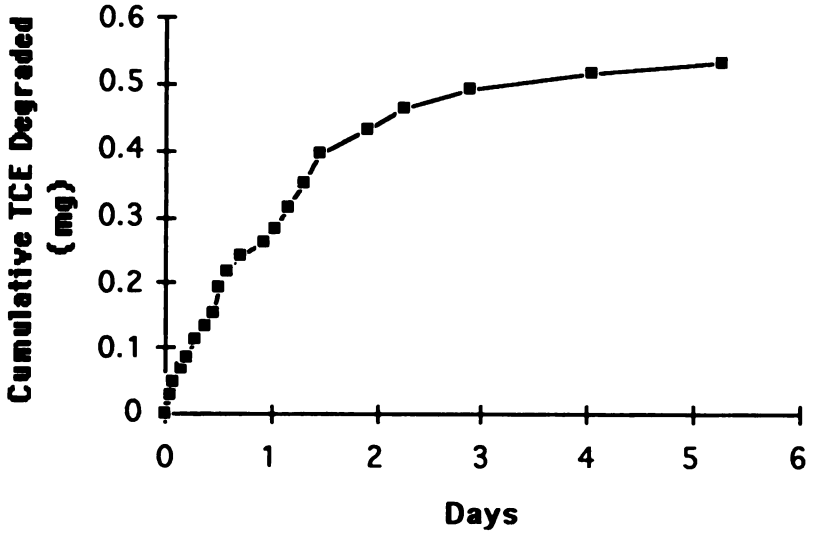

Figure 3.5 Cumulative TCE degradation

Figure 3.5 shows the degradation of TCE to be leveling off after about three days. The cumulative TCE degraded after five days was 0.53 mg. Dividing this mass by the cell mass of 15 mg gives an apparent transformation capacity of 0.036 mg TCE/mg cells.

These data can be compared with T_C obtained for the high concentration data set. For the high concentration data, the total TCE degraded was 0.89 mg TCE. Dividing this mass by the amount of cells in the bottle (16.2 mg) gives a T_C of 0.055 mg TCE/mg cells.

Finally, Tables 3.1 through 3.3 provide a summary of all the current models used to describe cometabolism for a wide range of TCE concentrations (covering three orders of magnitude). Nonlinear regression analysis was performed using Systat 5.1 application software (Systat, Inc.) Table 3.1 gives best fit parameter estimates for model 1 at five different initial concentrations. A key assumption for use of model 1 is that the decay term b is constant and therefore independent of substrate concentration. Accordingly, b was estimated from the high concentration data set and model 1c and used as a constant in evaluating the mid-range and low concentration data sets with models 1a and 1b. Model 1 also assumes that the maximum rate of substrate utilization, k, is independent of substrate concentration. Consequently, the value for k obtained using the high concentration data set was used to evaluate the mid-range data set in estimating K_S.

Table 3.2 gives best fit parameter estimates for model 2 at all concentrations. A key assumption for model 2 is that the transformation capacity and the maximum rate of substrate utilization remain constant and are independent of substrate concentration. Consequently, T_C and k were estimated from the high concentration data and used as a constant in evaluating model parameters at lower concentrations.

Table 3.3 gives the best fit parameter estimates for model 3 with varying initial concentrations. For comparison, the 'measured' values of T_C are also provided. For low concentrations, the measured transformation capacity could only be determined by continuous long-term respiking. In addition, model 3 was used to estimate the initial TCE concentration, S^o, and consistently underestimated the transformation capacity by choosing a lower S^o and

Data set and Model used	Model 1 - Fitting Parameters k'	k	Ks	b	R^2
Model 1b S°=.015-.017 mg/L	1.4±.23	N/A	N/A	9.03 (a)	.998±.001
Model 1a S°=.22-.25 mg/L	N/A	0.422 (b)	.257±.013	9.03 (a)	.98±.007
Model 1a S°=.91-.97 mg/L	N/A	0.422 (b)	.184±.034	9.03 (a)	.98±.002
Model 1c S°=5.5-5.7 mg/L	N/A	0.38±.01	N/A	9.03 (a)	.97±.005
Model 1c S°=10.2-12.5 mg/L	N/A	0.422±.06	N/A	9.03±.63	.94±.01

(a)= used average b from high concentration data to predict other parameters
(b)=used average K from high concentration data to predict other parameters
N/A= model did not estimate these parameters

Table 3.1 Evaluation of model 1 at five different TCE concentrations.

Data set and Model used	Model 2 - Fitting Parameters k'	k	Ks	Tc	R^2
Model 2b S°=.015-.017 mg/L	1.33±.24	N/A	N/A	.047 (a)	.997±.001
Model 2a S°=.22-.25 mg/L	N/A	0.422 (b)	0.29±.02	.047 (a)	.97±.008
Model 2a S°=1.1-1.2 mg/L	N/A	0.422 (b)	0.21±.04	.047 (a)	.98±.001
Model 2c S°=5.5-5.7 mg/L	N/A	0.406±.03	N/A	.047 (a)	.97±.006
Model 2c S°=10.2-12.5 mg/L	N/A	0.422±.06	N/A	.047±0.0	.94±.01

(a)= used average Tc from high concentration data to predict other parameters
(b)= used average b from high concentration data to predict other parameters
N/A= model did not estimate these parameters

Table 3.2 Evaluation of model 2 at five different TCE concentrations.

Data set	Model 3 - Fitting Parameters b	Tc	R^2	Measured Tc
S°=.015-.017 mg/L	0.083±.01	0.047(a)	0.73±.046	N/M
S°=.22-.25 mg/L	1.2±.15	0.047(a)	0.63±.016	N/M
S°=.91-.97 mg/L	5.114±.637	0.047(a)	0.91±.018	N/M
S°=5.5-5.7 mg/L	8.69±.64	0.047(a)	0.97±.006	0.043±.003
S°=10.2-12.5 mg/L	9.031±.635	0.047±0.0	0.944±.01	0.055±.0.0
Respike data S°=.12-.18 mg/L S°= .15-.23 mg/L	N/D	N/D	N/D	0.035 0.034

N/M= not measured
N/D= not determined

Table 3.3 Evaluation of model 3 and 'measured' T_c with five TCE concentrations.

leveling off higher than the data as shown in Figure 3.6

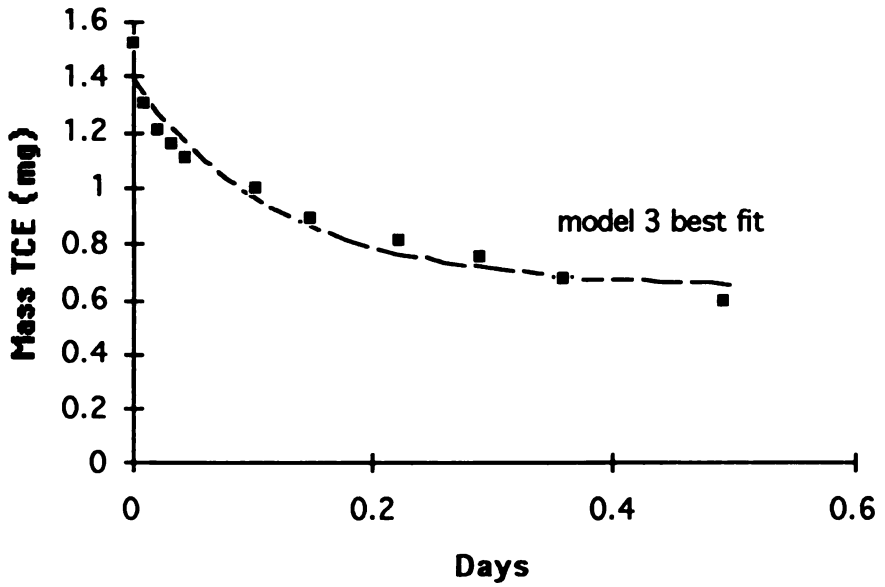

Figure 3.6 Typical degradation curve for high-range TCE concentration. The measures mass of TCE degraded is compared with the fit for model 3 (note that model 3 is the same as models 1c and 2c.)

It should be noted that due to the inaccuracy of a time zero measurement due to nonequilibrium between the headspace and liquid phase of a volatile compound at the initial injection of TCE, computing the 'measured' transformation capacity using the initial data point for each set of data would be inaccurate. Instead, the initial data point was obtained by averaging the controls for the experiment, which were injected with the same initial amount of TCE, and subtracting the remaining TCE once the degradation leveled off.

Discussion

There are some conceptual differences in the models of Table 1 which should help in their selection. In model 1, the loss of activity is not directly linked to transformation, whereas models 2 and 3 assume a direct linkage. In model 3, transformation rate is

independent of substrate concentration and depends only upon the rate of decay of transformation activity. This decay term and k were estimated at high TCE concentration and used at lower concentrations to predict the necessary parameters. These parameters were then used to compare the consistency of the model at all concentrations. From Table 3.1, dividing the k by the K_S predicted from model 1a at the medium TCE concentration of 0.91-0.97 mg/L, and using propagation of errors (Appendix D), gives a k' of 2.3 ±.097 L/mg-day. This value is almost twice as large as the value predicted by model 1b at the low TCE concentration of 1.4 L/mg-day. However, using the same procedure at the TCE concentration range of 0.22-0.24 mg/L gives a k' of 1.63±.15. This reveals some inconsistencies in model 1 over the range of data tested. As for transformation capacity, model 1 predicts increasing T_C with increasing TCE concentration. Since the apparent T_C at low concentrations was fairly close to T_C at high concentrations (shown using the respike data), model 1 does not appear to be appropriate for this data set.

In model 2, T_C is assumed to be independent of TCE concentration. For the evaluation of model 2, T_C and k were estimated at the high TCE concentration and used in the models for lower TCE concentration to predict the necessary parameters. From Table 3.2, dividing k by the K_S as predicted with model 2a, and using propagation of errors, gives a k' of 2.05 ±.096 L/mg-day (at the TCE concentration of 0.91-0.97 mg/L) which is again much larger than the 1.33 L/mg-day value predicted by model 1b at the lower concentration. However, the k' for the TCE concentration of 0.22-0.24 mg/L was 1.46±.12. Again this indicates problems with the consistency of model 2 over the concentration range tested.

The assumption behind model 3 is that both T_C and b are independent of TCE concentration. Using model 3, T_C was held constant and the decay term allowed to vary. From Table 3.3, the assumption that the decay term is constant does not seem to hold true for the data set. The term b varies from 9.031 d^{-1} at the high TCE concentration to .083 d^{-1} at the low TCE concentration with poor correlation coefficients at the lower end.

To evaluate the uniqueness of these non-linear parameter estimates and how important each one is over the concentration range tested, a sensitivity analysis was performed (Beck, 1977). The detailed analysis is performed in Appendix D, but in summary, most of the models gave unique parameters except for the low concentration data using model 3, and the low TCE concentration range of 0.22-0.24 mg/L using models 1a and 2a. This suggests that models 1a and 2a should report k' (k/Ks) rather than k and Ks at the concentration in question, and for model 3, another approach to modeling should be used at the lower concentration range, possibly the one used by Saez and Rittmann (1991) discussed below. However, since models 1 and 2 converge to model 3 at high TCE concentrations, and model 3 appears to be valid in this range, it will be used to model data obtained at TCE concentrations at or above 2 mg/L.

There are certain limitations of the models which should be considered. For model 2, the solution is discontinuous when F = 0. Real solutions can only be obtained when $F + S/TcX_O > 0$ which simplifies to $X_OTc > (S_O-S)$, indicating that the extent of transformation can not exceed the transformation capacity (Alvarez-Cohen, 1991a).

It should be noted that Saez and Rittmann (1991) used a different approach for parameter estimation with model 3. In this model, biomass is assumed to decay in a first order manner given by:

$$X = X_o e^{-bt} \qquad (3.1)$$

Saez and Rittmann (1991) linearized equation 3.1 and used biomass measurements to determine X_O and b. From there, equation 3.2 was linearized using the estimated values for X_O and b to determine T_C.

$$S = S_o - T_c X_o (1 - e^{-bt}) \qquad (3.2)$$

For this project, the time rate of change in biomass was not obtained, so data involving substrate concentration vs. time was fit to equation 6 using non-linear parameter estimation to solve for T_C and b.

CHAPTER 4

EFFECTS OF pH AND FORMATE ADDITION

Introduction

Several investigators have shown that environmental conditions play a key role in the degradation of TCE (Henry, 1991, Alvarez-Cohen, 1991b). These environmental factors enhance or reduce transformation rates or capacity. This chapter explores two environmental factors: the addition of formate, which provides additional reducing power for mmo oxidation of TCE, and pH effects. These factors may be helpful in the optimal operation and design of engineered systems and in the comparison of methanotroph enrichments from different sources.

Initially, the effects of formate addition at low TCE concentrations were investigated. Subsequently, high TCE concentrations were investigated at various pH values.

Materials and Methods

Culture preparation was as described in Chapter 2. For the formate experiments at neutral pH, 10 to 15 mL of mid-log phase cells were harvested and placed in a flask. Two different levels of sodium formate (Aldrich Chemical Co., Deerfield, Ill.), 2 mM and 20 mM, were added by weighing out the necessary amount of formate, adding it to the cell suspension, and diluting up to 100 mL with mineral media. This solution was then transfered into a 250 mL bottle and sealed with a Mininert®valve. Depending on the experiment, various amounts of TCE were added from the TCE saturated stock solution to the bottles. The bottles were t hen shaken vigorously for 15 seconds, sampled, placed on a rotary shaker, and periodically sampled thereafter.

For the pH experiments, the culture was grown at a neutral pH of 6.8, harvested and incubated in 250-mL bottles buffered at a desired pH. The liquid volume in the 250-mL bottles contained 50 mL of a universal buffer (Perin and Dempsey, 1979) with the remaining 50 mL consisting of a mixture of 42 mL of mineral media and 12 mL of culture to obtain final pH values between 5.5 and 10.

Results

Formate effects at low TCE concentrations were examined initially. Two different levels of formate were used: 2 and 20 mM. Samples without formate were also tested. The initial aqueous phase TCE concentration was approximately 0.19 mg/L. At these TCE concentrations, formate had no effect on the degradation rates. (Figure 4.1).

The next logical step was to determine whether formate addition had any effect at high TCE concentrations. For experiments run at high TCE concentrations (4.5 mg/L), 20 mM formate was used to ensure that transformation would be visible if formate made a difference.

With the addition of 20 mM formate, k increased from 0.145 to 2.4 mg TCE/mg biomass-day. In addition, the transformation capacity increased from 0.043 mg TCE/mg cells to 0.082 mg TCE/mg cells. This data was fit using model 3 and the results are shown in Figure 4.2. It should be noted that because the TCE concentration decreased to zero (and no additional TCE was added), the transformation capacity is underestimated.

Degradation parameters for pH of 5.5, 6.8, 8.3, and 10 were determined. Transformation rates decreased in the more basic region (near pH = 10). The acidic region of pH = 5.5 had slightly slower rates, and the neutral range between pH = 6.8 to 8.5 seemed to contain the fastest rates as shown in Figure 4.3. Table 4.1 provides a summary of the parameters obtained from this data when evaluated with model 1b. Due to the difficulty of obtaining consistent initial TCE concentrations at this level of TCE, data from triplicate samples were averaged.

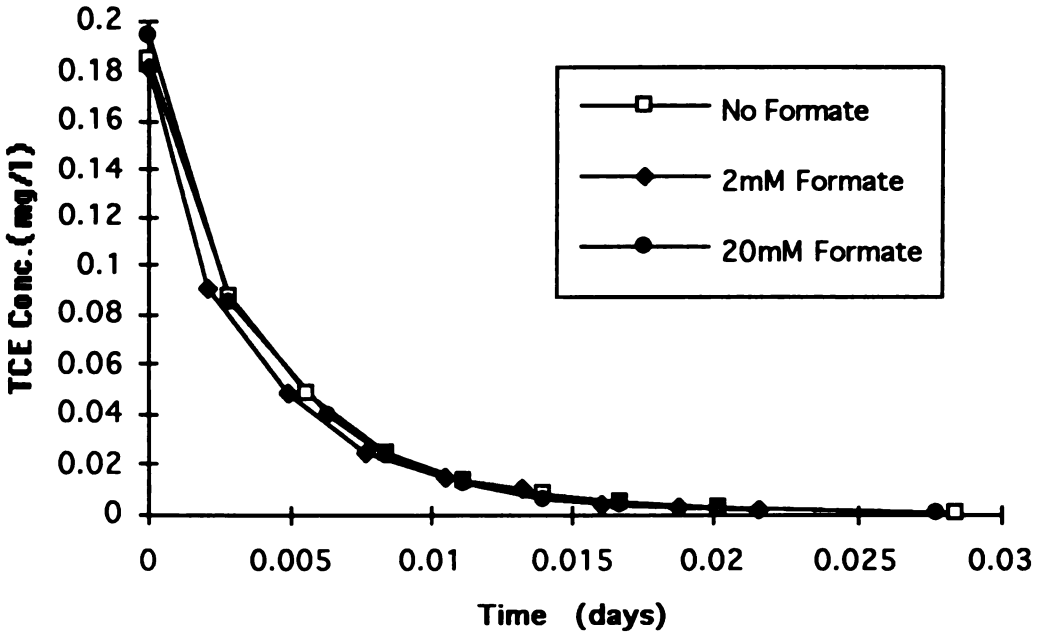

Figure 4.1. Effects of formate addition on TCE Degradation at low TCE concentrations (initial TCE concentration = 0.18 mg/L)

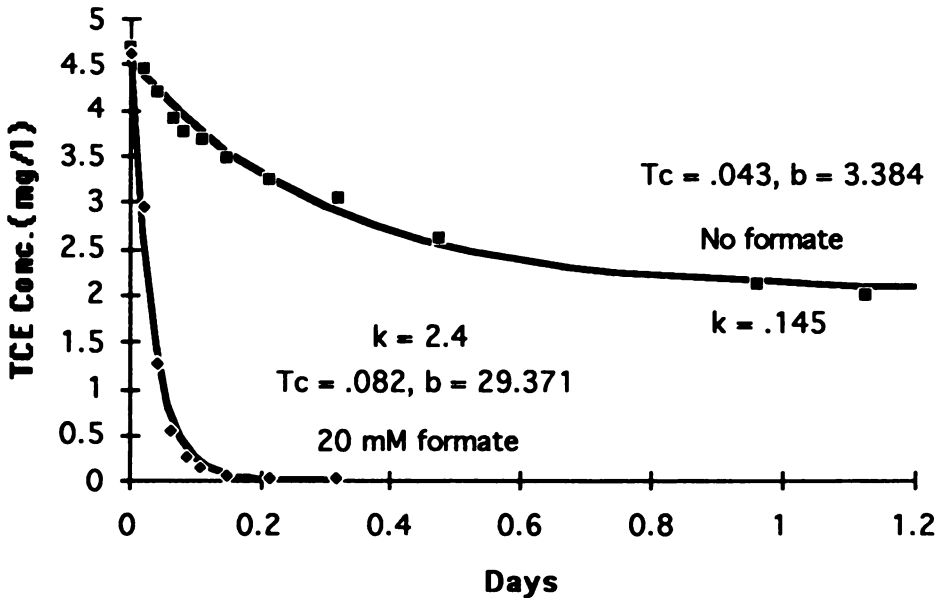

Figure 4.2. Effect of formate on TCE degradation at a high TCE concentrations. The Tc reported was obtained by parameter estimation.

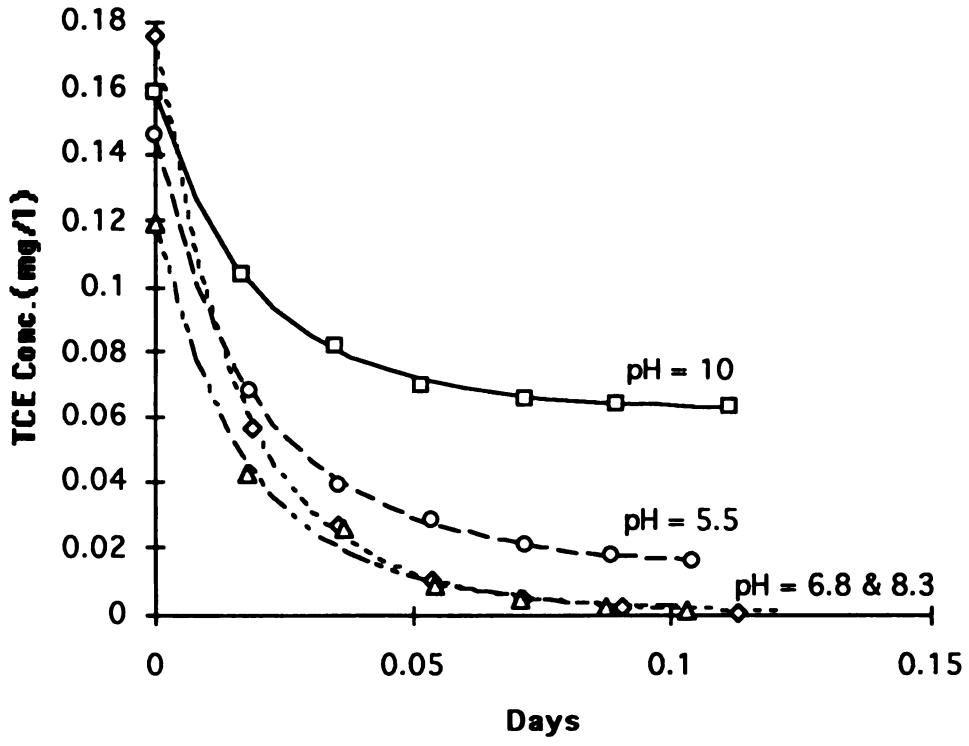

Figure 4.3. Effects of pH on TCE transformation at low TCE concentrations. (initial TCE concentration = 0.12-0.17 mg/L)

Table 4.1

Summary of pH Effects at Low TCE Concentration				
Start pH	End pH	R2	k'	b
5.6	5.5	1	1.061	20.47
6.82	6.78	0.995	1.225	9.682
8.32	8.2	1	1.357	8.605
9.98	9.86	0.999	0.698	35.64

The effects of pH were also evaluated at a higher TCE concentration of 5.0 - 5.5 mg/L. These results were modeled with model 3 and are shown in Figure 4.4 with a summary of the kinetic parameters listed in Table 4.2. Incubation pH had a dramatic effect on transformation

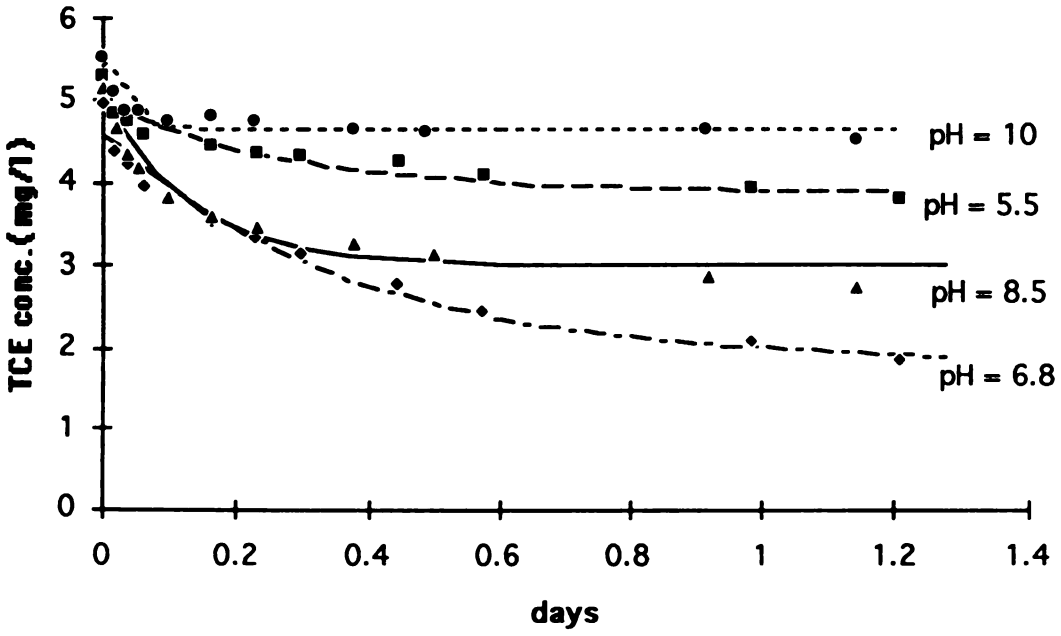

Figure 4.4. Effects of pH on TCE transformation at high TCE concentrations. (initial TCE concentration = 4.5 - 5.5 mg/L)

Table 4.2

Summary of pH Effects at High TCE concentrations (Triplicate Samples)					
pH (begin)	pH(end)	R2	Tc	b	k
5.47	5.41	0.86	0.0197	3.385	0.067
5.48	5.5	0.869	0.0212	2.969	0.063
5.48	5.41	0.917	0.0212	4.304	0.0912
6.86	6.86	0.976	0.0515	2.943	0.151
6.95	6.92	0.97	0.053	2.772	0.147
6.98	6.91	0.969	0.053	2.415	0.128
8.45	8.53	0.963	0.038	7.286	0.277
8.4	8.45	0.96	0.039	8.825	0.309
8.42	8.44	0.966	0.036	9.053	0.326
10.11	10.19	0.914	0.017	30.827	0.524
10.05	10.14	0.918	0.017	40.504	0.688
10.02	10.1	0.905	0.015	47.371	0.71

capacity. The effects of incubation pH were further evaluated by providing 20 mM formate with a high levels of TCE (5 mg/L). The results for pH 5.5, 6.8 and 10 are shown in Figures 4.5, 4.6, and 4.7 respectively. Again, all data were evaluated with model 3. From Figure 5, at pH = 5, the maximum specific rate of substrate utilization without formate was .0896 mg TCE/mg cells-day. While formate was provided this value increased to 0.392 mg TCE/mg cells-day. The transformation capacity almost tripled from 0.019 mg TCE/mg cells to 0.049 mg TCE/mg cells.

In one sample, illustrated in Figure 4.6, TCE was respiked at neutral pH in order to provide a better estimate for T_C. Formate addition increased transformation capacity from .034 mg TCE/mg cells to .139 mg TCE/mg cells. In the more basic region (pH = 10) formate addition did not increase TCE transformation rates or capacity (Figure 4.7).

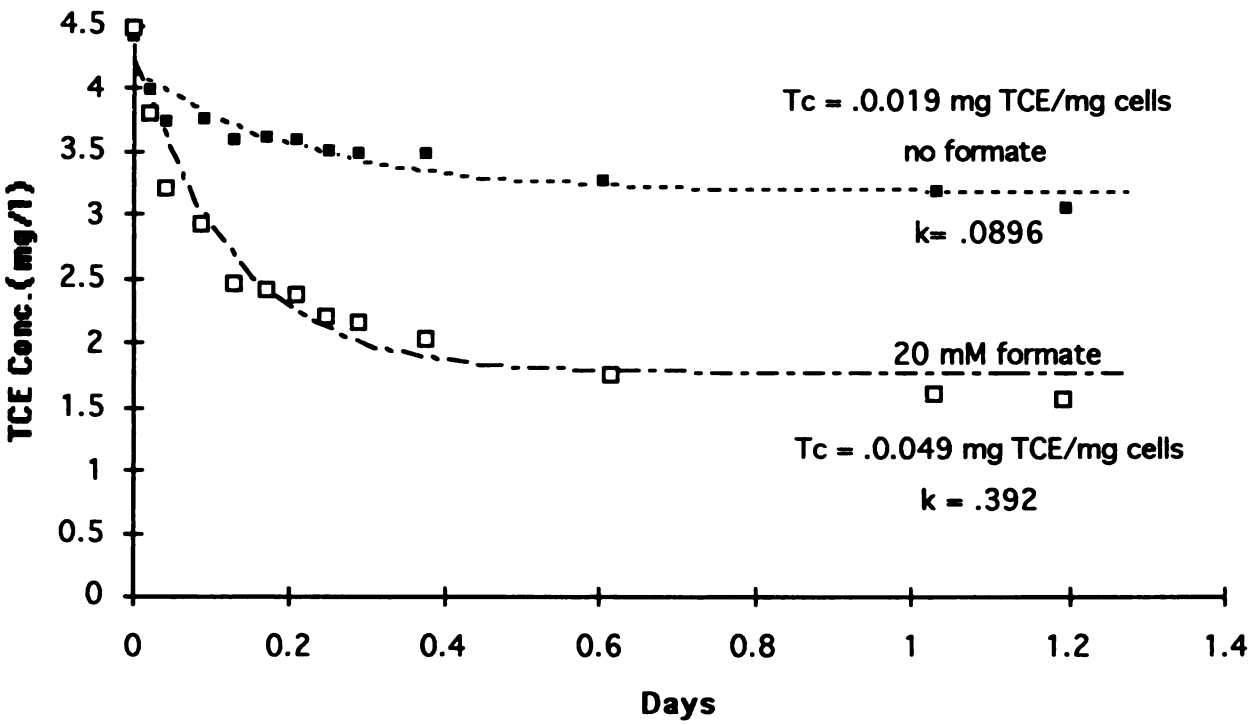

Figure 4.5. Formate effects at pH = 5.

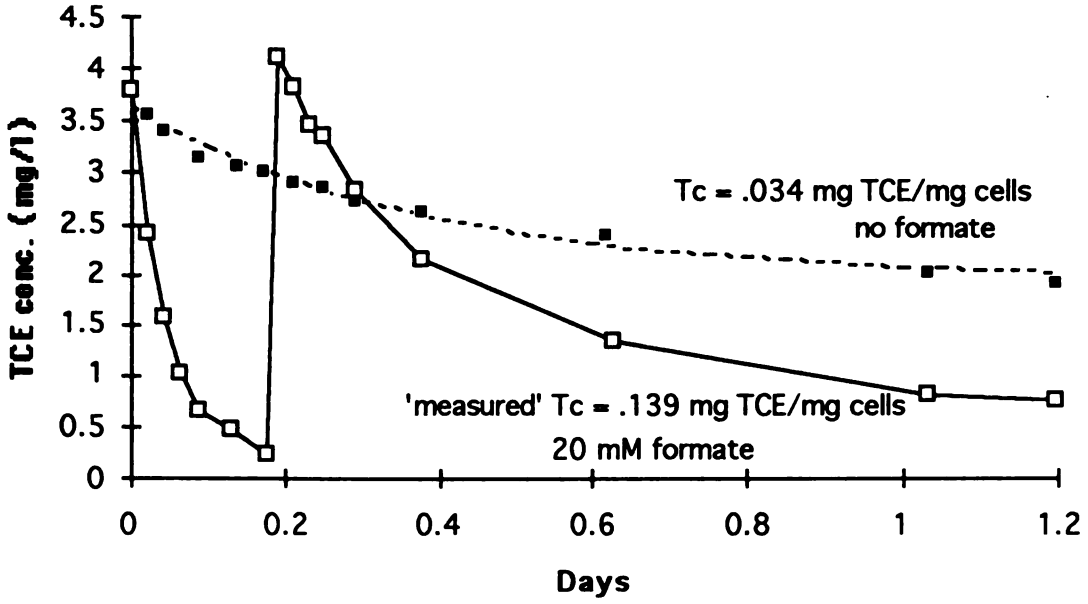

Figure 4.6. Formate effects at pH = 6.8.

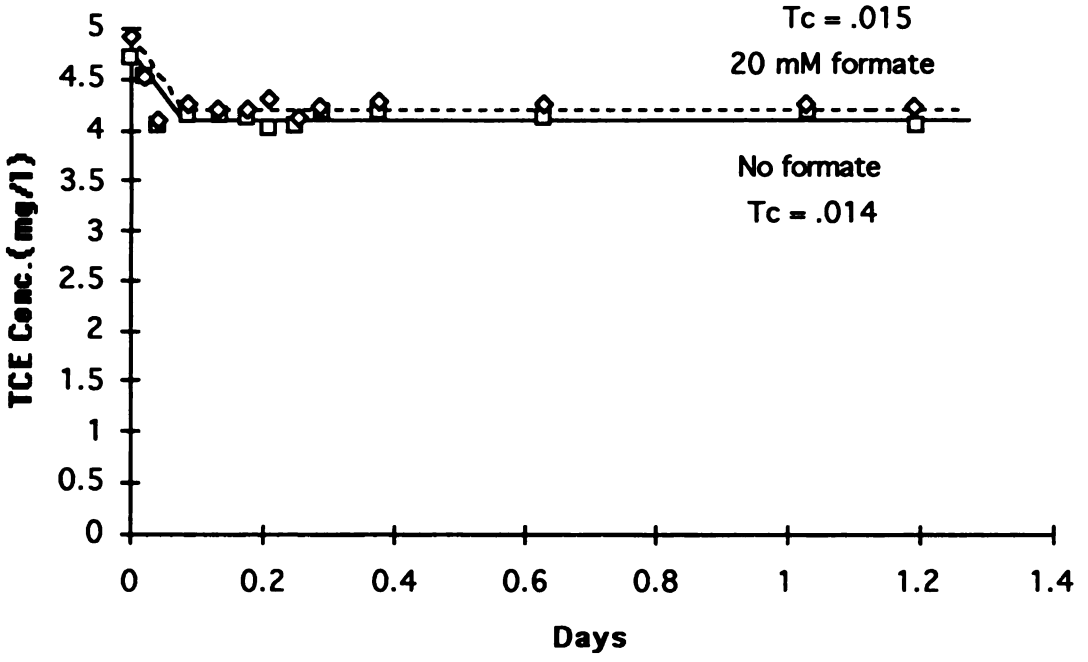

Figure 4.7. Formate effects at pH = 10.

Discussion

The results indicate that formate addition is helpful only under certain conditions. At low TCE concentrations, there appears to be enough reducing power available to degrade TCE. However, if respike experiments were performed at these low TCE levels with formate, the transformation capacity would probably increase. Additional TCE would require additional reducing power, which the formate could provide.

Formate addition at high TCE concentrations is definitely advantageous in the neutral pH range and also in the more acidic region of pH = 5.5 , but it does not enhance degradation at the more basic region of pH = 10.

Table 2 indicates that there is an increasing k with increasing pH. This could indicate an enzyme which increases activity at high pH but also looses activity at an accelerated rate at higher pH.

Based on the high TCE concentration data, transformation capacity appears to be a more accurate measure of capabilities of a culture than either the decay coefficient b or the maximum rate of substrate utilization k. At pH = 10, the k value is very high, but if the decay coefficient is also large, the overall efficiency of T_C is low.

CHAPTER 5

EFFECTS OF ENDOGENOUS DECAY

Introduction

As mentioned in Chapter 3, it has been suggested that current models to describing cometabolism of TCE should possibly by modified to distinguish between the loss in transformation activity caused by TCE toxicity and the loss cause by endogenous decay. In order to do this, however, it is necessary to understand the mechanism or mechanisms behind the loss of activity.

To facilitate part of this objective, an experiment was designed to study endogenous decay to see how this parameter would effect the transformation capacity of a culture over a certain period of time.

Materials and Methods

The culture was prepared as described in Chapter 2. For these studies, 30 mL of culture containing 15 mg cells and 70 mL of mineral media was transferred to five 250-mL bottles. Transformation capacity at time zero was measured by spiking one of the five bottles with 5.0 mg/L TCE and monitoring the disappearance over time. In order to measure the decline in transformation capacity over a 4 day period, the remaining bottles were incubated without TCE addition for 1, 2, 3, or 4 days prior to the measurement of transformation capacity. Once spiked with TCE, each bottle was measured for a certain period of time until the degradation ceased and the transformation capacity leveled off.

Results

The results from each day is shown in Figures 5.1 through 5.5. All data was evaluated using model 3 unless otherwise stated. As shown, transformation capacity decreased from .04 mg TCE/mg cells in day 1 to 0.0081 mg TCE/mg cells in day 5.

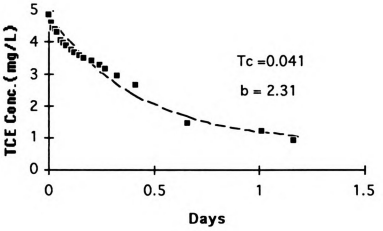

Figure 5.1. Aeration experiment for day 1.

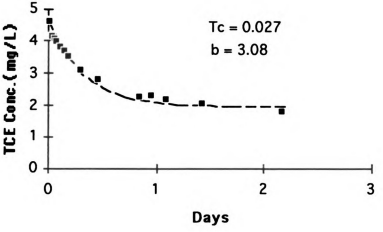

Figure 5.2. Aeration experiment from day 2.

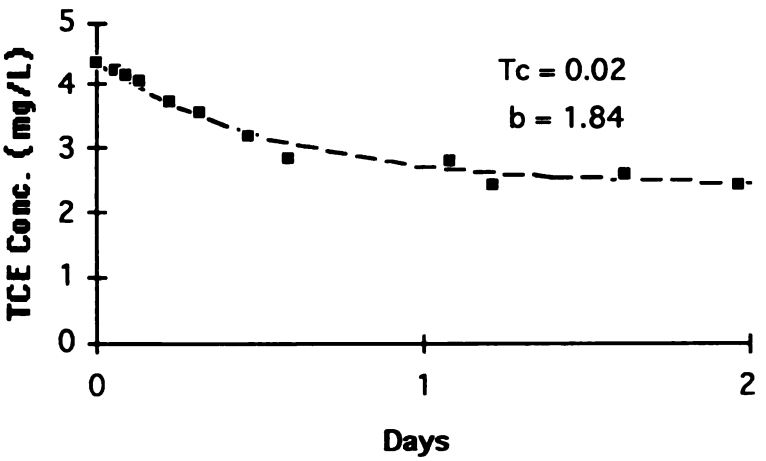

Figure 5.3. Aeration experiment for day 3.

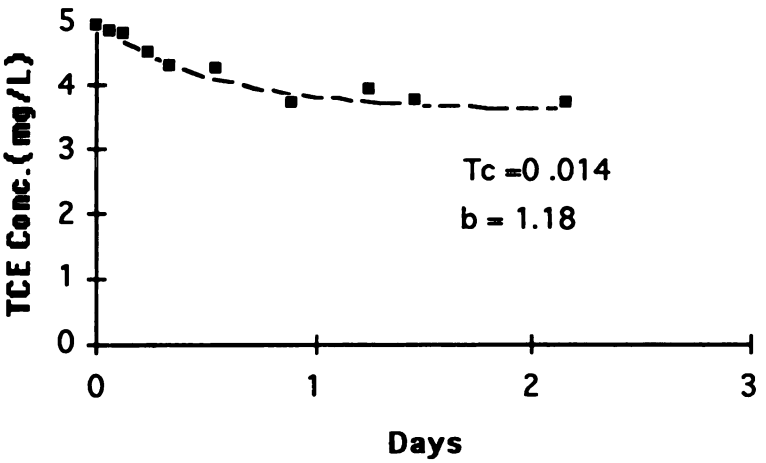

Figure 5.4. Aeration experiment for day 4.

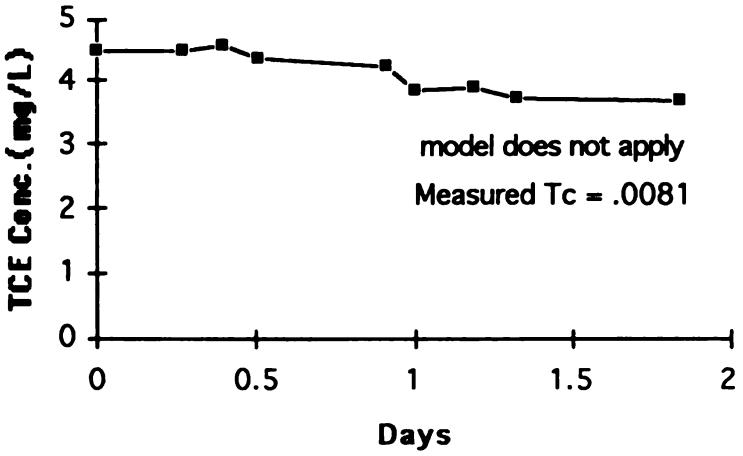

Figure 5.5. Aeration experiment for day 5.

As shown in Figure 5.5, model 3 was unable to predict a transformation capacity for the data because there was very little degradation, so the 'measured' transformation capacity was used for comparison.

When the individual transformation capacities for each day of aeration are plotted against time, there is an obvious trend which was modeled with equation 5.1 listed below.

$$T_C = T_{C(max)}^{0} e^{-bt} \qquad (5.1)$$

Figure 5.6 shows a graph of the data and the modeled equation. Using equation 5.1, the data gives a decay term of 0.381 day^{-1} and a T_C^o of 0.04 mg TCE/mg cells. It is necessary to keep in mind that the transformation capacity values reported for each day are a result of two separate processes: endogenous decay and, due to the high TCE concentration, toxicity. In order to compare this with low concentration data, an assumption was made that endogenous decay (aeration only) does not effect susceptibility to toxicity. In other words, the shape of the

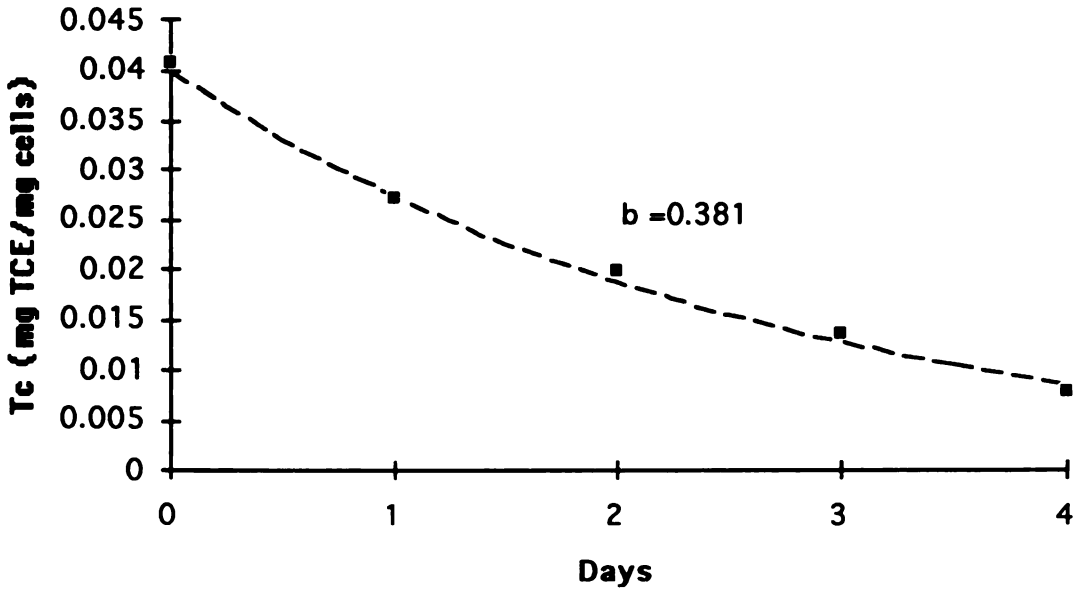

Figure 5.6. Transformation capacity lost over time due to aeration.

curve would remain the same whether at high or low TCE concentration, hence all the parameters estimated from this curve would be the same.

Discussion

If equation 5.1 is applied to the low concentration respike data shown in Figure 3.5, it is possible to calculate a T_c^o that takes into account endogenous decay. By using the above decay value for this culture of 0.381 day^{-1}, and using the individual spiked quantities of TCE at their respective times, equation 5.1 gives a T_c^o of 0.067 mg TCE/mg cells. Comparing this to the measured T_c of 0.035 mg TCE/mg cells (Chapter 3) which does not account for endogenous decay, it is evident that the T_c is drastically reduced by endogenous decay at low TCE concentrations (Figure 5.7).

Based on the above findings, it is likely that T_c is a function of the substrate concentration S, and that it's value increases at low S, if endogenous decay can be accounted

for (Figure 5.8).

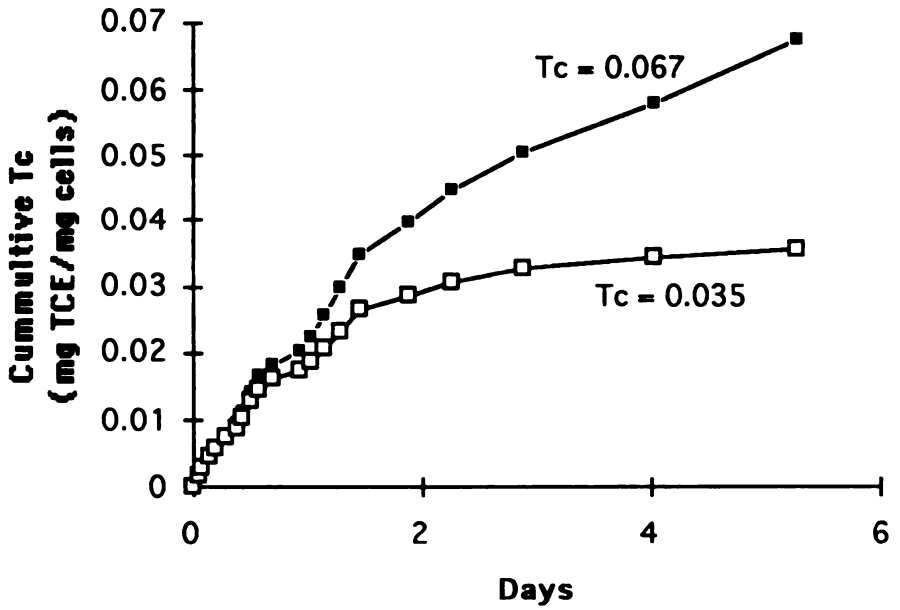

Figure 5.7. Transformation capacity for the five day respike experiment (Chapter 3) including endogenous decay effects ($T_C = 0.035$) compared to transformation capacity corrected for endogenous decay effects of 0.381 day^{-1} ($T_C = 0.067$).

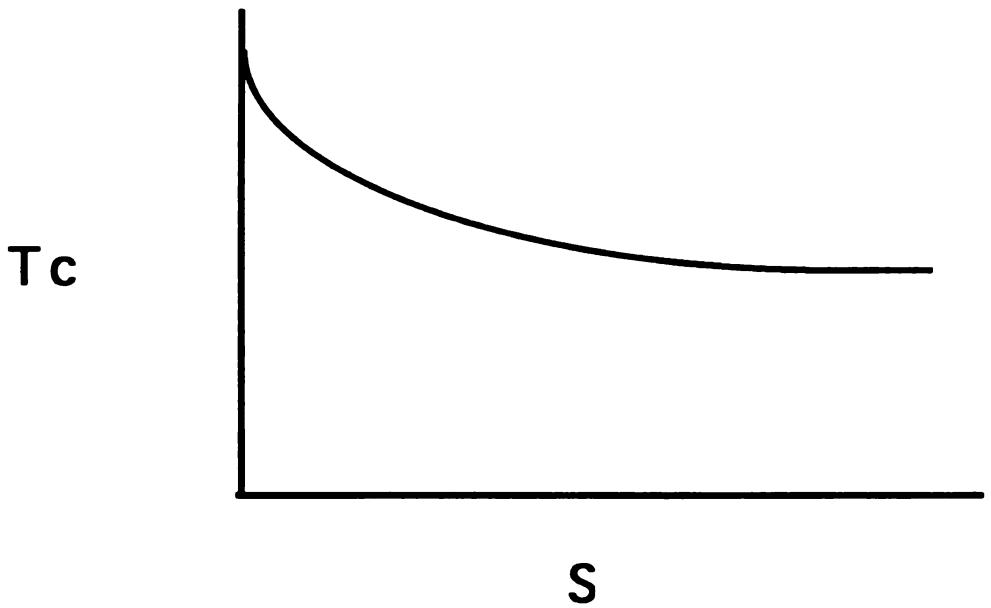

Figure 5.8. Proposed explanation of T_C vs. S based on above findings. (drawing of 'measured' T_C data for various TCE concentrations)

This finding indicates that the assumption behind model 2 that T_C does not change with time may appear to be valid only because endogenous decay plays such a large role at low TCE concentration. At low TCE concentrations, rates of TCE transformation are low, permitting more extensive endogenous decay during the transformation.

As indicated by Figure 5.8, the transformation capacity at low substrate concentration is quite large. This raises the question that if endogenous decay could be overcome, is there some threshold concentration of TCE below which damage to the cells does not occur? The idea of a threshold has long been debated by toxicologists but could be one possible approach to low level TCE modeling.

It is obvious that the loss of transformation activity of the cells with time is a key issue. Specific questions need to be answered as to how this activity is lost. Figure 5.9 gives suggestions as to how this may happen. Fundamental research and models are needed that will differentiate between all these potential activity sinks.

The models currently in use assume that the decay term indicates dead cells. This particular issue is quite important because if the cells are dead, it requires a certain amount of time to regrow them. However, if the cells are not dead, but exhausted and out of reducing power, then the time needed to rejuvenate them would be less than it takes to regrow them.

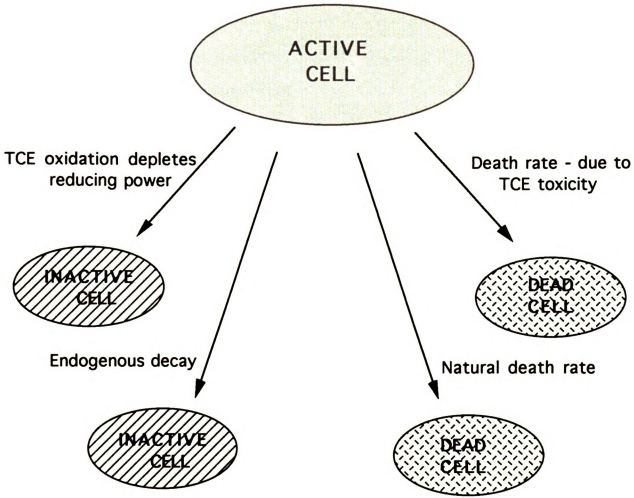

Figure 5.9. Pathways for potential loss of transformation activity.

CHAPTER 6

ENGINEERING APPLICATION

As discussed in Chapter 1, some researchers found formate addition to be beneficial to TCE transformation. In studies conducted, formate addition at low TCE concentration had no effect on degradation rates, but at high TCE concentration, the addition of formate provided readily available reducing power to enhance degradation.

High concentrations of TCE depleted the reducing power of cells as evidenced by the effect of formate addition. To prevent this, it is necessary to reduce the amount of TCE transformed per cell per unit time. This could be accomplished by reducing the amount of TCE that individual cells were exposed to. One way of doing this would be to increase cell density. It has been shown that increasing the cell density increased TCE transformation capacity of a mixed culture (Alvarez-Cohen, 1991b). Reducing liquid TCE concentrations could be accomplished by diluting process wastes with larger volume reactors or by removing TCE using absorptive materials. The addition of activated carbon reduced the concentration in solution and increased transformation capacity on a suspended culture of *M. trichosporium* OB3b (Oldenhuis *et al.*, 1991).

The pH of a system has also been shown to effect the transformation of TCE. A shift from pH 7 to 7.5 in a continuous recycled expanded bed reactor decreased TCE transformation by 85% (Phelps, *et al.*,1990). The pH also effects mineral nutrient availability (Stumm and Morgan, 1981) and the formation of transformation intermediates which may have some influence on TCE oxidation toxicity. The TCE epoxide rearranges in water to form formate, carbon monoxide, dichloroacetic acid and glyoxylic acid (Henschler *et al.*, 1979). The ratio of products formed depends upon the pH of the system, with CO and formate predominating at neutral regions and becoming more dominant at basic regions (Henschler *et al.*, 1979). It is interesting to note that CO has been shown to inhibit TCE degradation (Henry, 1991b). This

could explain the considerably reduced transformation capacity of a culture at high pH as opposed to neutral pH. In addition, some enzymes can be denatured at this high pH (pH = 10).

In order to provide optimal pH for a treatment system, the pH in the transformation reactor should be maintained in the neutral range of pH 6.8 to pH 8.5 for mixed culture MM1. Additional studies are needed to determine whether this conclusion can be generalized to other methanotroph cultures. If a waste tends to vary in pH, as is typical for industrial wastes, a pH control system would appear to be warranted.

Alvarez-Cohen showed that transformation capacity does not change with TCE concentration (model 2a). Chapter 5 indicates that this may appear to be true only because at low TCE concentration, the transformation rates are slow and endogenous decay reduces the transformation capacity of a culture. If it is true that T_C increases at low TCE concentration, then an operating system that exposed cells to lower TCE concentrations, such as a completely mixed reactor, may be most effective if the negative effects of endogenous decay can be reduced. Endogenous decay might be decreased by periodically feeding the cells methane, reducing the mean cells residence time, or alternating the level of oxygen exposure.

If the problem associated with differentiating cells that are dead and cells that are out of reducing power could be solved, this would have a major impact on processes that involve sequencing reactors. The question of how long to recharge cells to restore transformation capacity would is dependent on whether or not the cells are dead or exhausted.

Environmental conditions clearly play a role in the efficiency of methanotrophic degradation of TCE. Reducing power availability , mineral nutrient availability as a function of pH, endogenous decay and TCE concentration all play a role. All these factors must be taken in to account during the design and implementation of treatment systems. By optimizing these factors, the use of methanotrophs for the treatment of TCE and other chlorinated solvents would appear to have a promising future.

CHAPTER 7

CONCLUSIONS

Based on research described in this thesis, the following conclusions can be made for mixed culture MM1:

1. Formate addition increases transformation capacity at higher TCE concentrations (4.5-5 mg/L). At low TCE concentrations (.224-.25 mg/L) , formate addition had no apparent effect on the TCE degradation rate.

2. Basic and acidic incubations reduce transformation capacity of a culture.

3. The transformation capacity of a decaying culture decreases with time at a rate of 0.38 d^{-1}.

4. The transformation capacity of a culture at low TCE concentrations is artificially low because TCE transformation rates are slow compared to endogenous decay.

5. The assumptions underlying models 2 and 3 that transformation capacity does not change with TCE concentration appears to be incorrect. Model 2 might appear valid only because endogenous decay reduces transformation capacity, and the decrease is most significant at low TCE concentrations when TCE degradation rates are slowest.

6. Model 1 does not describe TCE transformation for the full range of TCE concentrations. Transformation capacity appears to increase rather than decrease with

decreasing substrate concentration if the transformation capacity is corrected for endogenous decay.

FUTURE WORK

1. Determine if increasing the reducing power with formate addition to a low TCE concentration respike experiment would increase the transformation capacity.

2. Determine if increasing the biomass concentration at all TCE concentrations increases the transformation capacity.

3. Determine the effect of DO concentration (or the partial pressure of oxygen) on endogenous decay.

4. Determine the effects of pH on cell growth.

5. Determine the relationship between methane utilization activity and TCE transformation activity.

6. Finally, a model is needed or experiments designed to distinguish between the cell death rate and the cell inactivation rate, and what processes are responsible for each.

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APPENDIX A

APPENDIX A

Whittenbury Mineral Medium

The recipe for NMS(nitrate mineral salts medium - whttenbury medium was as follows:

per Liter

1.0 g $MgSO_4 . 7H_2O$
1.0 g KNO_3
0.2 g $CaCl_2 . 2H_2O$
3.8×10^{-3} g FeEDTA
5×10^{-4} g $Na_2MoO_4 . 2H_2O$
1 mL trace element stock (below)

use deionized water and after autoclaving, add 10 mL per liter of autoclaved phosphate stock (below).

Trace element stock for NMS

per liter

500 mg $FeSO_4 . 7H_2O$
400 mg $ZnSO_4 . 7H_2O$
20 mg $MnCl_2 . 4H_2O$
50 mg $CoCl_2 . 6H_2O$
10 mg $NiCl_2 . 6H_2O$
15 mg H_3BO_3 (boric acid)
250 mg EDTA

use 1 mL per liter

Phosphate Stock for NMS

per liter

26 g KH_2PO_4
33 g Na_2HPO_4

use 10 ml per liter (autoclaved)

APPENDIX B

APPENDIX B

Methane Utilization

Materials and Methods

The culture was grown as describe in Chapter 2. During a growth cycle, the culture was harvested at beginning, middle and stationary phases of growth.

A 250-mL bottle containing 50 mL of mineral media and 50 mL of culture was sealed with a Mininert® cap. The mass of the culture added to each bottle varied considerable depending on the stage of growth. Using a 251 ft^3 cylinder of 99.5% pure methane, 5 mL of methane was drawn out and injected into the bottle using a 30-mL syringe equiped with a 22 gage 1.5 inch sterile needle. The bottle was then shaken for 25 seconds, after which a 0.7 mL headspace sample was taken with a 1.0 mL Precision-Lok gas syringe and injected into a HP 5890 II gas chromatograph equiped with a thermal conductivity detector (TCD). This was repeated until 7% of the methane was oxidized.

A calibration curve was generated for the data using 99% pure methane gas from a 14 liter cylinder of Scotty Analyzed Gases. The calibration curve was made up of at least five points which bracketed the data. Anywhere from 10 μL to 100 μL injections were made using a 100 μL Precision-Lok syringe.

In order to determine the amount of methane that was being injected into the GC for the calibration curve, a spreadsheet was devised which converted the volume of methane present into the number of moles injected based on the ideal gas law:

$$PV = n\,RT$$

Where:

P = pressure (101.326 kPa)

V = volume (varied)
R = gas constant (8.314 kJ.kmol.K)
T = temperature (298 K)
n = number of moles

The number of moles of methane was then converted into a mass of methane by dividing by the molecular weight. Using this calibration curve, the headspace data for the sample in the 250 mL bottle was converted into a mass of methane present. To determine the concentration of methane in the bottle, the mass was divided by the injection volume which was 0.7 mL(C_G). The concentration of the methane present in the liquid (C_L) was determined using Henry's constant of 27.2 at 21°C for mass transfer equilibrium of methane (Mackay, 1981):

$$H_c = \frac{C_G}{C_L}$$

Therefore, the total mass of methane present at any time can be calculated as:

$$M(t) = C_G V_G + C_L V_L$$

A linear regression of the total mass of methane (M(t)) versus elapsed time was run to obtain the methane utilization rate, dM/dt. A sample output of the spreadsheet is shown in Table B-1.

Table B.1

METHANE UTILIZATION SPREADSHEET

Methane Utilization Rate for 08-29-91, Culture Monitoring (at 8:00 am)

16 = molecular mass of methane in g/mole

CALIBRATION CURVE

Volume (mL)	mass (mg)	area
0.01	0.006544	7580
0.02	0.013088	32157
0.03	0.019632	47975
0.04	0.026176	91952
0.05	0.03272	112141

SAMPLE ANALYSIS

k = maximum rate of substrate (methane) consumption in days-1
M(t) = time total mass of methane present at time t

100 = volume of the aqueous solution in mL
150 = volume of the headspace in mL
0.7 = volume of the samples tested in mL
27.2 = Henry's Constant for methane
760 = cell concentration in mg/L (from dry weight)

time (min)	area	mass (mg)	headspace conc. (mg/L)	aqueous conc. (mg/L)	M(t)
0	70097	0.0263	37.6094	1.3827	5.7797
5.8	63218	0.0241	34.4254	1.2656	5.2904
10.8	59882	0.023	32.8813	1.2089	5.0531

Methane Utilization = -975.3 mg/L.d

APPENDIX C

APPENDIX C

MIXED CULTURE MM1

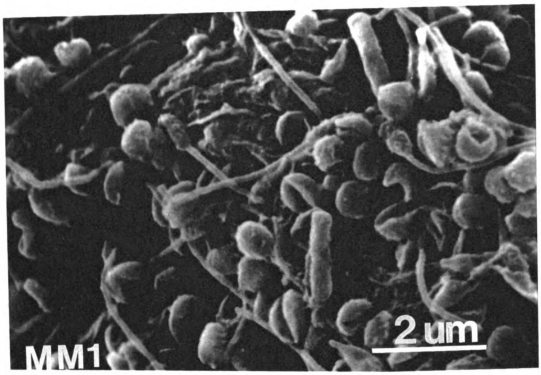

Figure C.1. Scanning electron micrograph (SEM) of mixed culture MM1.
(Courtesy of S. Henry)

APPENDIX D

APPENDIX D

PROPAGATION OF ERROR

Originally taken from J. Topping, Error of Observation and Their Treatment:

If the function is a division :

$$R = \frac{A}{C}$$

Then the absolute error of the calculated result is given by the difference of the individual relative absolute errors, i.e.

$$e_R = \left(\frac{\alpha}{A} - \frac{\gamma}{C}\right)R$$

where:

α = the absolute error associated with A.

γ = the absolute error associated with C.

SENSITIVITY ANALYSIS

The sensitivity equations were derived by taking the equations associated with each model and taking the first derivative of the dependent variable with respect to the parameter of interest (e.g. dS/dk'). This was then multiplied by their respective parameter to yield consistent units. Since the dependent variable is S, those models that did not have a direct solution for S were solved using implicit differentiation. If there was a lack of proportionality or no linear relationship between the curves, then the experimental conditions gave unique parameter estimates over the concentration range tested.

Figure D.1 evaluates models 1b and 2b for the TCE concentration range of 0.015-0.017 mg/L. The lack of proportionality between the three curves indicate that these are unique solutions under the conditions of the experiment. It should be noted that in Figure D.1 (A), that the equation for k' is multiplied by 10. This indicates that for the indicated substrate concentration, model 1b is relatively insensitive to changes in k'. In addition, Figure D.1 (B) indicates that model 2b is extremely insensitive to changes in Tc since it was multiplied by 1000.

Figure D.2 shows a high proportionality between k and K_S for both models 1a and 2a at the TCE concentration range of 0.22-0.24 mg/L, suggesting that unique estimates for k and K_S may not have been obtained. In this case, it would be preferable to lump the two parameters k and K_S into a single parameter k' ($=k/K_S$) as in model 1b and 2b.

Figure D.3, evaluates models 1a and 2a for the TCE concentration of 0.91-0.97 mg/L. The figure indicates that both models yield unique solutions under the conditions applied during the experiment.

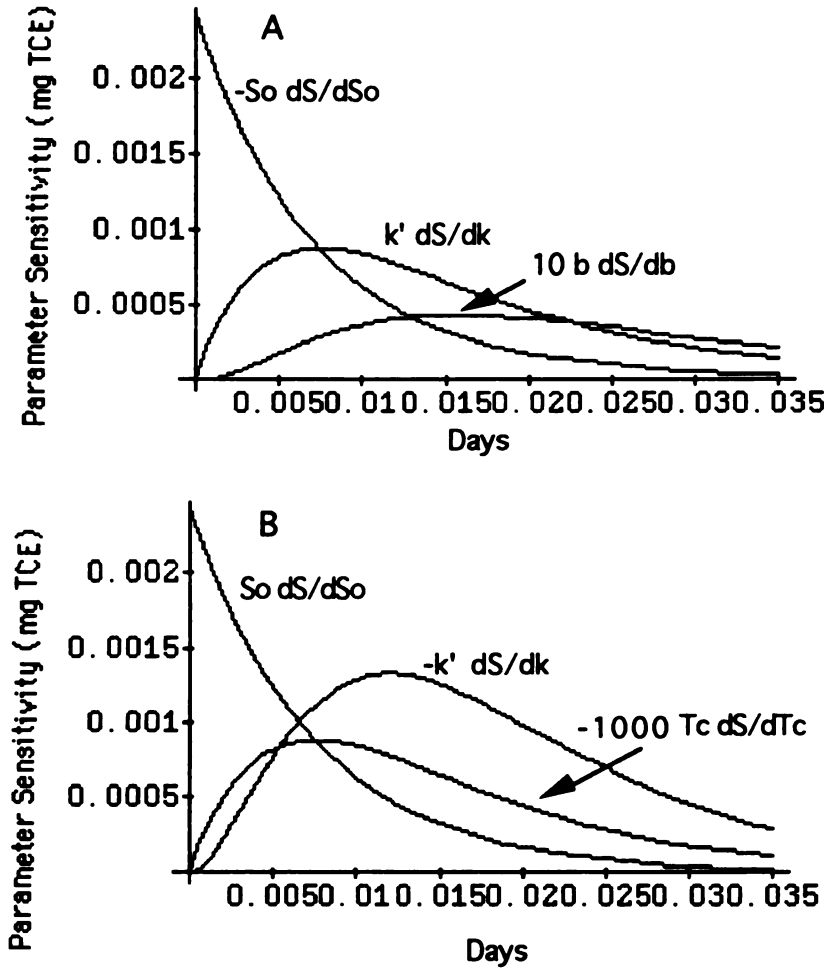

Figure D.1 Sensitivity analysis for model 1b and 2b for the TCE concentration range of 0.015-0.017 mg/L (mass ~0 0024 mg TCE). (A) Model 1b, k'=1.4, b=9.031, So=.0024, Xo=15. (B) Model 2b, k'=1.33, Tc=.0467, So=.0024, Xo=15.

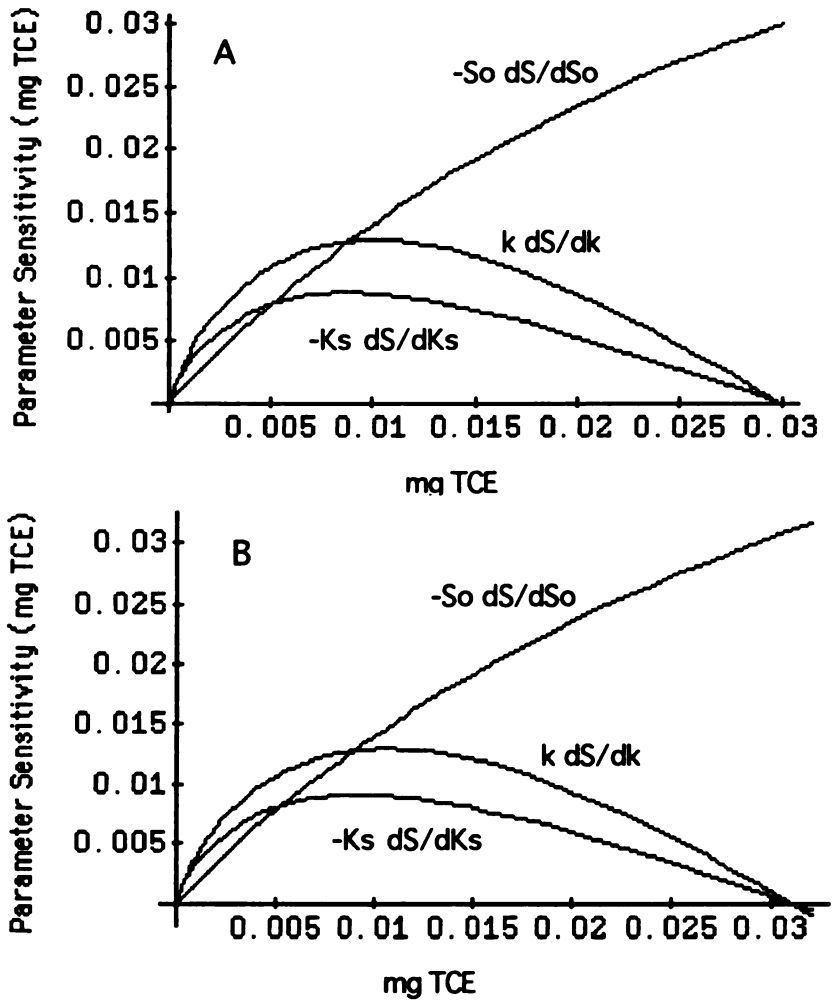

Figure D.2 Sensitivity analysis of models 1a and 2a at a TCE concentration of 0.22-0.24 mg/L (mass~.033 mg TCE. (A) Model 1a,So=.032, Xo=16.2, k=.4215, b=9.031, Ks=.257. (B) Model 2a, So=.032, Xo=16.2, k=.4215, Tc=.0467, Ks=.288.

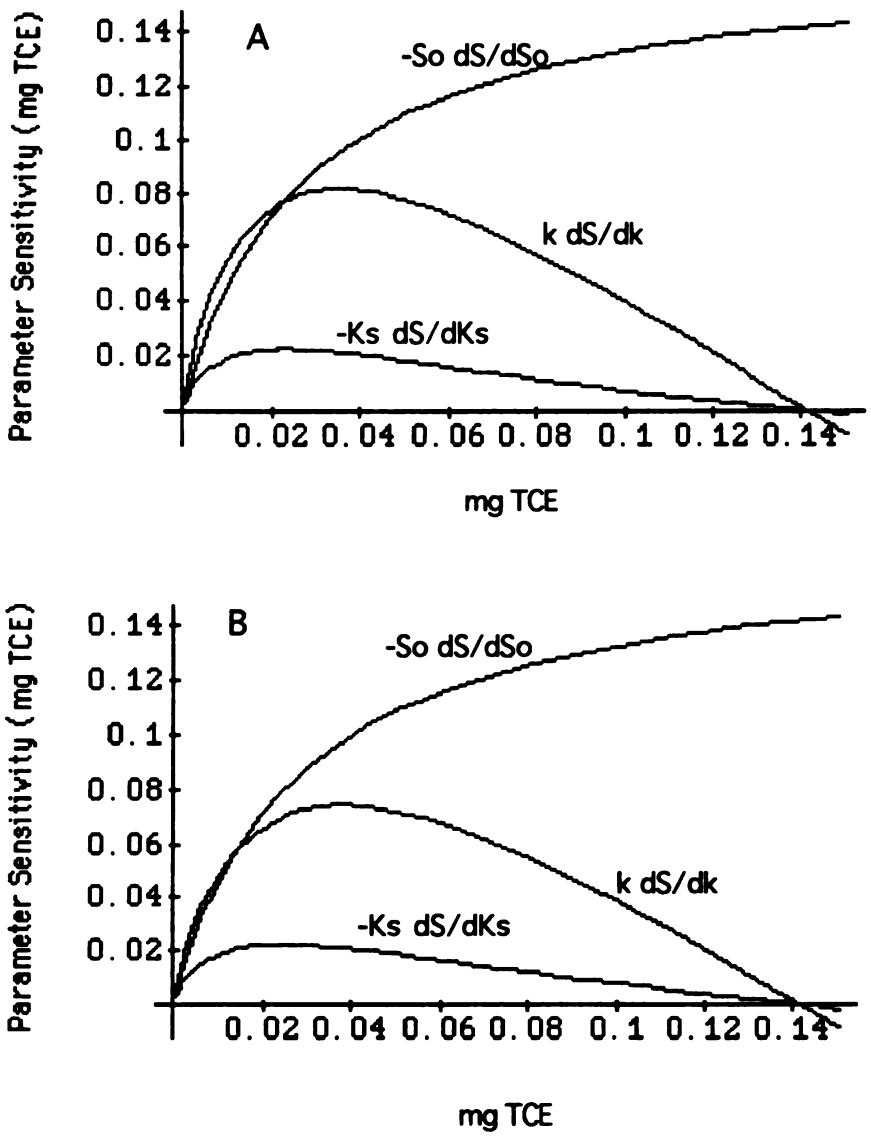

Figure D.3. Sensitivity analysis for model 1a and 2a for the TCE concentration of 0.91-0.97 mg/L (mass = .146 mg TCE). (A) Model 1a, So=.146, Xo=15.6, b=9.031, Ks=.184, k=.4215. (B) Model 2a, So=.146, Xo=15.6, Tc=.0467, Ks=.206, k=.4215.

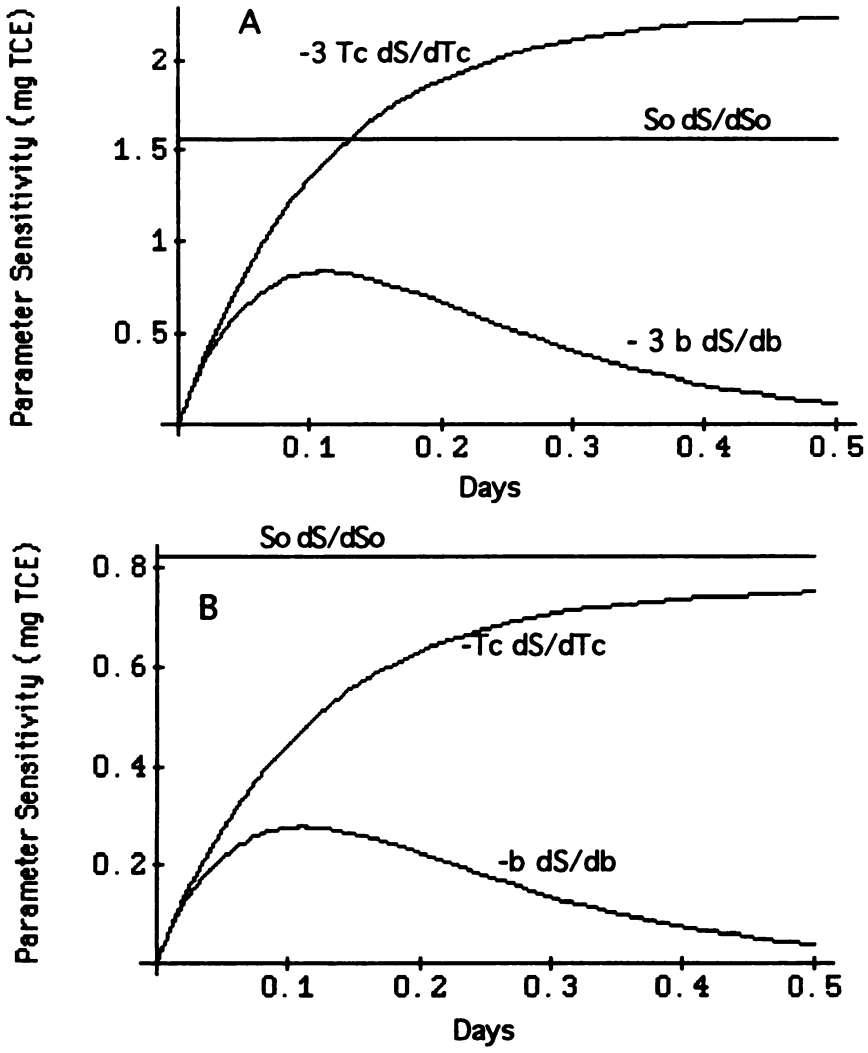

Figure D.4. Sensitivity analysis for model 3 for the higher TCE concentration ranges of 10.2-12.5 mg/L (mass~1.56 mg TCE) and 5.5-5.7 mg/L (mass~.83 mg TCE). (A) Model 3, So=1.56, Tc=.0467, b=9.031, Xo=16.2. (B) Model 3, So=.83,b=8.69, Tc=.0467,Xo=16.2.

From Figure D.4 which evaluates model 3 at the higher initial TCE concentration range of 10.2-12.5 mg/L and 5.5-5.7 mg/L, it is also evident that the lack of proportionality indicates unique solutions under these experimental conditions. However, comparing this to Figure D.5, which evaluates model 3 at the lower TCE concentrations of 0.91 mg/L down to 0.015 mg/L, shows a marked difference. In Figure D.5, there is a direct proportionality between the curves at these lower TCE concentrations. This indicates that unique estimates of both b and T_C may not have been obtained.

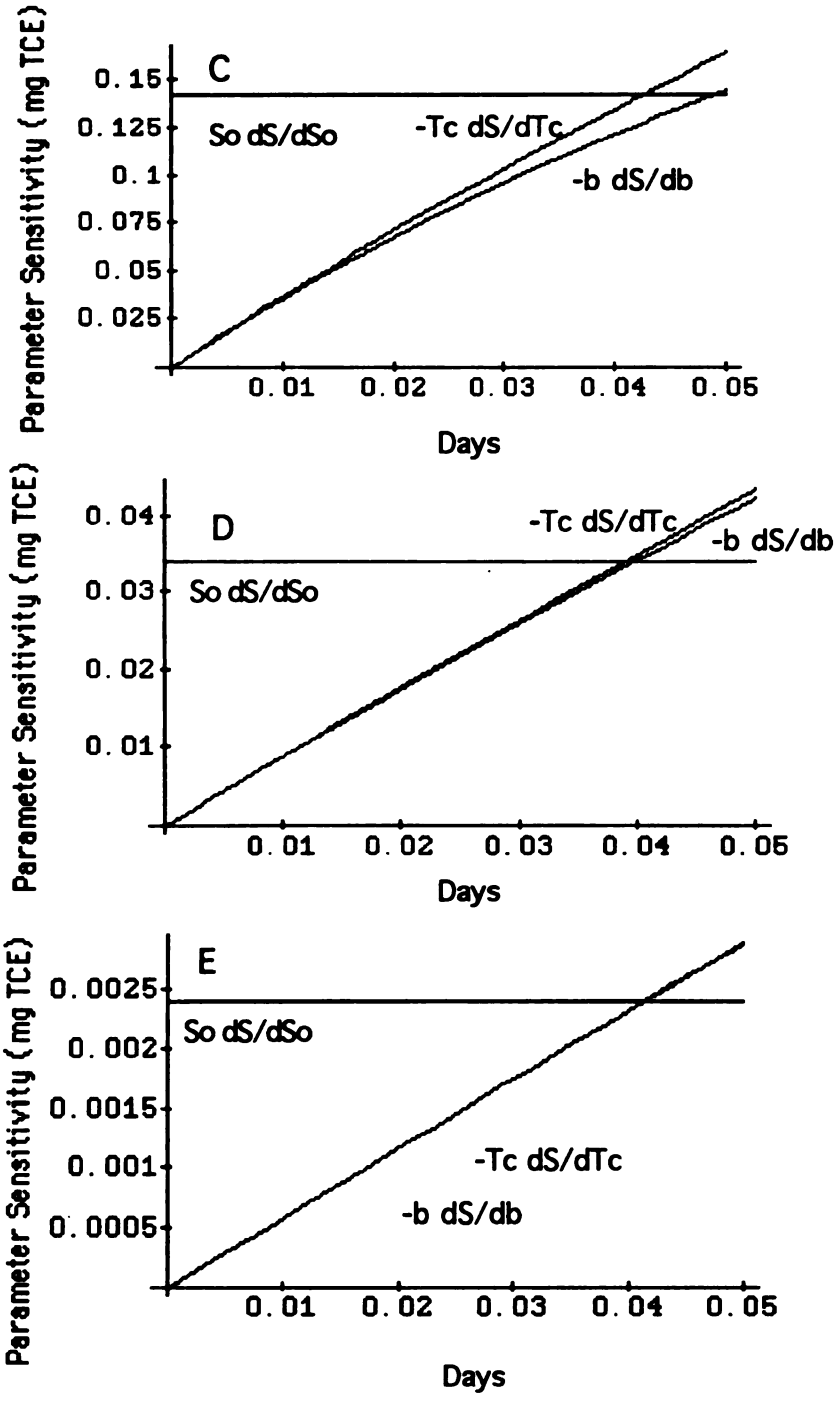

Figure D.5. Sensitivity analysis for model 3 at the lower initial TCE concentrations of 0.91-0.97 mg/L (mass~.142 mg TCE), 0.22-0.24 mg/L (mass ~.034 mg TCE), and .015-.017 mg/L (mass ~ .0024 mg TCE). (C) So=.142, Xo=15.6, Tc=.0467, b=5.114. (D) So=.034, Xo=15.6, Tc=.0467, b=1.187. (E) So=0.0024, Xo=15.6, Tc=.0467, b=.083.