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KINETICS CHARACTERIZATION OF COMETABOLIZING COMMUNITIES AND ADAPTATION TO NONGROWTH SUBSTRATE

By

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ABSTRACT

KINETICS CHARACTERIZATION OF COMETABOLIZING COMMUNITIES AND ADAPTATION TO NONGROWTH SUBSTRATE

By

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Many compounds of environmental and toxicological significance are transformed by cometabolism. For routine engineering application of cometabolism, several issues must be addressed. First, a generally accepted kinetic model is needed to describe transformation of growth and nongrowth substrates. Second, methods are needed to evaluate model parameters. Finally, changes in the cometabolic activity of a community due to long term, repeated exposure to nongrowth substrates need to be investigated.

An unstructured model for cometabolism is presented and verified experimentally in this research. The model includes the effects of cell growth, endogenous cell decay, product toxicity, and competitive inhibition with the assumption that cometabolic transformation rates are enhanced by reducing power obtained from oxidation of growth substrates. A theoretical transformation yield is used to quantify the enhancement resulting from oxidation. A systematic method for evaluating model parameters is described. The applicability of the model is evaluated by comparing experimental data for methanotrophic cometabolism of TCE with model predictions from independently measured model parameters. Propagation of errors is used to quantify errors in parameter estimates and in the final prediction. The model predicts TCE and methane transformation successfully for a wide range of concentrations of TCE (0.5 - 9 mg/L) and methane (0.05 - 6 mg/L). The

model was also sucessfully applied for the simplified case of nongrowth substrate transformation of HCFCs and HFCs by resting cells.

To describe adaptive changes within cometabolizing communities, the verified cometabolism model was analyzed in terms of the "fitness" concept. Fitness was quantified in term of measurable kinetic parameters. The selection gradient for each parameter was defined as the partial derivative of fitness with respect to that parameter. The gradient indicates the selection acting on each fitness component, with other components held constant. It appears possible to use selection gradients as criteria for the stability of a community under a given perturbation.

Finally, a methanotrophic mixed culture and a phenol-degrading culture were repeatedly exposed to different levels of TCE. Changes of community structure were monitored and the effects of TCE exposure were evaluated. Various phenotypic parameters were measured to monitor change within each community. Molecular methods of community analysis (ARDRA and DGGE) were used to monitor shifts in microbial community structure. The results indicate that the phenol-degrading community is very stable despite repeated exposure to TCE. Greater instability in a methanotrophic mixed culture is predicted and observed. A stable community is able to maintain its structure and performance under nongrowth substrate exposure. Based on the results of this study, a diverse community seems to have higher stability.

To the Memory of My Father,
張新永

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NOMENCLATURE

English symbols

b	first-order endogenous decay constant (d^{-1})
C	concentration of nongrowth substrate (mg nongrowth substrate/L)
C_0	initial concentration of nongrowth substrate (mg nongrowth substrate/L)
C_∞	final concentration of nongrowth substrate at t = ∞ (mg nongrowth substrate/L)
G_P	Selection gradient for fitness component, P
H_{cc}	Henry's constant of nongrowth substrate (-)
H_{cs}	Henry's constant of growth substrate (-)
k_c	maximum specific rate of utilization of the nongrowth substrate (mg nongrowth substrate/mg cells-d)
k_s	maximum specific rate of utilization of growth substrate (mg growth substrate/mg cells-d)
k_c'	$= k_c / K_c$, second order rate coefficient of utilization of the nongrowth substrate (L/mg cells-d)
K_{ic}	inhibition coefficient indicating the effect of nongrowth substrate concentration on growth substrate utilization rate (mg nongrowth substrate/L)
K_{is}	inhibition coefficient indicating the effect of growth substrate concentration on nongrowth substrate utilization rate (mg growth substrate/L)
K_c	half-saturation coefficient of nongrowth substrate (mg nongrowth substrate/L)
K_s	half-saturation coefficient of growth substrate (mg growth substrate/L)
M_c	mass of nongrowth substrate (mg)
M_s	mass of growth substrate (mg)
m	Malthusian parameter
q_c	specific rate of utilization of nongrowth substrate (mg nongrowth substrate/mg cells-d)
q_s	specific rate of utilization of growth substrate (mg growth substrate/mg cells-d)
S	concentration of growth substrate (mg growth substrate/L)
t	time (d)
$(T_c)_{obs}$	$= -q_c/\mu$, observed transformation capacity (mg nongrowth substrate/mg cells),
T_c	theoretical transformation capacity in the absence of endogenous decay (mg nongrowth substrate/mg cells)
$(T_y)_{obs}$	$= q_c / q_s$, observed transformation yield (mg nongrowth substrate/mg growth substrate)

T_y theoretical transformation yield (mg nongrowth substrate/mg growth substrate)
V_G volume of gas phase (L)
V_L volume of liquid phase (L)
W fitness
X active organism concentration (mg cells/L)
X_0 initial concentration of active organism (mg cells/L)
Y_m maximum yield or true growth yield (mg cells/mg growth substrate)
Y observed yield (mg cells/mg growth substrate)

Greek symbols

μ specific growth rate of organism (day^{-1})
μ_m maximum specific growth rate of organism (day^{-1})
μ_0 specific growth rate of organism of the ancestor community (day^{-1})

CHAPTER 1

INTRODUCTION

The phenomenon of cometabolism was first reported by Leadbetter and Foster (1959). Since then, many microbial cometabolizing populations have been identified. Many compounds transformed by cometabolism are toxic and, therefore, of environmental concern because of deliberate or inadvertent release into waters and soils. Cometabolism may be a useful tool for removal of such contaminants, particularly those not readily catabolized, from natural environments and engineered systems. Quantitative understanding of cometabolic transformations will enable rational engineering design.

COMETABOLIC TRANSFORMATION

Under aerobic conditions, a number of organic compounds are transformed by cometabolism. These transformation are typically mediated by aerobic organisms possessing nonspecific oxygenase activity capable of oxidizing hydrocarbons as growth substrates and other compounds as nongrowth substrates. Transformation of growth substrate yields carbon or energy for the organisms; transformation of nongrowth substrate wastes the energy and reducing reserves of the cell. In the latter case, the transformation is termed cometabolic. This work focuses on monooxygenase activity. Monooxygenases (MO) cometabolically attack a broad range of compounds, including halogenated aliphatic compounds (RX):

$$RX + O2 + 2e- + 2H+ \xrightarrow{MO} RXO + H2O$$

In a true sense, cometabolism is not metabolism (energy yielding) but fortuitous transformation of a compound by pathways that do not yield energy to the organisms. Enhancing these fortuitous side-reactions is a goal of engineered transformation using microorganisms. However, this goal may conflict with the primary objective of the microbe: the use of electrons for growth and respiration. The "dilemma" that microbes face in cometabolism is shown in Figure 1.1. In normal metabolism, the fraction of electrons used for energy generation (f_e) plus the fraction of electrons used for synthesis (f_s) will equal one. In cometabolism, a fraction of the electrons removed from the electron donor may also be used in cometabolic reactions (f_{co}). Since the energy and the products of the transformation are unavailable for microbial use, f_s+f_e will decrease in the presence of a compound that is cometabolized. Thus, the successful removal of contaminants by cometabolism depends, at least in part, upon the electron donor requirements and the efficiency with which electrons can be directed to cometabolic transformation.

ADAPTATION OF COMETABOLIZING COMMUNITIES

Cometabolic transformation is a complex phenomenon, especially when both growth and nongrowth substrates are simultaneously present. Significant declines in methane conversion rates by methanotrophs following exposure to TCE are observed for both resting and formate-fed cells, suggesting toxic effects caused by TCE or its transformation products (Alvarez-Cohen and McCarty 1991a,b). The presence of toxic transformation products can be expected to have some impact on the development of microbial communities during long-term exposure to nongrowth substrate (Figure 1.2). Changes in populations are likely related to the level of exposure to nongrowth substrate, turnover of transformation products and utilization of growth substrate.

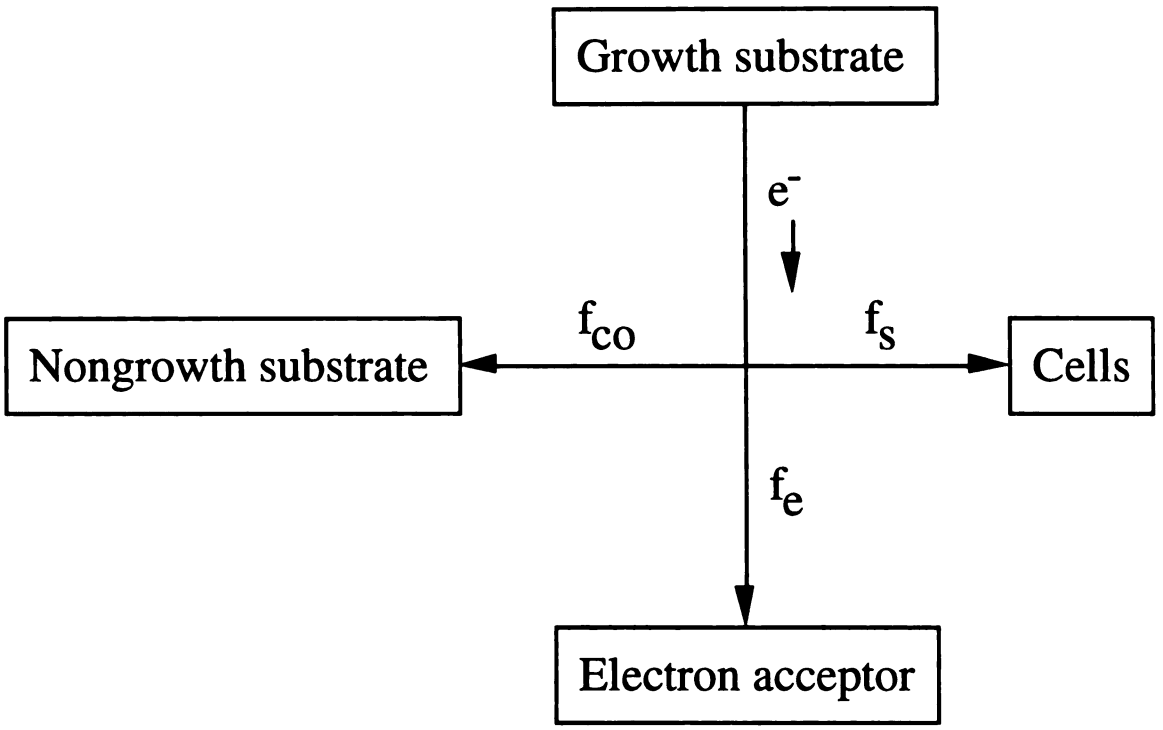

Figure 1.1 Flow of reducing power in cometabolizing system

The accumulation of stable nongrowth substrate breakdown products in pure cultures indicates that pure cultures are not able to mineralize nongrowth substrates. However, research shows that many mixed cultures and communities can achieve mineralization. Since pure cultures with oxygenases typically suffer from product toxicity, other populations capable of utilizing these toxic products may play an important role in detoxification. Under conditions of prolonged or repeated exposure to nongrowth substrate, selection pressures may favor shifts in the microbial community structure so as to enhance detoxification.

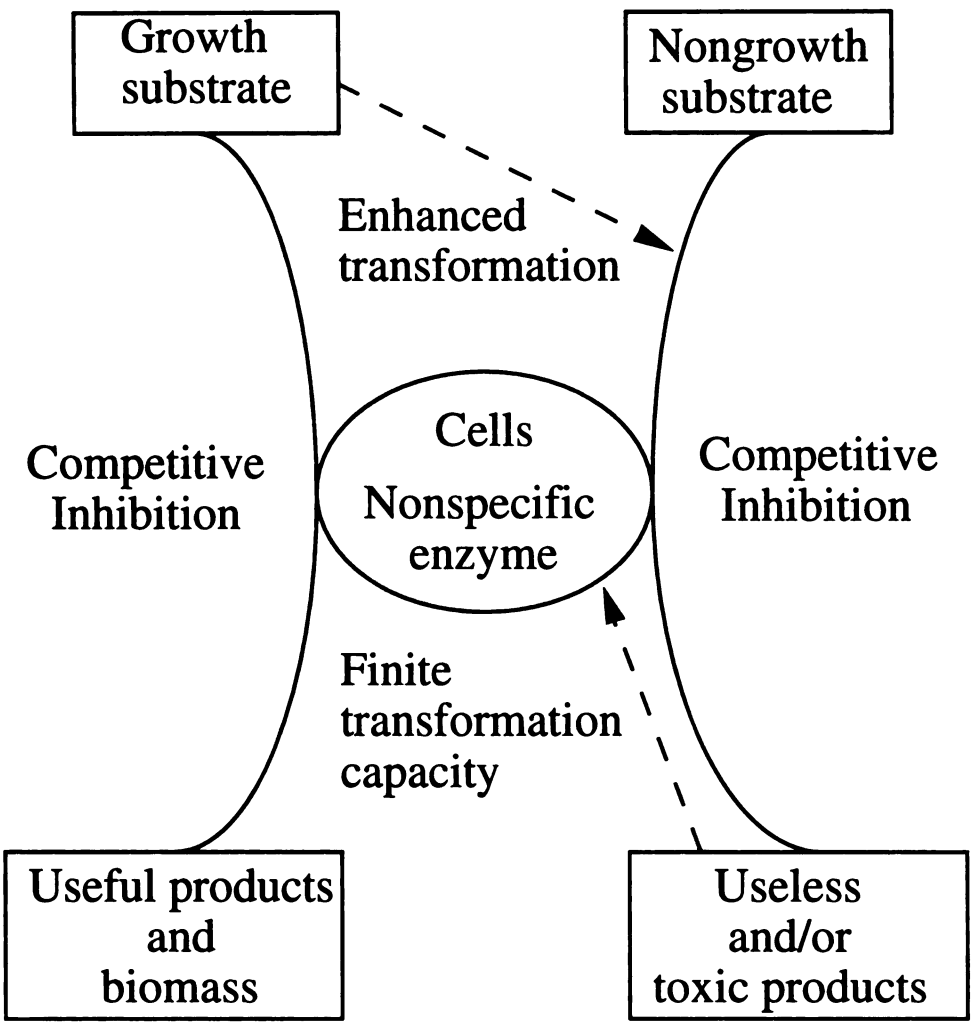

Figure 1.2 General scheme of cometabolic transformation. Dashed lines indicate the effects of substrates or products on transformation.

Different reactor environments may also have different effects on the adaptation of cometabolizing microbial communities. Communities in batch and plug flow reactors are exposed to high concentration of halogenated compound whereas continuous well-mixed systems are exposed to low concentrations continuously. Consequently, selective pressures in batch or plug flow reactors might be expected to favor cometabolizing communities with higher rate of transformation of halogenated compounds.

RESEARCH OBJECTIVES

The objectives of this research are to evaluate and verify a model for cometabolic transformation of nongrowth substrate and transformation of growth substrates and to use this model to characterize adaptive changes during long-term exposure to nongrowth substrate. The hypothesis are:

1. Cometabolism can be quantified using a model based on saturation kinetics and incorporating terms for the loss of microbial biomass caused by endogenous decay, depletion of cofactors and product toxicity.
2. Under conditions of long term periodic exposure to nongrowth substrate, total biomass will be negatively affected by cometabolism, but can recover by means of changes in community structure. These changes can be characterized by phenotypic, morphological and genotypic parameters.
3. Exposure to nongrowth substrate forces selective changes in the community and enhanced nongrowth substrate degradation as secondary populations capable of detoxifying reaction products become more prevalent.
4. Simple cometabolizing communities derived from growth upon a single rate-limiting substrate can be treated as a single population in terms of fitness or growth. Both genotypic and phenotypic adaptation within populations will contribute to phenotypic changes of the whole community. For the characterization of the community, the phenotypic properties evaluated will be apparent values for the whole community.

5. Adaptive changes of community structure can be described in term of a fitness parameter defined as the ratio of specific growth rate during nongrowth substrate exposure to specific growth rate before exposure to nongrowth substrate. The value of fitness at any instant will be the total result of phenotypic adaptation of the community. The sensitivity of change in each phenotypic property to fitness can also be evaluated as a measure of community stability.

OUTLINE OF THE THESIS

This work was conducted in three phases. The first phase, in chapters 3 and 4, entails presentation and verification of a model for cometabolism kinetics. In chapter 3, the model is verified for resting cells using transformation of HCFC/HFCs by a methanotrophic mixed culture as a model system. In chapter 4, the model is extended to growing cells and verified experimentally for the methanotrophic mixed culture with trichloroethylene as the nongrowth substrate. In second phase of this work (chapter 5), the model verified in chapters 3 and 4 is analyzed in terms of " fitness " concept to describe adaptive changes within cometabolizing communities, Fitness is quantified in term of measurable kinetic parameters. The final phase in chapters 6 and 7 focuses on long-term adaptations of cometabolic model systems in response to long-term repeated exposure to nongrowth substrate. A methanotrophic mixed culture in a chemostat and a phenol-degrading community in a sequencing batch reactor were chosen as model systems representing extreme cases for adaptation. The engineering significance of this work is described in chapter 8. Finally, the dissertation concludes with a summary of the most important results and possible future studies.

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CHAPTER 2
BACKGROUND

COMETABOLISM PRINCIPLES

General concept

Many compounds of environmental and toxicological significance are transformed by cometabolism. In this study, cometabolism is defined as transformation of a nongrowth substrate that depends upon the previous or concurrent utilization of a growth or nongrowth substrate (Criddle 1993; Horvath 1972). A growth substrate is defined as an electron donor that supports growth. An energy substrate is defined here as an electron donor that provides reducing power and energy for the transforming population, but does not by itself support growth.

The term "co-oxidation" was first used to described cometabolism because the original observations all involved oxidations. Subsequently, reductive transformations were discovered that did not facilitate growth of the transforming organisms but still depended upon the concurrent or previous utilization of a growth or energy substrate. These "co-reduction" reactions led to the use of the broader term cometabolism. It now appears that certain cometabolic transformations are also hydrolytic. Thus, in addition to the well known examples of co-oxidation, we now recognize the potential for "co-reductions" and "co-hydrolyses". All of the known co-oxidations occur only under obligate aerobic conditions, while most co-reductions occur under anaerobic conditions

Cometabolism is important for many transformations, including some polynuclear aromatic hydrocarbons, halogenated aliphatic and aromatic hydrocarbons, and pesticides. Many of these compounds express toxic properties and are environmental concern. This phenomenon has been observed so frequently that it appears to represent a very important type of microbial metabolism (Table 2.1). Many microbial species exhibit the phenomenon of cometabolism. Table 2.2 lists those microorganisms which have been clearly shown to possess transformation by cometabolism.

Table 2.1 Organic compounds subject to cometabolism and accumulated products

Compound	Product
Ethane	Acetic acid
Propane	Propionic acid, acetone
Butane	Butanoic acid, methyl ethyl ketone
m-Chlorobenzoate	4-Chlorocatechol, 3-chlorocatechol
o-Fluorobenzoate	3-Fluorocatechol, fluoroacetate
2-Fluoro-4-nitrobenzoate	2-Fluoroprotocatechuic acid
4-Chlorocatechol	2-Hydroxy-4-chloro-muconic semialdehyde
3, 5-Dichlorocatechol	2-Hydroxy-3,5-dichloro-muconic semialdehyde
3-Methylcatechol	2-Hydroxy-3-methyl-muconic semialdehyde
o-Xylene	*o*-Toluic acid
p-Xylene	*p*-Toluic acid, 2, 3-dihydroxytoluic acid
Pyrrolidone	Glutamic acid
Cicerone	Cinerolone
naphthalene	salicyclic acid
n-Butylbenzene	Phenylacetic acid
Ethylbenzene	Phenylacetic acid
n-Propylbenzene	Cynnamic acid
p-Isopropyltoluene	*p*-Isopropylbenzoate
n-Butyl-cyclohexane	Cyclohexaneacetic acid
2, 3, 6-Trichlorobenzoate	3, 5-Dichlorocatechol
2, 4, 5-Trichlorophenoxy acetic acid	3, 5-Dichlorocatechol
p-*p*'-Dichlorodiphenyl methane	*p*-Chlorophenylacetate
1, 1-Diphenyl-2, 2, 2-trichloroethane	2-Phenyl-3, 3, 3-trichloropropionic acid
1, 1, 1-Trichloroethene	1, 2-dichloroethene
Trichloroethylene	Trichloroacetate, 2,2,2-trichloro-ethanol, dichloroacetate

Source: (1) Horvath 1972; (2) Dalton and Stirling 1982

Table 2.2 Microorganisms exhibiting the phenomenon of cometabolism

Microorganism
Acetobacterium woodii
Achromobacter sp.
Acinetobacter sp.
Arthrobacter sp.
Aspergillus niger
Azotobacter chroococcum
Azotobacter vinelandii
Bacillus megaterium
Bacillus sp.
Brevibacterium sp.
Clostridium sp.
Flavobacterium sp.
Hydrogenomonas sp.
Methylomonas sp.
Microbacterium sp.
Micrococcus cerificans
Micrococcus sp.
Nitrosomonas europaea
Nocardia erythropolis
Nocardia sp.
Pseudomonas sp.
P. fluorescens
P. methanica
P. putida
Rhodococcus sp.
Streptomyces aureofaciens
Trichoderma viride
Vibrio sp.
Xanthomonas sp.

Source: (1) Horvath 1972;
(2) Dalton and Stirling 1982 ; (3) Criddle 1993

Energy requirement

Many aerobic cometabolic reactions are catalyzed by non-specific oxygenase enzymes that use O_2 as the electron acceptor and NADH as the reducing energy source to oxidize both growth and nongrowth substrates (Colby et al. 1977; Fox et al. 1990; Nelson et al. 1987; Wackett et al. 1989). These enzymes are the methane monooxygenases (MMO) of methanotrophs (Fox et al. 1990; Oldenhuis et al. 1989; Tsien et al. 1989), ammonia monooxygenases of nitrifiers (Arciero et al. 1989; Hyman et al. 1988; Rasche et al. 1990;

Vannelli et al. 1990), propane monooxygenases (Arciero et al. 1989; Hyman et al. 1988; Vannelli et al. 1990; Wackett et al. 1989), certain toluene mono- and dioxygenases, and certain phenol monooxygenases (Winter et al. 1989; Zylstra et al. 1989). After the initial oxidation step, growth substrates are further degraded to regenerate reducing energy (NADH), which promotes more substrate oxidation. However, the oxidation of nongrowth substrate in the absence of growth substrate can cause the depletion of NADH in cells since NADH is not regenerated. Thus, energy or reducing power must be present to transform the nongrowth substrate. Transformation can not be sustained if growth substrate is not supplied continuously or intermittently.

Substrate interaction

In the metabolism of multiple substrates, competitive inhibition is frequently reported. A number of substrate interactions have been observed during hydrocarbon degradation by cometabolism involving monooxygenases and dioxygenases. Saéz and Rittmann (1991, 1993) reported that batch experiments on the simultaneous utilization of phenol and 4-chlorophenol by *Pseudomonas putida* PpG4 demonstrated 4-chlorophenol inhibited the oxidation of growth substrate and the cometabolic degradation of 4-chlorophenol was proportional to the rate of phenol oxidation. Competitive inhibition between phenol and trichloroethylene (TCE) was also observed for the degradation by *Pseudomonas cepacia* G4(Folsom et al. 1990). Studies on TCE degradation by methanotrophs (Alvarez-Cohen and McCarty 1991a; Anderson and McCarty 1994; Broholm et al. 1992; Chang and Alvarez-Cohen 1995a; Saéz and Rittmann 1993) indicated that competitive inhibition was generally present between methane (growth substrate) and TCE (nongrowth substrate). Chang et al. (1993) also revealed competitive inhibition and cometabolic patterns using paired substrates (benzene, toluene, and *p*-xylene). Some research also concluded that the degradation of nongrowth substrates is enhanced in the presence of growth or energy

substrate (Chang and Alvarez-Cohen 1995a; Chang et al. 1993; Criddle 1993; Saéz and Rittmann 1993).

Product toxicity

Several examples indicate that cometabolism by pure cultures does not typically result in the mineralization of nongrowth substrates (Table 2.1). Horvath (1971) reported that cometabolism of 2, 3, 6-trichlorobenzoate resulted in accumulation of 3, 5-dichlorocatechol and development of a toxic environment for the cells. Several researchers have shown that stable and toxic TCE breakdown products accumulate in pure cultures of methanotrophs. This suggests that methanotrophic bacteria in isolation are not be able to effectively mineralize TCE (Henry and Grbić -Galić 1990; Little et al. 1988; Oldenhuis et al. 1989). Significant declines in methane conversion rates following exposure to TCE were observed for both resting and formate-fed cells, suggesting toxic effects caused by TCE or its transformation products (Alvarez-Cohen and McCarty 1991a; Alvarez-Cohen and McCarty 1991b).

Cometabolic community

The products of cometabolic transformation accumulate in pure cultures, but, in a mixed culture, they are typically used by other microorganisms. As a result, cometabolic transformations are key initiatory reactions in pathways that ultimately result in the complete degradation of many environmental pollutants. Some research shows that methanotrophic mixed cultures have advantages for complete degradation of TCE. Since methanotrophs suffer from product toxicity in TCE transformation, heterotrophs in mixed culture may play an important role in detoxification. Heterotrophic bacteria in the methanotrophic mixed cultures apparently can degrade most of the water-soluble breakdown products from ^{14}C-TCE, decreasing levels of water-soluble radiolabel and

increasing production of $^{14}CO_2$ (Little et al. 1988). Futhermore, Uchiyama and co-workers reported that a heterotrophic bacterium in a methanotrophic mixed culture, *Xanthobacter autotrophicus*, can can oxidize dichloroacetic and glyoxylic acid completely and can reduce trichloroacetic acid levels (Uchiyama et al. 1992). These results indicate that heterotrophic bacteria in microbial communities play an important role in complete degradation of nongrowth substrates. Another good example is the initial cometabolic transformation of PNAs, which is typically followed by a series of degradation steps leading to CO_2. The initial step is not mediated by the same organisms as the mineralization steps.

QUANTIFICATION OF COMETABOLISM

Cometabolism by resting cells

Nongrowth substrates can be transformed by resting cells in the absence of growth substrates. Under these conditions, cells utilize nongrowth substrate in the absence of growth substrates. Transformations of growth substrate by cells can generally be described using a saturation kinetic expression:

$$q_s = k_s\left(\frac{S}{K_s + S}\right) \qquad (1)$$

Transformations of nongrowth substrate by resting cells can also be described using saturation kinetics:

$$q_c = k_c\left(\frac{C}{K_c + C}\right) \qquad (2)$$

If the substrate concentration is low ($S << K_s$, $C << K_c$), the specific transformation rate is directly proportional to the substrate concentration:

$$q_s = k_s' S \tag{3}$$

$$q_c = k_c' C \tag{4}$$

At high substrate concentration ($S >> K_s$, $C >> K_c$), the specific rate of substrate transformation is independent of substrate concentration($q_s = k_s$, $q_c = k_c$).

In some cases, a growth substrate inhibits its own transformation at high concentration. To describe this situation, Haldane kinetics is often used:

$$q_s = \frac{k_s S}{K_s + S + \frac{S^2}{K_i}} \tag{5}$$

As discussed previously, loss of cometabolic transformation activity can occur as a result of endogenous decay and product toxicity. To evaluate the loss of cell activity during the transformation of nongrowth substrate, first order decay of biomass is usually used:

$$\frac{dX}{dt} = -bX \tag{6}$$

To account for the loss of transformation activity in resting cells caused by a depletion of reducing power (in the absence of growth or energy substrate) and by product toxicity, the concept of "transformation capacity" is applied. Transformation capacity was first defined as (Alvarez-Cohen and McCarty 1991a):

$$(T_c)_{obs} = \frac{dC}{dX} \tag{7}$$

Alvarez-Cohen and McCarty assumed that biomass transformation capacity was equal to the mass of nongrowth substrate ultimately degraded divided by the initial biomass used. Criddle (1993) defined a "theoretical" biomass transformation capacity by correcting for losses caused by endogenous decay. The later definition represents a theoretical maximum value in the absence of external reducing power.

Most models proposed to describe transformations of nongrowth substrate under resting cell condition are a combination of Eq. (2), (6), and (7). Criddle (1993) proposed a model that unified the earlier models. This model assumed that nongrowth substrate is degraded with saturation kinetics and endogenous decay and that loss of cometabolic activity can be attributed to product toxicity are incorporated into cell decay term. A summary of these models is shown in Table 2.3.

Cometabolism by growing cells

Earlier cometabolic models presented in the literature focus on resting cells, yet frequently, both the rates and extent of cometabolism are enhanced in the presence growth substrate and energy substrates. Competitive inhibition between growth and nongrowth substrates (Folsom et al. 1990; Strand et al. 1990) or between multiple nongrowth substrates (Alvarez-Cohen and McCarty 1991c) is also observed. When there is competitive inhibition between the growth substrate and the nongrowth substrate, $(K_s)_{obs}$ and $(K_c)_{obs}$ replace K_s and K_c respectively in Eq. (1) and Eq. (2), where:

$$(K_s)_{obs} = K_s(1+\frac{C}{K_{ic}}) \tag{8}$$

$$(K_c)_{obs} = K_c(1+\frac{S}{K_{is}}) \tag{9}$$

Transformations of growth and nongrowth substrate by cells under the conditions of competitive inhibition can generally be described as follow:

Table 2.3 Summary of cometabolic transformation model by resting cells

Model	Differential equations for substrate utilization rate	Integrated form	Ref.
1a	$-\frac{dC}{dt}=\frac{k_c CX}{K_c+C}$ and $\frac{dX}{dt}=-bX$ so $-\frac{dC}{dt}=\frac{k_c CX_0}{K_c+C}e^{-bt}$	$K_c \ln(\frac{C}{C_0})+C-C_0=\frac{-k_c X_0}{b}(1-e^{-bt})$	1,2
1b $C<<K_c$	$-\frac{dC}{dt}=k_c^{'}CX$ and $\frac{dX}{dt}=-bX$ so $-\frac{dC}{dt}=k_c^{'}CX_0e^{-bt}$	$C=C_0e^{(\frac{k_c^{'}X_0}{b}(e^{-bt}-1))}$	2,3
1c $C>>K_c$	$-\frac{dC}{dt}=k_cX$ and $\frac{dX}{dt}=-bX$ so $-\frac{dC}{dt}=k_cCX_0e^{-bt}$	Same as model 3 where $(T_c)_{obs}=\frac{k_c}{b}$	4
2a	$-\frac{dC}{dt}=\frac{k_cCX}{K_c+C}$ and $\frac{dC}{dX}=(T_c)_{obs}$ so $-\frac{dC}{dt}=\frac{k_cC(X_0-\frac{1}{(T_c)_{obs}}(C_0-C))}{K_c+C}$	$t=\frac{1}{k_c}((\frac{K_c}{C_0/(T_c)_{obs}-X_0})\ln\{\frac{CX_0}{FC_0}\}$ $+(T_c)_{obs}\ln\{\frac{X_0}{F}\})$ where $F=X_0-\frac{1}{(T_c)_{obs}}(C_0-C)$	5
2b $C<<K_c$	$-\frac{dC}{dt}=k_c^{'}CX$ and $\frac{dC}{dX}=(T_c)_{obs}$ so $-\frac{dC}{dt}=k_c^{'}C(X_0-\frac{1}{(T_c)_{obs}}(C_0-C))$	$C=C_0\frac{F^{'}e^{-k_c^{'}F^{'}t}}{X_0-\frac{C_0}{(T_c)_{obs}}e^{-k_c^{'}F^{'}t}}$ where $F^{'}=X_0-\frac{C_0}{(T_c)_{obs}}$	4
2c $C>>K_c$	$-\frac{dC}{dt}=k_cX$ and $\frac{dC}{dX}=(T_c)_{obs}$ so $-\frac{dC}{dt}=k_c(X_0-\frac{1}{(T_c)_{obs}}(C_0-C))$	Same as model 3 where $(T_c)_{obs}=\frac{k_c}{b}$	4
3	$\frac{dX}{dt}=-bX$ and $\frac{dC}{dX}=(T_c)_{obs}$ so $-\frac{dC}{dt}=bT_cX_0e^{-bt}$	$C=C_0-(T_c)_{obs}X_0(1-e^{-bt})$	6
4	$-\frac{dC}{dt}=\frac{k_cCX}{K_c+C}$ and $\frac{dX}{dt}=-bX-\frac{1}{T_c}(\frac{k_cCX}{K_c+C})$	No integrated form	4

Reference: (1) Galli and McCarty 1989; (2) Schmidt et al. 1985; (3) Criddle et al. 1990; (4) Criddle 1993; (5) Alvarez-Cohen and McCarty 1991a; (6) Saéz and Rittmann 1991.

$$q_s = k_s\left(\frac{S}{K_s\left(1+\frac{C}{K_{ic}}\right)+S}\right) \tag{10}$$

$$q_c = k_c\left(\frac{C}{K_c\left(1+\frac{S}{K_{is}}\right)+C}\right) \tag{11}$$

To describe the observed enhancement in the rate of cometabolism in the presence of growth substrate, modification of Eq (10) or Eq (11) were proposed. Chang and Alvarez-Cohen (1995a) proposed a general model that includes reducing energy explicitly as a limiting reactant during cometabolism. They applied the model to describe degradation of TCE by methanotrophs. The effects of reducing power were separated from toxicity effects and quantified by supplying the cells with formate. For methanotrophs, formate provides energy as NADH, but does not support growth. Thus, the experimental approach of Chang and Alvarez-Cohen is somewhat specific to organisms for which energy substrates can be identified that do not support growth.

By contrast, Criddle (1993) introduced a term, the growth substrate transformation capacity, or theoretical transformation yield, to quantify the enhancement of cometabolism resulting from oxidation of the growth substrate:

$$q_c = (T_y q_s + k_c)\left(\frac{C}{K_c\left(1+\frac{S}{K_{is}}\right)+C}\right) \tag{12}$$

Use of Eq. (12) does not require explicit quantification of reducing power and may be applied to growth and energy substrates. Eq. (12) is related to the Luedeking-Piret (LP) model that is commonly used in fitting product formation data from many different fermentation (Bailey and Ollis 1986). In the LP model, $q_p = \alpha\mu + \beta$, where q_p = specific

rate of product formation, α = the growth-associated product yield, and β = nongrowth-associated product formation rate. The LP model can be modified to apply to cometabolism. Consequently, $q_c = (\alpha\mu + \beta) / Y_{p/s}$, where $Y_{p/s}$ =the mass of product formed by cometabolism of a unit mass of nongrowth substrate. The same result is derived with Eq. (12) when $C >> K_c$. Thus, the LP model is obtained as a limiting case of Eq. (12).

The most widely used model of cell growth and decay is the Monod expression as modified by Herbert et al (1956):

$$\mu = Y_m q_s - b = \frac{Y_m k_s S}{K_s + S} - b \qquad (13)$$

For cells that are growing, decaying, and simultaneously carrying out cometabolic transformation, Eq. (13) is an inadequate description of growth. A modification proposed by Criddle (1993) and used by Anderson and McCarty (1994) and by Chang and Alvarez-Cohen (1995a) is:

$$\mu = Y_m q_s - b - \frac{q_c}{T_c} \qquad (14)$$

Eq(14) indicates that an increase in the rate of cometabolism causes a decrease in the specific growth rate of a cometabolizing population. While this appears to be true for pure cultures, it may not be true for mixed cultures where organisms capable of using the products of cometabolism for growth are frequently present. Eq(10), (12), and (14) provide the framework for a complete kinetic description of cometabolism by growing or resting cells and were used throughout this research. A summary of these models is shown in Table 2.4.

Table 2.4. Summary of cometabolic transformation model by growing cells

Model	Differential equations for substrate utilization rates and growth rate		Ref.
1	$q_s = -\frac{1}{X}\frac{dS}{dt} = \frac{k_s S}{K_s + S}$	$q_c = -\frac{1}{X}\frac{dC}{dt} = \frac{k_c(\frac{C}{K_c})}{(1+\frac{S}{K_s}+\frac{C}{K_c})}$	1
2	$q_s = -\frac{1}{X}\frac{dS}{dt} = \frac{k_s S}{K_s(1+\frac{C}{K_c})+S}$ $\mu = \frac{1}{X}\frac{dX}{dt} = Yq_s - b$	$q_c = -\frac{1}{X}\frac{dC}{dt} = \frac{k_c C}{K_c(1+\frac{S}{K_s})+C}$	2
3	$q_s = -\frac{1}{X}\frac{dS}{dt} = \frac{\frac{k_s S}{(1+\frac{z_1^{n1}}{K_1})}}{K_s(1+\frac{z_2^{n2}}{K_2})+S+\frac{S^2}{K_h}}$ $\mu = \frac{1}{X}\frac{dX}{dt} = Yq_s - b$	$q_c = -\frac{1}{X}\frac{dC}{dt} = \alpha q_s + \beta b$	3
4	$q_s = -\frac{1}{X}\frac{dS}{dt} = \frac{k_s S}{K_s(1+\frac{C}{K_c})+S}$ $X = X_0 - \frac{1}{(T_c)_{obs}}(C_0 - C)$	$q_c = -\frac{1}{X}\frac{dC}{dt} = \frac{k_c C}{K_c(1+\frac{S}{K_s})+C}$	4
5	$q_s = -\frac{1}{X}\frac{dS}{dt} = \frac{k_s S}{K_s(1+\frac{C}{K_c})+S}$ $\mu = \frac{1}{X}\frac{dX}{dt} = Yq_s - b - \frac{q_c}{(T_c)_{obs}}$	$q_c = -\frac{1}{X}\frac{dC}{dt} = \frac{k_c C}{K_c(1+\frac{S}{K_s})+C}$	5
6	$q_s = -\frac{1}{X}\frac{dS}{dt} = (\frac{R}{K_R+R})(\frac{K_s S}{K_s(1+\frac{C}{K_c})+S})$ $\mu = \frac{1}{X}\frac{dX}{dt} = Yq_s - b - \frac{q_c}{T_c}$	$q_c = -\frac{1}{X}\frac{dC}{dt} = (\frac{R}{K_R+R})(\frac{k_c C}{K_c(1+\frac{S}{K_s})+C})$	6
7	$q_s = -\frac{1}{X}\frac{dS}{dt} = \frac{k_s S}{K_s(1+\frac{C}{K_{ic}})+S}$ $\mu = \frac{1}{X}\frac{dX}{dt} = Y_m q_s - b - \frac{q_c}{T_c}$	$q_c = -\frac{1}{X}\frac{dC}{dt} = (T_y q_s + k_c)(\frac{k_c C}{K_c(1+\frac{S}{K_{is}})+C})$	7

Reference: (1) Strand et al 1990; (2) Broholm et al 1992; (3) Saéz and Rittmann 1993; (4) Alvarez-Cohen and McCarty 1991a; (5)Anderson and McCarty 1994; (6) Chang and Alvarez-Cohen 1995a; (7) Criddle 1993.

MODEL MICROBIAL SYSTEMS FOR COMETABOLISM

Several aerobic bacteria with non-specific oxygenase activity are capable of oxidizing halogenated hydrocarbons. Microorganisms possessing this ability include toluene-oxidizing bacteria (Nelson et al. 1987; Wackett and Gibson 1988), methane-oxidizing bacteria (Little et al. 1988; Oldenhuis et al. 1989; Tsien et al. 1989), ammonia-oxidizing bacteria (Arciero et al. 1989; Hyman et al. 1988; Rasche et al. 1990; Vannelli et al. 1990), and propane-oxidizing bacteria (Wackett et al. 1989). The enzymes which have been implicated in catalyzing halocarbon oxidations are toluene mono- and dioxygenase (Winter et al. 1989; Zylstra et al. 1989), methane monooxygenase (Fox et al. 1990; Oldenhuis et al. 1989; Tsien et al. 1989), ammonia monooxygenase (Arciero et al. 1989; Hyman et al. 1988; Rasche et al. 1990; Vannelli et al. 1990), and propane monooxygenase (Wackett et al. 1989), respectively. A variety of non-specific oxygenases that attack TCE in aerobic environments are listed in Table 2.5.

As discussed previously, a diverse range of cometabolizing activities are present in the environment. Non-specific oxygenase activities within communities are the focus of this work. Such activity can be found in many hydrocarbon-degrading communities, the most well studied of which is the methanotrophs. Accordingly, a simple well-defined methanotrophic community was selected as a model cometabolizing community for this work. The second community selected was a phenol-degrading enrichment. This community was chosen because of its high growth rates, its high transformation capacity for TCE, and ease of handling of phenol in laboratory studies. Additional discussion of both methanotrophic and phenol-degrading communities are provided in the following sections.

Table 2.5 Examples of cometabolic transformation of trichloroethylene

Microorganisms	Growth substrate	References
Pseudomonas cepacia strain G4	phenol, toluene, o-cresol phenol	1, 2 3, 4
Pseudomonas putida F1	toluene	5, 6
Strain 46-1	toluene	7, 8
Methylosinus trichosporium OB3b	methane methanol formate	9, 10 11 12
Methylocystis sp. strain M	methane	13 14, 15
Mycobacterium vaccae JOB5	propane	16
Nitrosomonas europaea	ammonia	17, 18, 19
Xanthobacter strain Py 2	propylene	20, 21
Genetically engineered *Escherichia coli*	toluene	21

References: (1) Nelson et al. 1986; (2) Nelson et al. 1987; (3) Folsom et al. 1990; (4) Folsom &Chapman 1991; (5) Nelson et al. 1988; (6) Wackett & Gibson 1988; (7) Fox et al. 1990; (8) Little et al. 1988; (9) Oldenhuis et al. 1989; (10) Oldenhuis et al. 1991; (11) Tsien et al. 1989; (12) Newman & Wackett 1991; (13) Nakajima et al. 1992; (14) Uchiyama et al. 1989; (15) Uchiyama et al. 1992; (16) Wackett et al. 1989; (17) Arciero et al. 1989; (18) Hyman et al. 1988; (19) Rasche et al. 1991; (20) Ensign et al. 1992; (21) Zylstra et al. 1989.

Methanotrophic communities

Methanotrophs are classified into two major groups depending on their internal cell structure and carbon assimilation pathway. Type I organisms assimilate one-carbon compounds via a unique pathway, the ribulose monophosphate cycle, whereas Type II organisms assimilate C-1 intermediates via the serine pathway. The requirement for O_2 as a reactant in the initial oxidation of methane explains why all methanotrophs are obligate aerobes, whereas some organisms using methanol as electron donor can grow anaerobically (with nitrate or sulfate as electron acceptor). Both groups of methanotrophs contain extensive internal membrane systems, which appear to be related their methane-

oxidizing ability. Type I methanotrophs are characterized by internal membranes arranged as bundles of disk-shaped vesicles distributed throughout the organism whereas Type II methanotrophs possess paired membranes running along the periphery of the cell. Type I methanotrophs are also characterized by a lack a complete tricarboxylic acid cycle (the enzyme α-ketoglutarate dehydrogenase is absent), whereas Type II methanotrophs possess a complete cycle. In addition, most Type II methanotrophs can fix molecular nitrogen whereas Type I organisms do not. The classification and characteristics of methanotrophic bacteria are listed in Table 2.6.

The methylotrophs are capable of growth on a variety of organic compounds; however, they cannot use methane as carbon and energy sources. All methanotrophs can grow on methane, many are also able to utilize methanol and formaldehyde, and a few can use a wider range of organic compounds. Most methanotrophs are obligate methylophiles, which means that they are incapable of growth on compounds that contain carbon-carbon bonds. The responsible enzyme of methanotrophic bacteria, monooxygenase, catalyzes the incorporation of one oxygen atom from molecular oxygen into methane to produce methanol. The lack of substrate specificity of the monooxygenase enzyme enables it ability to oxidize a broad range of compounds, including halogenated aliphatic compounds. Monooxygenases can hydroxylate many alkanes and aromatic compounds and form epoxides from alkenes (Semprini et al. 1992). The epoxides are unstable and hydrolyze to acids. Since some products of these reactions are not further metabolized by methanotrophs, a community of microorganisms is necessary for mineralization.

Table 2.6 Tentative classification Scheme for Methanotrophic Bacteria*

Characteristic	Group I	GroupX	Group II
Morphology	Straight rod	Coccus	Straight, curved or pear-shaped rod
Membrane arrangement			
Bundles of vesicular disks	+	+	-
Paired peripheral membranes	-	-	+
Motility	±	-	±
Resting stage	Azotobacter-type cyst	Azotobacter-type cyst	Lipid cyst or terminal exospore
Rosette	-	-	+ (most strains)
Major carbon assimilation pathway	Rump	Rump	Serine
Autotrophic CO_2 fixation	-	+	-
Complete TCA cycle	-	-	+
Nitrogenase	-	+	+
Isocitrate dehydrogenase			
NAD^+ and $NAD(P)^+$ specific	+	-	–
NAD^+ specific	-	+	-
$NAD(P)^+$ specific	-	-	+
Glucose-6-dehydrogenase	+($NADP^+$ – specific)	+($NADP^+$ – specific)	-***
6-Phosphogluconate dehydrogenase	+($NADP^+$ – specific)	+($NADP^+$ – specific)	-
Predominant fatty acid carbon-chain length	16**	16	18
Growth at 45°C	Variable	+	–
Mol% G+C of DNA	50-54	62.5	61.7-63.1

* Green 1992.

Kinetic coefficients for methane utilization and TCE transformation reported in literature are summarized in Table 2.7 - 2.8. Although units have been standardized for purposes of comparison, differences in experimental protocol and methods of reporting sometimes made an accurate and complete comparison impossible. Clearly, broadly accepted unstructured models of cometabolism and standardized experimental protocols are needed to enable fair comparison of organisms from different sources and assist in the design and operation of engineering systems.

Table 2.7 Kinetic coefficients of methane utilization

Culture	Y mg cells/ mg CH_4	k_s mg CH_4/ mg cell-day	K_s mg CH_4/L	k_s' L/mg cell-day	b day^{-1}	Refs.
Methanotrophic mixed culture	0.34 (obs.) 0.51 (true)	1.13	0.67			Strand et al. 1990
Methanotrophic mixed culture	0.2[a]	1.72[a]	0.2 (Ki=12 mg/L for TCE)		0.12	Broholm et al. 1992
Methanotrophic mixed culture	0.33	0.94	1.07		0.18	Chang &Alvarez-Cohen 1995a
Methanotrophic mixed culture	0.35					Alvarez-Cohen &McCarty 1991a
Methylosinus trichosporium OB3b		8.37	1.47	7.49		Oldenhuis et al. 1991
Methanotrophic mixed culture					0.14	Henry & Grbić -Galić 1991
Methylomonas sp. MM2	0.26-0.73	0.16-1.63				Henry & Grbić -Galić 1990

a. biomass based on protein content.

Table 2.8 Kinetic coefficients of TCE cometabolic transformation by methanotrophic communities

Culture/growth substrate	k_c mg TCE/ mg cell-day	K_c mg TCE/ L	k_c' L/mg cell-day	$(T_y)_{obs}$ mg TCE/ mg growth substrate	$(T_c)_{obs}$ mg TCE/ mg cell	Refs.
Methanotrophic mixed culture/ methane			0.009			1
Methanotrophic mixed culture/ methane			0.011[a]			2
Methanotrophic mixed culture/ methane	1.03 (- formate) 4.17 (+formate)	3.84 (- formate) 6.95 (+formate)			0.05 (- formate) 0.1 (+formate)	3
Methanotrophic mixed culture/ methane				0.017 (- formate) 0.034 (+formate)	0.05 (- formate) 0.1 (+formate)	4
Methanotrophic mixed culture/ methane	0.84 (- formate) 4.8 (+formate)	0.69 (- formate) 7.9 (+formate)			0.043 (-formate) 0.061 (+formate)	5
Methanotrophic mixed culture/ methane	5.1 (+formate)	7.3 (+formate)		0.013	0.036	6
Methanotrophic mixed culture/ methane	0.84	1.5			0.042	7
Methylosinus trichosporium OB3b/ methane	41.6 (+formate)	18.1 (+formate)				8
Methylosinus trichosporium OB3b/ methane	54.9	19.1	2.88			9
Methylomonas sp. MM2/ methane	0.046-0.29	0.51-1.35	0.003-0.86			10
Methanotrophic mixed culture/ methane			0.61			10
sMMO from *Methylosinus trichosporium* OB3b/methane	64	4.83				11

a. biomass based on protein content.

b. References: (1) Strand et al. 1990; (2) Broholm et al. 1992; (3) Chang and Alvarez-Cohen 1995a; (4) Chang and Alvarez-Cohen 1995b; (5) Alvarez-Cohen and McCarty 1991a; (6) Alvarez-Cohen and McCarty 1991b; (7) Alvarez-Cohen and McCarty 1991c; (8) Brusseau et al. 1990; (9) Oldenhuis et al. 1991; (10) Henry and Grbić -Galić 1990; (11) Fox et al. 1990.

Phenol-oxidizing bacteria

Another group of microorganism that exhibit cometabolic oxygenase activity are bacteria that degrade aromatic compounds. aromatic-oxidizing microorganisms usually grow faster than methanotrophs by one or two orders of magnitude. The faster growth kinetics and

relative ease of addition of aromatic compounds give aromatic-oxidizing microorganisms certain pratical advantages in reactor systems. Some *Pseudomonas* species produce aromatic oxygenases that can degrade halogenated alkenes, including TCE (Nelson et al. 1987; Wackett and Gibson 1988). Other research established that *Pseudomonas putida* PpG4 would utilize phenol as growth substrate and cometabolize 4-chlorophenol (Saéz and Rittmann 1991; Saéz and Rittmann 1993). Previous results (Gottschalk 1986) supported that the initial step of 4-chlorophenol degradation, a monooxygenase-mediated attack on 4-chlorophenol, requiring O_2 and NADPH as cosubstrates. Phenol oxidation supplies the electrons needed to regenerate the NAPDH cosubstrate. Phenol and toluene are typical inducing agents for this activity. In aromatic oxygenase systems, TCE is degraded to formate, carbon monoxide, and glyoxylic acid in pure culture(Wackett and Householder 1989; Winter et al. 1989). *P. cepacia* G4, degraded TCE to CO_2, Cl^- and unidentified, nonvolatile products (Nelson et al. 1987; Nelson et al. 1986).

Microorganisms with phenol- or toluene-degrading ability include bacteria, such as *Pseudomonas* (Beltrame et al. 1980; Yang and Humphrey 1975), *Nocardia* (Rizzuti and Augueliaro 1982), and *Bacillus* (Buswell 1975); yeast, such as *trichosporon* (Gaal and Neujahr 1979); and multicellular fungi, such as *Fusarium* (Anselmo et al. 1985). Although many of these cultures (*Pseudomonas* strain G4, *Pseudomonas putida* F1, *Pseudomonas putida* B5, *Pseudomonas putida* PpF1) can transform TCE (Nelson et al. 1987; Nelson et al. 1986; Nelson et al. 1988; Wackett and Gibson 1988), the ability to degrade aromatic compounds does not always correlate with the ability to degrade TCE. The MMO system of the methanotrophs appears to be somewhat more consistent in its ability to degrade TCE although the rates of oxidation vary substantially.

Phenol-oxidizing microorganisms have demonstrated effective transformation of *cis*- and *trans*- dichloroethylene and trichloroethylene in laboratory and in-situ field studies

(Hopkins et al. 1993a). The phenol-oxidizing microorganisms appear to have a much higher capacity to degrade trichloroethylene than the methanotrophs. Trichloroethylene degradation of 90 percent has been achieved with 99.8 percent removal of injected phenol. Separate laboratory studies suggest that the addition of noncompetitive external reducing power may significantly increase the transfomation potential. Trichloroethylene transformation capacities were enhanced by the addition of aliphatic compounds, the greatest enhancement being with formate or lactate (Hopkins et al. 1993b). Kinetic coefficients for phenol utilization and TCE transformation reported in the literature are summarized in Table 2.9 - 2.10.

Table 2.9 Kinetic coefficients of phenol utilization

Culture	Y mg cells/ mg phenol	μ_m 1/hr	k_s mg phenol/ mg cell-hr	K_s mg phenol/ L	K_i mg phenol/ L	Refs.
Mixed culture	NA	0.131-0.363		5-266	142-1199	1
Mixed culture	0.7-0.9	0.66		16.5	634.4	2
P. putida sp	0.55	0.119		5.27	377	3
P. putida		0.53-1.84		0.5-1.23	8-20	4
Mixed culture		0.326		19.2	229	5
Mixed culture (non filament)	0.545	0.260		24.5	173	6
Mixed culture (filaments)	0.616	0.223		5.8	934	6
Mixed culture		0.21		630.41		2
Mixed culture	0.45	0.117		245		7
Mixed culture			0.07			2
Mixed culture			0.011-0.030			8
P. fluorescens			0.08			9
Nocardia			0.37			9
P. cepacia G4			2.6	0.8	43	10
Mixed culture	0.55					11
Mixed culture			0.051-0.135			12

Refences: (1) D'Adamo et al. 1984; (2) Auteinrieth et al. 1991; (3) Kotturi et al. 1991; (4) SoKol 1988; (5) Szetela and Winnicki 1981; (6) Pawlowsky and Howell 1973; (7) Beltrame et al. 1980; (8) Tischler and Eckenfelder 1969; (9) Rizzuti and Augueliaro 1982; (10) Folsom et al. 1990; (11) Chang and Alvarez-Cohn 1995b; (12) Shih et al. 1996.

Table 2.10. Kinetic coefficients of TCE cometabolic transformation by phenol-oxidizing bacteria

Culture/growth substrate	k_c mg TCE/ mg cell-day	K_c mg TCE/ L	k_c' L/mg cell-day	$(T_y)_{obs}$ mg TCE/ mg growth substrate	$(T_c)_{obs}$ mg TCE/ mg cell	Refs.
Mixed culture/ phenol				0.11 (-phenol) 0.01 (+phenol)	0.24 (-phenol) 0.03 (+phenol)	1
Mixed culture/ phenol				0.017 (-phenol) 0.019 (+phenol)	0.031 (-phenol) 0.034 (+phenol)	2
Mixed culture/ phenol			0.12-0.20			3
Mixed culture/ phenol	0.10	0.35	0.0026-0.11			4
Pseudomonas cepacia G4/ phenol	0.74	0.40				5
Pseudomonas putida F1/ toluene			0.0162			6

a. biomass based on protein content.
b. References: (1) Hopkins et al. 1993a; (2) Chang and Alvarez-Cohen 1995b; (3) Coyle et al. 1993; (4) Shih et al. 1996; (5) Folsom et al. 1990; (6) Wackett and Gibson 1988.

MODEL NONGROWTH SUBSTRATES

Halogenated hydrocarbons containing one or two carbon atoms constitute a significant fraction of the hazardous substances from industrial , domestic, and agricultural sources. These compounds tend to be mobile and persistent in soil and groundwater. Some have the potential for ozone depletion. For this work, the model compounds studied as nongrowth substrates were selected hydrochlorofluorocarbons (HCFCs), a hydrofluorocarbons(HFC), and trichloroethylene(TCE). The ban on CFCs has promoted the widespread use of HCFCs and HFCs. The presence of hydrogen makes HCFCs and HFCs more susceptible to tropospheric oxidation than the CFCs, and thus less likely to migrate into the stratosphere. To date, there is relatively little information on the fate of HCFCs or HFCs in aquatic environments. Trichloroethylene is a commonly detected

groundwater contaminant and is classified as a priority pollutant by the U. S. Environmental Protection Agency. A detailed discussion of these model compounds and their known properties for biodegradation is provided in the following sections.

HCFC/CFCs

Chlorofluorocarbons (CFCs) are widely used refrigerants and aerosols in industry and domestic life. Over the past decade, they have been implicated as agents of depletion of stratospheric ozone and as contributors to global warming (Molina and Rowland 1974)(Molina & Roland 1974; Rowland & Molina 1975). As a result, worldwide production of CFCs will be banned under the terms of Montreal Protocol. Nevertheless, CFCs will continue to be released into the environment due to past production and continued use. In aerobic aquatic environments, CFCs are recalcitrant, but they are transformed anaerobically (Denovan and Strand 1992; Lesage et al. 1992; Lovely and Woodward 1992; Semprini et al. 1992).

The ban on CFCs has inspired a major research effort to assess two classes of CFC substitutes - the hydrochlorofluorocarbons (HCFCs) and the hydrofluorocarbons (HFCs). HCFCs and HFCs are one- and two-carbon aliphatics, similar in structure and physical properties to the CFCs, but containing one or more hydrogen atoms. The presence of hydrogen makes HCFCs and HFCs more susceptible to tropospheric oxidation than the CFCs, and thus less likely to migrate into the stratosphere. To date, there is relatively little information on the fate of HCFCs or HFCs in aquatic environments. Lessage et al. (1992) reported transformation of HCFC-123a to HCFC-133 and HCFC-133b under methanogenic conditions. DeFlaun et al. (DeFlaun et al. 1992) reported aerobic transformation of three HCFCs and one HFC by *Methylosinus trichosporium* OB3b. The properties and purity of chemicals used as model nongrowth substrates in this work are summarized in Table 2.11.

Table 2.11 Properties and purity of HCFCs and HFC evaluated in this work.

Compound	Chemical name	Solubility in water,wt%, @25°C	Boiling point,°C, @760mm Hg	Density, g/cm^3, @25°C	Purity, %
HCFC-22	chlorodifluoro-methane	0.30	-40.8	1.194	99.9788
HCFC-142b	1-chloro-1,1-difluoroethane	0.14	-9.2	1.108	99.9609
HCFC-123	1,1-dichloro-2,2,2-trifluoroethane	0.21*	27.9	1.48*	**
HFC-134a	1,2,2,2-tetrafluoro-ethane	**	-26.2	1.206	99.8483

*@ 21.1°C ; **Data unavailable from manufacturers.

Trichloroethylene

Trichloroethylene (TCE) has been widely used in industry for many years as a popular dry cleaning solvent, an excellent degreasing agent, an extraction agent in decaffeinating coffee, and in several other ways. Because of improper handling, inadequate disposal techniques, or accidental spillage, it is commonly found in soil and groundwater near industrial sites (Barbash and Roberts 1986; Verschueren 1983). The presence of TCE and other low-molecular-weight chlorinated aliphatic hydrocarbons in groundwater threatens drinking water supplies (Roberts et al. 1982) and endangers human health because of the toxicity and suspected or demonstrated carcinogenicity of these chemicals (Miller and Guengerich 1983). In 1976, TCE was included on the EPA list of hazardous substances. It has become the subject of extensive governmental regulation. Moreover, TCE is partially degraded anaerobically to vinyl chloride, which is more toxic than TCE and is a known carcinogen (Parsons et al. 1984; Vogel and McCarty 1985). Therefore, TCE is the most frequently reported contaminant at hazardous waste sites on the National Priority List of the U.S. Environmental Protection Agency .

Trichloroethylene ($Cl_2C=CHCl$) is a synthetic, chlorinated organic chemical that fulfills all requirements for the degreasing solvent. It has high solvency for oils, greases, waxes, tars, resins, lubricants, and coolants generally found in the metal-processing industry. TCE is only slightly soluble in water (about 1100 ppm at 77°F) and forms an azeotrope with water, resulting in a mixture with a lower boiling point and vapor density. It is considered to be a highly volatile compound and favors environmental partitioning to the air rather than water. TCE is destroyed by photooxidation in the atmosphere, with a half-life of about one day.

Table 2.12 General information and properties of trichloroethylene[1]

Property	Value
Chemical Abstracts Service (CAS) number	79-01-6
Chemical formula	C_2HCl_3
Molecular weight	131.40
Physical state	Colorless liquid
Boiling point	86.7°C
Melting point	-73°C
Density	1.4 g/mL at 25°C
Vapor pressure	77 mm Hg at 25°C
Water solubility	1 g/L at 20°C
Henry's constant (dimensionless)[2]	0.392 at 25°C
Log octanol/ water partition coefficient	2.29
Odor threshold	0.5 mg/L in water;
	2.5-900 mg / m^3 in air
Air concentration conversion factor	5.46 mg / m^3=1 ppm

References: (1) Ware 1988; (2) Gossett 1987.

Methanotrophic transformation of trichloroethylene

In 1985, Wilson and co-workers reported on the possibility of aerobic oxidation of TCE by soil microorganisms that were provided natural gas as a primary source of energy (Wilson and Wilson 1985). Since them, the ability of methane-utilizing bacteria to cometabolize TCE has been reported and confirmed by several researchers (Fliermans et al. 1988; Fogel et al. 1986; Little et al. 1988). It is generally believed that the enzyme methane monooxygenase (MMO) oxidizes TCE to epoxides, which spontaneously hydrolyzes to

glyoxylate and dichloroacetate under acidic conditions, and carbon monoxide and formate under basic conditions (Henry and Grbić -Galić 1986; Henschler et al. 1979; Little et al. 1988; Miller and Guengerich 1982). Glyoxylate and dichloroacetate are oxidized to carbon dioxide by heterotrophic bacteria. Formate and carbon monoxide are oxidized to carbon dioxide by methanotrophs.

Later, researchers reported that besides products resulting from epoxide hydrolysis, intramolecular halide or hydride migration can occur yielding 2,2,2-trichloroacetaldehyde (chloral hydrate) (Fox et al. 1990). Chloral hydrate can be reduced further to trichloroethanol or oxidized to trichloroacetic acid (Newman and Wackett 1991). Chloral is toxic and may be responsible for the product toxity observed during TCE transformation by methanotrophs. Trichloroacetate degraded slowly in one methanotrophic mixed culture (Uchiyama et al. 1989). Formation of significant levels of Trichloroacetate and epoxide degradation products by *Methylocystis* sp. strain M indicates that chlorine migration and epoxide formation proceed in parallel (Nakajima et al. 1992). Other research also shows that these pathways proceed in parallel in *Methylocystis trichosporium* OB3B (Fox et al. 1990; Newman and Wackett 1991; Oldenhuis et al. 1989). Taken together, these reports suggest that both pathways occur simultaneously in type II methanotrophs. On the other hand, reports published to date suggest that type I methanotrophs transform exclusively by the epoxide pathway (Henry and Grbić -Galić 1986; Little et al. 1988). Methanotrophs and the products produced via transformation pathways are summarized in Table 2.13.

Table 2.13 TCE transformation and product formation by methanotrophs

Methanotroph	Transformation path	Products reported	Reference
Type I, *Methylomonas* sp. strain MM2	_	FA*, GA*, DCAA*	Henry & Grbić -Galić 1986
Type I, strain 46-1	1#	GA, DCAA	Little *et al.* 1988
Type II, *Methylosinus trichosporium* OB3b	1&2#	Chloral, TCetOH*	Oldenhuis *et al.* 1989
Soluble MMO from *Methylosinus trichosporium* OB3b	1&2	FA, GA, CO, DCAA, Chloral	Fox *et al.* 1990
Methylosinus trichosporium OB3b	1&2	FA, GA, CO, DCAA, Chloral, TCAA*, TCetOH	Newman and Wackett 1991
-Methylococcus capsulatus, -Methylosinus, sporium, -Methylosporovibrio methanica 81Z	_	Chloral	Newman and Wackett 1991
Type II, *Methylocystis* sp. strain M	1&2	FA, GA, DCAA, Chloral, TCAA, TCetOH	Nakajima *et al.* 1992
Type II, *Methylocystis* sp. strain M + *Xanthobacter autotrophicus* DA4	1&2	TCAA	Uchiyama *et al.* 1992

*FA: formic acid
GA: glyoxylic acid
DCAA: dichloroacetic acid
TCetOH: 2,2,2-trichloroethanol
TCAA: trichloroacetic acid
#Path 1: epoxidation
Path 2: chloride migration

Recent studies indicate that TCE transformation capacity is not only a function of the availability of reducing power, but also of the specific cometabolized compound and the toxicity of its transformation products (Alvarez-Cohen and McCarty 1991a; Henry and Grbić -Galić 1991a; Wackett and Householder 1989). Formate addition resulted in increased initial specific TCE transformation rates and elevated transformation capacity. Significant declines in methane conversion rates following exposure to TCE were observed for both resting and formate-fed cells, suggesting toxic effects caused by TCE or its transformation products (Alvarez-Cohen and McCarty 1991a; Alvarez-Cohen and McCarty

1991b). Not many researchers have reported on the toxicity of the specific products. Oldenhuis and co-workers suggested that TCE epoxide can be expected to bind covalently to proteins and nucleic acids. Other possible reactive metabolites that might bind irreversibly are chloral, dichloroacetyl chloride, and formyl chloride (Oldenhuis et al. 1991). Organisms or communities capable of degrading a large amounts of TCE should possess detoxification systems or populations that degrade these compounds.

The presence of toxic transformation products can be expected to have some impacts on the development of microbial communities during long-term TCE exposure. Changes in the populations are likely related to the level of TCE exposure, turnover of transformation products and utilization of growth substrate. Lackey et al. (1994) used total-recycle expanded-bed bioreactors to evaluate the degradation potential of TCE by a microbial consortium. Ester-linked phospholipid fatty acid profiles (PLFAME) were used to monitor the change of TCE-affected community during short-term perturbation. The results showed that a propane-utilizing bacterial biomaker increased as TCE was degraded and propane consumed. However, the relationship between community structure and extent of TCE exposure was not clear for these short-term exposures.

MODEL REACTOR SYSTEMS FOR COMETABOLISM

In order to study the development of microbial communities, a culture or community of microorganisms must be grown under defined conditions. Reactor configurations that have been evaluated include: completely-stirred tank reactor (Coyle et al. 1993; Landa et al. 1994), fed-batch reactor (Strand et al. 1990), fixed-bed reactors (Strand et al. 1991; Strandberg et al. 1989), expanded-bed reactor(Lackey et al. 1994; Phelps et al. 1990) and multi-stage systems(Alvarez-Cohen and McCarty 1991d; Folsom and Chapman 1991). This work focuses on dispersed growth systems.

Three basic modes of dispersed growth culture are widely used. Simple batch systems are used to study substrate-sufficient growth with maximum specific growth rate. Continuous culture in chemostats permits full control over specific growth rate with a given environment or conversely, the environment may be varied with the specific growth rate held constant. A unique feature of chemostats is that a time-independent steady state can be attained which enables one to determine the relationship between microbial behavior (genetic and phenotypic expression) and the environmental conditions. The last basic mode is fed-batch systems. In such systems, the culture is provided with a substrate feed which permits substrate-limited growth with a decreasing specific growth rate.

The three basic reactor systems described in last section affect the process parameters differently. Characteristically, batch systems show the four phases of growth (lag, logarithmic, stationary, and decline). In a chemostat culture at steady state, all the environmental factors are constant. In a variable volume fed-batch system in the "quasi steady state" all the environmental factors are virtually constant except the growth-limiting substrate; in constant volume fed-batch system with substrate-limiting growth, all nutrient concentrations vary throughout the cycle.

Several model systems have been considered for the evaluation of cometabolism of TCE. The cases here are much different from the traditional growth study involving only growth substrates. In most cases, both growth and nongrowth substrates were injected into model systems. Thus, the interactions between both substrates are important. The questions discussed in these research can be divided into three categories: verification of kinetic models, evaluation of reactor operations and monitoring of development of microbial communitines.

The presence of toxic transformation products can be expected to have some impacts on the microbial community structure during long-term TCE exposure. Changes in the populations are likely related to the level of TCE exposure, turnover of transformation product and utilization of growth substrate. These factors will have different effects in simple mixed cultures compared to complex communities and in batch reactors compared to continuous reactors. In batch reactors, for example, the microbial community is exposed to a range of growth and nongrowth substrate concentrations. This may select for a more diverse community with "specialist" organisms that occupy a variety of niches created by substrate concentration gradients. In contrast, a chemostat favors selection of specific populations at a fixed specific growth rate. This can be expected to result in a less diverse culture. Since nongrowth substrates are not mineralized by cometabolizing species and since heterotrophs are known to play important roles in detoxification, more diverse cultures should have advantages for cometabolism.

As discussed previously, batch system and chemostat represent two extremes for cometabolizing nongrowth substrate. One purpose of this work is to assess the effect of TCE exposure on microbial communities. Therefore, both systems were chosen as model systems for this study. Compared to conventional batch reactors, sequencing batch reactors (SBRs) have several advantages for cometabolic transformations. An SBR can alternate between periods of growth on growth substrates and periods of cometabolism of nongrowth substrate, eliminating the possibility of competitive inhibition for enzymes between growth and nongrowth substrates. SBRs also have a great ability to periodically change environmental condition, selecting or enriching specific microbial populations. Thus, SBRs offer a better model environment to study the dynamic changes of microbial communities than conventional batch system.

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CHAPTER 3

KINETICS OF COMETABOLISM BY RESTING CELLS*

ABSTRACT

This research investigated the potential for methanotrophic biotransformation of three HCFCs -- chlorodifluoromethane (HCFC-22); 1-chloro-1,1-difluoroethane (HCFC-142b); and 1,1-dichloro-2,2,2-trifluoroethane (HCFC-123); and one HFC -- 1,2,2,2-tetrafluoroethane (HFC-134a). All of these compounds were biotransformed to differing degrees by methanotrophic mixed culture MM1. Rates of transformation were obtained by monitoring disappearance of the target compounds from the headspace in batch experiments. Henry's constants were determined over a range of conditions to enable estimation of the intrinsic rates of transformation. Intrinsic rates of transformation were obtained by combining a second order rate expression with an expression describing loss of transformation activity due to either endogenous decay or product toxicity. For HCFC-123 and HFC-134a, the independently measured endogenous decay rate for mixed culture MM1 (0.594/day) was sufficient to account for the observed loss of transformation activity with time. However, the endogenous decay rate did not account for the loss of transformation activity for HCFC-22 and HCFC-142b. A model based on product toxicity provided a reasonable representation of the loss of transformation activity for these compounds. The order of reactivity was HCFC-22 > HCFC-142b > HFC-134a > HCFC-123, with second order rate coefficients of 0.014, 0.0096, 0.00091, and 0.00054 L/mg-day, respectively. Transformation capacities for HCFC-22 and HCFC-142b were 2.47

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and 1.11 μg substrate/mg biomass, respectively.

INTRODUCTION

Cometabolic transformation of nongrowth substrates under resting cell conditions is a specific case for cometabolism. Under this condition, reducing power or energy reserve is consumed for nongrowth substrate transformation with little or no energy return to microorganisms in the community. Several researchers have chosen resting cell conditions to study cometabolic transformation because interaction between growth and nongrowth substrates is not present. Kinetic models for cometabolic transformation by resting cells have been developed by several researchers (Alvarez-Cohen and McCarty 1991a; Criddle et al. 1990; Galli and McCarty 1989; Saéz and Rittmann 1991; Schmidt et al. 1985). Criddle (1993) proposed a model that unified the earlier models. This model assumed that nongrowth substrate is degraded with saturation kinetics and endogenous decay and that loss of cometabolic activity due to product toxicity can be incorporated into the cell decay term.

This chapter focuses on the methanotrophic biotransformation of three HCFCs (HCFC-22: chlorodifluoromethane, HCFC-142b: 1-chloro-1,1-difluoroethane, and HCFC-123: 1,1-dichloro-2,2,2-trifluoroethane) and one HFC (HFC-134a: 1,2,2,2-tetrafluoroethane) in a defined methanotrophic mixed culture. The unified model was used to predict the cometabolic transformation of HCFCs/HFC. The headspace method was used to monitor disappearance of target compounds. To obtain intrinsic kinetic data using this method, Henry's constants are needed. Because these constants were not available for the compounds studied, Henry's constants were first determined. Disappearance of the target compounds was then monitored in methanotrophic mixed cultures. Henry's constants were measured over a range of ionic strengths to enable use of these measurements in environments beyond those of the present study, such as seawater. Knowledge of

methanotrophic transformations should assist in selecting environmentally acceptable HCFCs and HFCs, modeling environmental fate, developing treatment technologies for fugitive manufacturing emissions, and remediating future wastewater and groundwater contamination.

Rationale for experimental work

Chlorofluorocarbons (CFCs) are widely used refrigerants and aerosols in industry and domestic life. Over the past decade, they have been implicated as agents of depletion of stratospheric ozone and as contributors to global warming (Molina and Rowland 1974; Rowland and Molina 1975). As a result, worldwide production of CFCs will be banned under the terms of Montreal Protocol. Nevertheless, CFCs will continue to be released into the environment due to past production and continued use. In aerobic aquatic environments, CFCs are recalcitrant, but they are transformed anaerobically (Denovan and Strand 1992; Lesage et al. 1992; Lesage et al. 1990; Lovely and Woodward 1992; Semprini et al. 1992).

The ban on CFCs has inspired a major research effort to assess two classes of CFC substitutes - the hydrochlorofluorocarbons (HCFCs) and the hydrofluorocarbons (HFCs). HCFCs and HFCs are one- and two-carbon aliphatics, similar in structure and physical properties to the CFCs, but containing one or more hydrogen atoms. The presence of hydrogen makes HCFCs and HFCs more susceptible to tropospheric oxidation than the CFCs, and thus less likely to migrate into the stratosphere. To date, there is relatively little information on the fate of HCFCs or HFCs in aquatic environments. Lesage et al. (1992) reported transformation of HCFC-123a to HCFC-133 and HCFC-133b under methanogenic conditions. DeFlaun et al. (1992) reported aerobic transformation of three HCFCs and one HFC by *Methylosinus trichosporium* OB3b.

MATERIALS AND METHODS

Chemicals

HCFC-22, HCFC-142b and HFC-134a were obtained from Asahi Glass Co., LTD. (Yokohama, Japan). HCFC-123 was obtained from Allied-Signal, Inc. (Morristown, NJ, USA). All chemicals used in media preparation were ACS grade, and all water used was 18 megaohm resistance or greater.

Analytical techniques

The study compounds were analyzed by withdrawing 0.5 mL of headspace from the test bottles using a Precision gas-tight syringe and injecting the sample onto a Perkin Elmer 8500 Gas Chromatograph (GC) equipped with a squalene packed column and a flame ionization detector. The GC was operated isothermally at 90°C with helium as carrier. Concentrations were obtained from an external standard calibration curve bracketing the concentration range of interest.

Measurement of Henry's constants

The modified EPICS procedure (Gossett 1987) was used to determine Henry's constants for each of the target compounds. Pure compounds were dissolved in methanol as stock solutions. To examine possible cosolvent interferences, five serum bottles with same amounts of HCFC-134 but with different methanol content (0 to 5%) were prepared. The result showed no significant cosolvent effect for methanol levels below 2%. Therefore, all subsequent measurements were conducted under this condition. For each compound, Henry's constant was measured in six 158.8 ml serum bottles: three containing 100 milliliters of distilled water, and three containing 25 milliliters. Both sets of bottles were sealed with Teflon/rubber septa and aluminum crimp caps. HCFCs/HFC solutions were injected into each bottle using a 0.5 mL gas-tight syringe. The bottles were then incubated

in an inverted position for 24 hrs at the desired temperature (6, 12, 22, 30 and 40°C, all ±0.2°C) on a temperature-controlled shaker, and headspace samples were analyzed by gas chromatography. To assess the effects of ionic strength on the Henry's constant, six serum bottles were filled with 100 mL solution, each with different concentration of KCl (0, 0.2, 0.4, 0.6, 0.8, 1.0 M). These bottles were then analyzed by headspace gas chromatography.

Culture conditions

Mixed culture MM1, a methanotrophic enrichment obtained from aquifer material at Moffett Field, California, was used for these experiments (Henry and Grbić -Galić 1991a). This culture is a stable consortium consisting of one methanotroph and three or four heterotrophs containing predominantly Grain-negative pleomorphic coccobacilli and prosthecates as well as some Grain-negative bacilli and cocci. The methanotroph in the mixed culture expresses soluble MMO similar to that of *Methylosinus trichosporium* OB3b under similar growth conditions (Henry and Grbić -Galić 1991a).

Mixed culture MM1 was grown in Whittenbury Mineral Medium containing (per liter of deionized water): 1.0 g of $MgSO4 \cdot 7H_2O$, 1.0 g of KNO_3, 200 mg of $CaCl_2 \cdot 2H_2O$, 3.8 mg of FeEDTA, 0.5 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg of $FeSO_4 \cdot 7H_2O$, 0.4 mg of $ZnSO_4 \cdot 7H_2O$, 0.02 mg of $MnCl_2 \cdot 4H_2O$ 0.05 mg of $CoCl_2 \cdot 6H_2O$ 0.01 mg of $NiCl_2 \cdot 6H_2O$, 0.015 mg of H_3BO_3, 0.25 mg of EDTA, 260 mg of KH_2PO_4, and 330 mg of Na_2HPO_4. One liter of culture was grown at room temperature (~21°C) in a continuously stirred 4-liter bottle supplied 30% methane in air at 68 mL/min. Growth curves were monitored and as stationary phase approached, approximately 10 mL of culture was transferred to a 1 liter of fresh Whittenbury Medium. Cells were harvested in mid-log growth phase for biotransformation experiments.

Batch biotransformation experiments

HCFCs/HFC degradation studies were performed using 158.8 mL serum bottles sealed with Telfon/rubber septa and aluminum crimp caps. These bottles were incubated with 100 mL of of Whittenbury Mineral Media plus culture. An appropriate amount (measured as dry weight) of mixed culture MM1 was added to each test bottle. Some bottles were autoclaved after cell addition (autoclaved cell controls) and others were filled with 100 mL pure water (water controls). HCFCs or HFC solutions (dissolved in water) were added to each bottle using Precision gas tight syringes, then vigorously shaken upside-down on a rotary shaker (250 rpm). Headspace samples were periodically analyzed by GC as described previously.

Modeling transformation of HCFCs/HFCs

To quantify the cometabolic transformation of HCFCs and the HFC studied, a second order rate expression was combined with an expression describing loss of activity due to endogenous decay (b) and product toxicity (q_c / T_c):

$$q_c = k_c' C_L \tag{1}$$

$$\mu = \frac{dX/dt}{X} = -b - \frac{q_c}{T_c} \tag{2}$$

where:

q_c = specific rate of transformation (mg substrate/mg cell-d)

k_c' = second order rate coefficient (L/mg cell-d)

C_L = liquid phase concentration of the substrate (mg/L)

μ = specific growth (or decay) rate (d^{-1})

X = active organism concentration (mg/L)

b = endogenous decay coefficient (d^{-1})

T_c = theoretical or true biomass transformation capacity (mg substrate/mg cell)

The endogenous decay term b includes loss of activity caused by cell death and by depletion of reducing power required for monooxygenase activity. A more extensive discussion of these processes and of Eq. (1) and (2) is provided by Criddle (1993). For batch transformation of a volatile cometabolic substrate, a mass balance at equilibrium gives:

$$-\frac{dM_c}{dt} = q_c X V_L \tag{3}$$

$$M_c = C_L(V_L + H_c V_G) \tag{4}$$

where:

M_c = mass of substrate (mg)

H_c = Henry's constant (-)

V_L = liquid volume (L)

V_G = gas volume (L)

Batch cometabolic transformation can be described by Eq. (1) - (4). These equations can be solved simultaneously using a Runge-Kutta algorithm. Two simplifying cases should be noted. The first occurs when product toxicity is absent or insignificant, $b >> q_c / T_c$ and $\mu = -b$. For this case, Eq. (1) - (4) can be combined and integrated to give the mass of substrate M_c as a function of time:

$$M_c = M_{c0} e^{\left\{\frac{k_c' A X_0}{b}\left(e^{-bt} - 1\right)\right\}} \tag{5}$$

where:

M_{c0} = initial mass of substrate (mg)

X_0 = initial active organism concentration (mg/L)

$A = V_L/(V_L + H_c V_G)$

The second simplifying case is also obtained when product toxicity is the dominant factor causing loss of transformation activity. For this case, $q_c / T_c >> b$, and $\mu = q_c / T_c$ and Eq. (1) - (4) can be combined and integrated to give:

$$M = M_{c0} \frac{F e^{(-k_c AFt)}}{X_0 - \frac{C_{L0}}{T_c} e^{(-k_c AFt)}} \qquad (6)$$

where:

C_{L0}= initial concentration of substrate in the aqueous phase (mg/L)

$F = X_0 - C_{L0}/T_c$

Disappearance of the target compounds was modeled with both Eq. (5) and (6). Kinetic parameters were estimated by nonlinear regression using Systat 5.1 (Systat, Inc.). For all modeling with Eq. (5), an endogeous decay rate b of 0.594/day was assumed. This value was independently obtained by Clowater (1992) for loss of trichloroethylene (TCE) transformation activity in aerated batch cultures of mixed culture MM1. Cultures of MM1 were aerated in the absence of methane and periodically assayed to determine the TCE transformation rate. The endogenous decay coefficient b was then computed as the slope taken from a plot of the logarithm of specific TCE transformation rate vs. aeration time.

RESULTS

Henry's Law constants

Measured Henry's Law constants are provided in Table 3.1, along with coefficients of variation. With two exceptions, all coefficients of variation were less than 6%. The effects

of temperature on Henry's constant followed the van't Hoff relationship (Gossett 1987). Results from a linear regression of ln H vs. T^{-1}(H in m^3-atm/mole; T in K) are provided in Table 3.2. Salting-out coefficients are listed in Table 3.3. Henry's constants were relatively insensitive to salinity. For the most sensitive compound studied (HCFC-22), the ionic strength must exceed 0.35 M to cause a greater than 10% increase in the apparent Henry's constant.

HCFCs/HFC transformation rates

Figures 3.1-3.4 illustrate the methanotrophic transformation of the target compounds. All four compounds were degraded to different degrees over the concentration range studied (900-3000 μg/L). Model fits obtained using equations 5 and 6 are also illustrated in Figures 3.1-3.4. Estimates for the kinetic parameters used to describe the transformation of each compound are summarized in Table 3.4 and 3.5.

Table 3.1 Measured values of Henry's constant vs. temperature.

compound	temperature ℃	Hc (-)	H, m^3.atm/mol	CV*, %
HCFC-22	6	0.622	0.0142	8.00
	12	1.277	0.0298	5.36
	22	1.679	0.0406	5.57
	30	2.358	0.0586	3.87
	40	3.535	0.0907	1.99
HCFC-142b	6	1.390	0.0318	4.33
	12	1.749	0.0409	8.24
	22	2.432	0.0588	4.75
	30	3.213	0.0798	2.24
	40	3.926	0.101	2.83
HCFC-123	6	0.571	0.0131	3.26
	12	0.825	0.0193	2.96
	22	1.057	0.0256	4.26
	30	1.463	0.0364	5.02
	40	1.979	0.0508	1.19
HFC-134a	6	1.190	0.0272	5.92
	12	1.528	0.0357	2.47
	22	2.067	0.0500	0.99
	30	2.199	0.0546	3.86

*Percent coefficient of variation = 100(SD/mean). Triplicate measurements were performed for each compound and temperature.

Table 3.2 Temperature regression for Henry's constant*.

	H=exp(A-B/T)		
Compound	A	B	r^2
HCFC-22	11.66	4387	0.956
HCFC-142b	7.363	3011	0.995
HCFC-123	7.805	3373	0.990
HFC-134a	5.714	2588	0.979

*Computed using values given in Table 3.1. Units of Henry's constant are m^3-atm/mole; T is in degrees Kelvin.

Table 3.3 Salting-out coefficients (22°C)*.

	log γ=kI	
Compound	k, L/mole	r^2
HCFC-22	0.118	0.996
HCFC-142b	0.0838	0.960
HCFC-123	0.0860	0.997
HFC-134a	0.0761	0.972

*Based upon measurements from 0 to 1.0 M KCl solution. Salting-out coefficients were determined by plotting $\log_{10}$ (activity coefficient) vs. ionic strength: $\log_{10}\gamma = kI$ where: γ = activity coefficient (-), k = salting-out coefficient (L/mole), I=ionic strength (M).

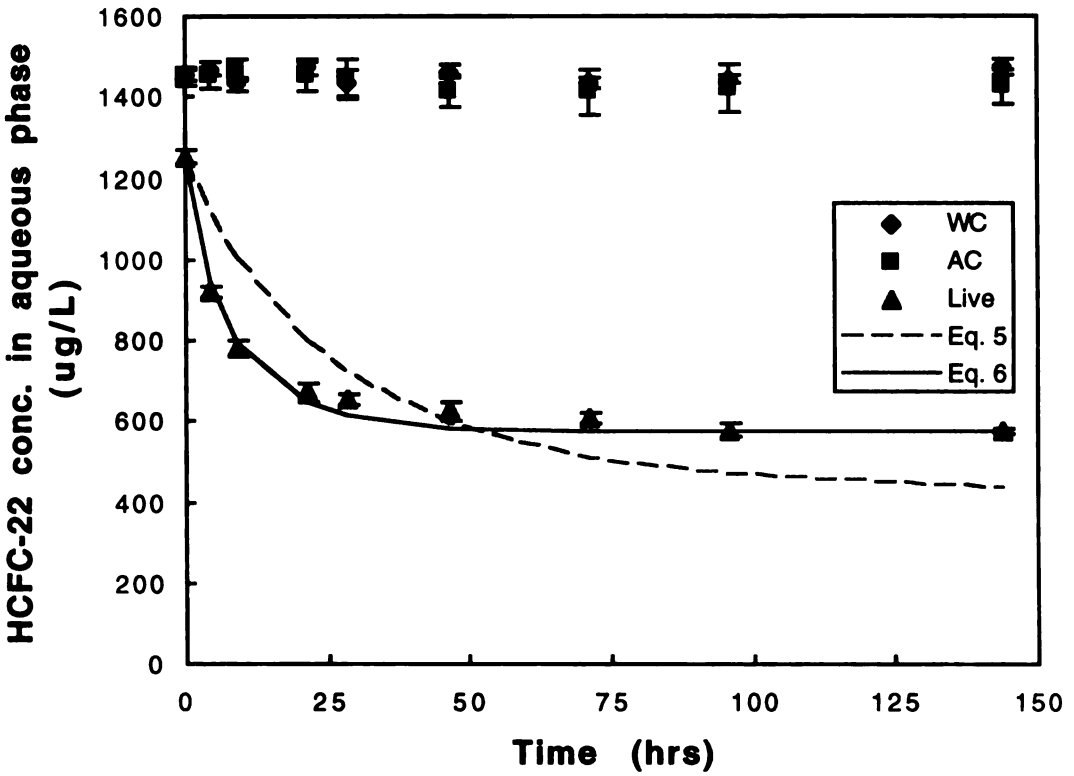

Figure 3.1 Biotransformation of HCFC-22 by methanotrophic mixed culture MM1. Fitting parameters are summarized in Tables 3.4 and 3.5. Error bars give standard deviations for three samples. WC = water control (no cells), AC = autoclaved control, LIVE = 275 mg/L MM1 (dry weight).

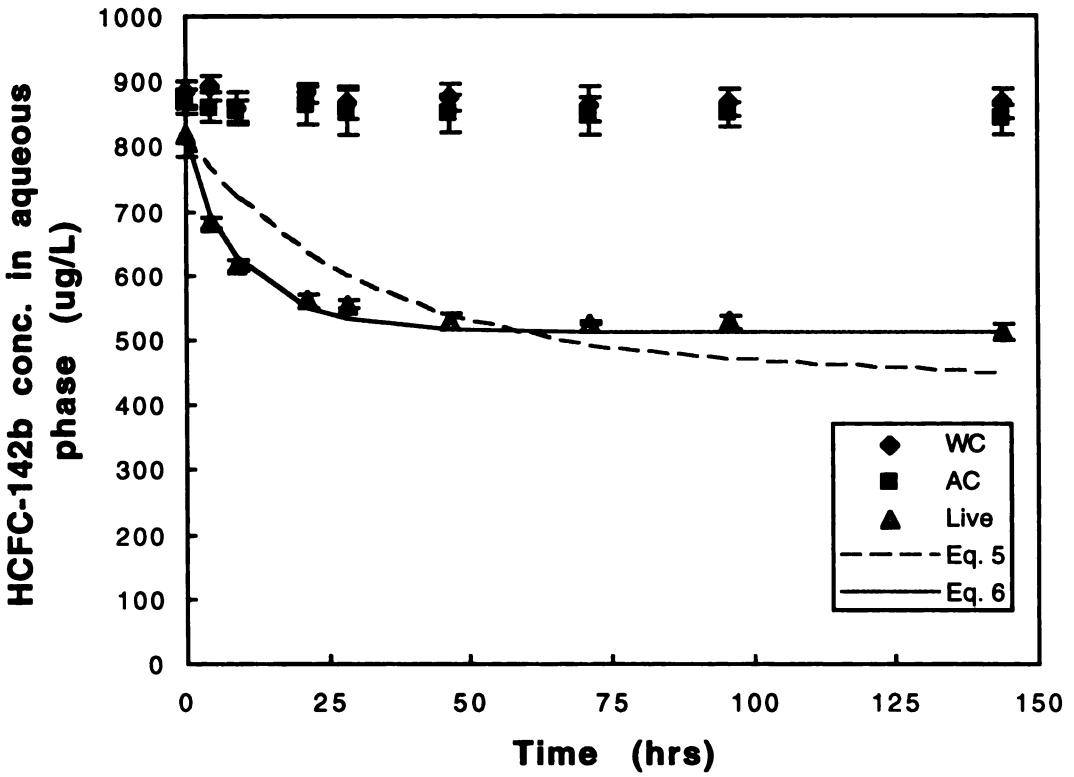

Figure 3.2 Biotransformation of HCFC-142b by methanotrophic mixed culture MM1: model fit based on equation 5 and 6, respectively. Fitting parameters are summarized in Tables 3.4 and 3.5. Error bars give standard deviations for three samples. WC = water control (no cells), AC = autoclaved control, LIVE = 275 mg/L MM1.

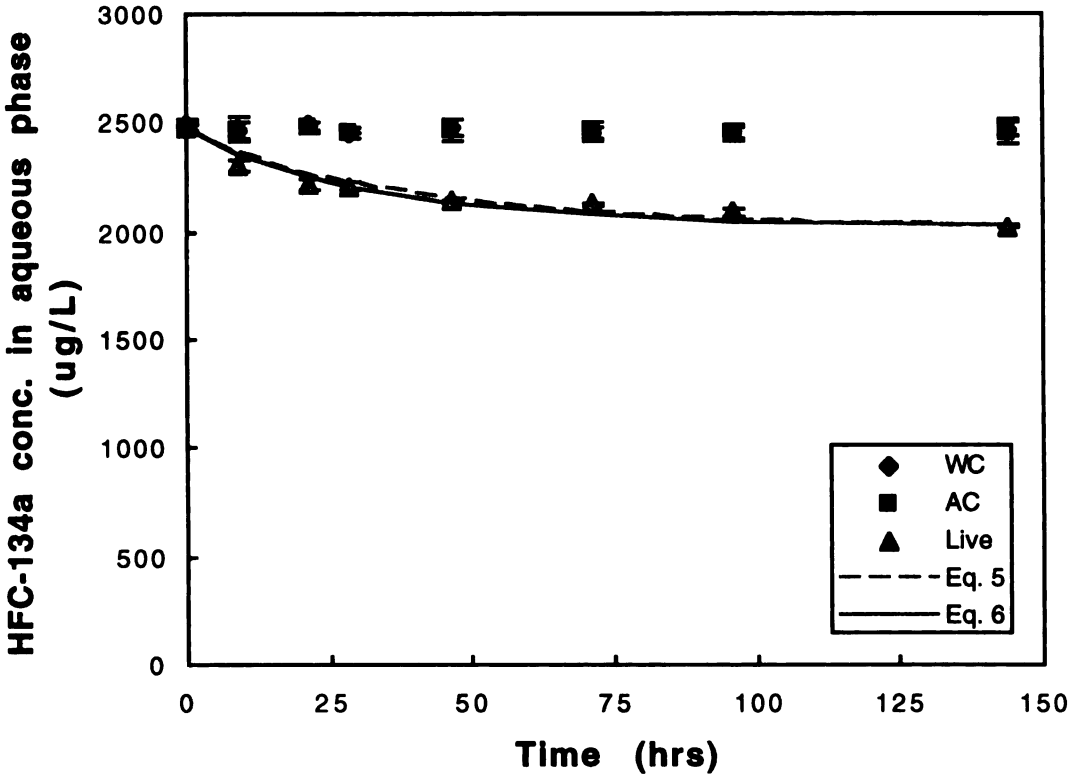

Figure 3.3 Biotransformation of HFC-134a by methanotrophic mixed culture MM1. Fitting parameters are summarized in Tables 3.4 and 3.5. Error bars give standard deviations for three samples. WC = water control (no cells), AC = autoclaved control, LIVE = 275 mg/L MM1.

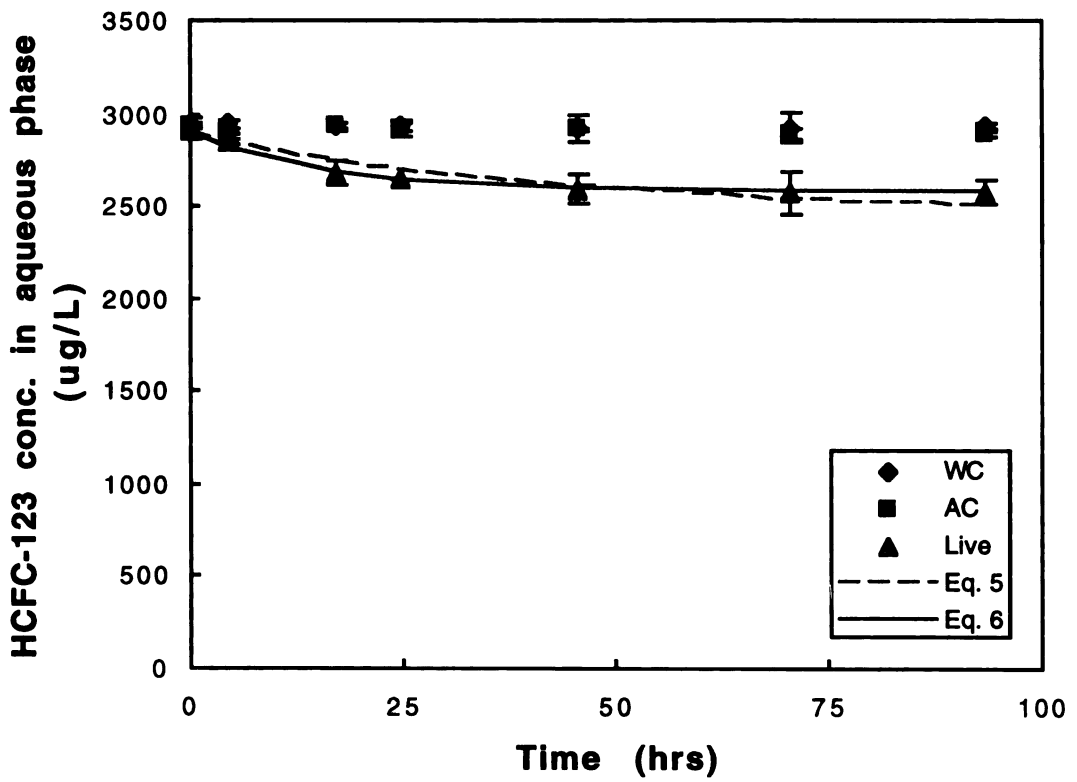

Figure 3.4. Biotransformation of HCFC-123 by methanotrophic mixed culture MM1. Fitting parameters are summarized in Tables 3.4 and 3.5. Error bars give standard deviations for three samples. WC = water control (no cells), AC = autoclaved control, LIVE = 275 mg/L MM1.

Table 3.4 Kinetic coefficients for HCFC/HFC transformation by methanotrophic mixed culture MM1. Best fit for the parameters of equation 5: comparison with TCE.

Compound	b (1/day)	k' (L/mg-day)[a]	Correlation coefficient r^2
HCFC-123	0.594[b]	0.00054 ±0.00014	0.888
HFC-134a	0.594[b]	0.00091 ±0.00002	0.915
HCFC-142b	0.594[b]	0.0030 ±0.0002	0.580
HCFC-22	0.594[b]	0.0043 ±0.0001	0.611
TCE	0.594[b]	1.4±0.23[c]	0.998[b]

[a] Determined by triplicate samples at 95% confidence interval.
[b] Independently determined by Clowater (1992).
[c] Clowater (1992)

Table 3.5 Kinetic coefficients for HCFC/HFC transformation by methanotrophic mixed culture MM1. Best fit for the parameters of equation 6: comparison with TCE.

Compound	T (μg substrate/mg cell)[a]	k' (L/mg-day)[b]	Correlation coefficient r^2
HCFC-123	1.166 ±0.264	0.00090 ±0.00011	0.934
HFC-134a	1.636 ±0.051	0.0011 ±0.0001	0.937
HCFC-142b	1.113 ±0.068	0.0096 ±0.0016	0.981
HCFC-22	2.468 ±0.050	0.014 ±0.002	0.984
TCE	47±0[c]	1.33±0.24[c]	0.997[c]

[a b] Determined by triplicate samples at 95% confidence interval.
[c] Clowater (1992).

DISCUSSION

Different degrees of transformation were obtained for the compounds studied. DeFlaun et al. (1992) reported that HCFC-123, HCFC-142b, HFC-134a were not degraded by the pure culture *Methylosinus trichosporium* OB3b. In the present study, HCFC-123 and HFC-134a degraded slowly, while HCFC-142b was transformed at a somewhat higher rate by mixed culture MM1. A possible explanation for the difference between this work and the DeFlaun study is that the MM1 methanotroph may possess enzymes with greater reactivity toward HCFCs and HFCs. It is also possible that the heterotrophs present in mixed culture MM1 facilitated transformation. Uchiyama (1992) found that TCE was mineralized to a greater extent by a mixed culture containing heterotrophs.

Pathways of transformation for the compounds evaluated in this study are not known. Presumably, in each case, oxygen is inserted at the carbon-hydrogen bond yielding an alcohol intermediate. Halogenated alcohols undergo further hydrolysis and elimination in aqueous systems giving rise to a variety of products. For HFC-134a, one of the possible products is trifluoroacetic acid. Trifluoroacetic acid is thought to be stable in aqueous environments, and it is an expected oxidation product in the troposphere. Analysis of the culture medium by ion chromatography after completion of HFC-134a transformation revealed a peak at a run time corresponding to that of trifluoroacetic acid. Additional analysis is needed to confirm this tentative identification.

For two of the target compounds - HCFC-123 and HFC-134a, use of Eq. (5) and the independently measured endogenous decay rate of 0.594 day^{-1} provided a reasonable fit to the data. It should be noted, however, that the model based on product toxicity (Eq. (6)) also fit the data well, indicating that conclusions about the mechanism for loss of transformation activity cannot be based on model fit alone.

As shown in Table 3.4, Eq. (5) provided a poor fit for HCFC-22 and HCFC-142b indicating that, for these compounds, another mechanism (besides endogenous decay) apparently contributes to the loss of transformation activity with time. As shown in Figures 3.1 and 3.2, Eq. (6) provided a good fit to these data. Thus, product toxicity may explain the loss of transformation activity for these compounds.

To assist in the interpretation of data, the transformation of the targeted fluorocarbons was compared with trichloroethylene (TCE). TCE is a useful bench mark for comparison because many researchers have evaluated methanotrophic transformation of TCE, and there is an extensive dataset on its transformation kinetics. As indicated by Table 3.5, rates of transformation for all of the fluorinated compounds studied were considerably slower than rates of transformation for TCE. Second order rate coefficients were 100 to 1000 times smaller for mixed culture MM1. Transformation capacities for HCFC-142b and HCFC-22 were ten to twenty times smaller than the values reported for TCE. The rapid loss of activity for HCFC-22 seems reasonable inasmuch as this compound is structurally similar to chloroform, a compound previously known to exhibit product toxicity in methanotrophic mixed cultures (Alvarez-Cohen and McCarty 1991c). For chloroform, the toxic byproduct is believed to be carbonyl chloride (phosgene). An analogous carbonyl may be formed from HCFC-22. Alvarez-Cohen and McCarty (1991c) reported a chloroform transformation capacity of 6.5 μg/mg cell, a value somewhat higher than that observed for HCFC-22.

The results of this work suggest that methanotrophic transformation is not likely to be a significant sink for the removal of HFCs and HCFCs globally. As indicated in Table 3.5, the fastest second order rate observed in this study was 0.014 L/mg cell-day for HCFC-22. Typical microbial densities in the soil and marine environment are on the order of 10^6 - 10^7 organisms per cm^3 of soil or water. Assuming that active methanotrophs constitute about

1% of these communities, first order rates of 10^{-10} to 10^{-9} s^{-1} would be possible for HCFC-22. In order to have a significant affect (>2-3%) on global lifetime estimates, first order rates exceeding 10^{-8} s^{-1} are required (Rodriguez et al. 1991).

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CHAPTER 4

KINETICS OF COMETABOLISM BY GROWING CELLS*

ABSTRACT

An unstructured model for cometabolism is presented and verified experimentally for a defined methanotrophic mixed culture. The model includes the effects of cell growth, endogenous cell decay, product toxicity, and competitive inhibition with the assumption that cometabolic transformation rates are enhanced by reducing power obtained from oxidation of growth substrates. A theoretical transformation yield is used to quantify the enhancement resulting from oxidation. A systematic method for evaluating model parameters is described. The applicability of the model is evaluated by comparing experimental data for methanotrophic cometabolism of TCE with model predictions from independently measured model parameters. Propogation of errors is used to quantify errors in parameter estimates and in the final prediction. The model successfully predicts TCE and methane transformation successfully for a wide range of concentrations of TCE (0.5 - 9 mg/L) and methane (0.05 - 6 mg/L).

INTRODUCTION

Many compounds of environmental and toxicological significance are transformed by cometabolism. In this study, cometabolism is defined as transformation of a nongrowth substrate by cells that are growing in the presence of growth substrate or by resting cells in the absence of growth substrate (Criddle 1993; Horvath 1972). With oxidative

* This study was submitted to biotechnology and bioengineering, Jun. 1996.

cometabolism, the growth substrate may compete with nongrowth substrate for positions at the enzyme active site, hindering transformation of the nongrowth substrate. However, in the absence of the growth substrate, the ability to sustain cometabolic transformation is eventually exhausted. Loss of transformation capacity may also result from damage to cellular material caused by toxic transformation products.

Recent studies indicate that cell inactivation can be quantified and incorporated into kinetic models by introducing a parameter termed biomass transformation capacity. This concept has been applied to quantify degradation kinetics for serveral oxgenase-expressing cultures with a range of chlorinated compounds (Alvarez-Cohen and McCarty 1991a; Chang and Alvarez-Cohen 1995b; Chang et al. 1993; Chang and Criddle 1995; Hopkins et al. 1993b). Alvarez-Cohen and McCarty (1991a) assumed that biomass transformation capacity was equal to the mass of nongrowth substrate ultimately degraded divided by the initial biomass used. Criddle (1993) defined a "theoretical" biomass transformation capacity by subtracting the effect of endogenous decay. The later definition represents a theoretical maximum value in the absence of external reducing power. Studies indicate that transformation capacity is not only a function of the availability of reducing power, but also of the specific cometabolized compound and the toxicity of its transformation products (Alvarez-Cohen and McCarty 1991c; Henry and Grbić -Galić 1991a; Wackett and Householder 1989).

Several models have been proposed to describe the cometabolic transformations of nongrowth substrate in the absence of growth substrate, many of which have been reviewed by Criddle (1993). Saéz and Rittmann (1991; 1993), for example, linked biomass decay with transformation of nongrowth substrate. Models have also been proposed to describe cometabolic degradation in the presence of growth substrates. Broholm et al. (1992) and Strand et al. (1990) modeled the interaction between growth

and nongrowth substrates by competitive inhibition, neglecting product toxicity and reducing power effects. Anderson and McCarty (1994) proposed a biofilm model that incorporated both product toxicity and competitive inhibition, but did not account for reducing power limitations. Recently, Chang and Alvarez-Cohen (1995a) proposed a general model that includes reducing energy explicitly as a limiting reactant during cometabolism. They applied the model to describe degradation of TCE by methanotrophs. The effects of reducing power were separated from toxicity effects and quantified by supplying the cells with formate. For methanotrophs, formate provides energy as NADH, but does not support growth. Thus, the experimental approach of Chang and Alvarez-Cohen is somewhat specific to organisms for which energy substrates can be identified that do not support growth.

In this paper, a general model is presented that combines the effects of cell growth, endogenous cell decay, product toxicity, and competitive inhibition with the assumption that cometabolic degradation rates are enhanced by reducing power obtained from oxidation of growth substrates. The proposed model does not require use of energy substrates, such as formate. A theoretical transformation yield is used to quantify the enhancement of cometabolism resulting from oxidation of the growth substrate (Chang et al. 1993; Criddle 1993). A systematic method for evaluating model parameters is developed. The applicability of the model is evaluated by comparing experimental data for methanotrophic cometabolism of TCE with model predictions from independently measured model parameters.

Rationale for experimental system

The experimental system selected for investigation in this work was methanotrophic transformation of trichloroethylene. In 1985, Wilson and co-workers reported aerobic oxidation of TCE by soil microorganisms provided natural gas as a primary source of

energy (Wilson and Wilson 1985). Since then, the ability of methane-utilizing bacteria to cometabolize TCE and other chlorinated organic solvents has been firmly established by many researchers (Fliermans et al. 1988; Fogel et al. 1986; Little et al. 1988). The enzyme methane monooxygenase (MMO) oxidizes TCE to an epoxide, which spontaneously degrades to intermediates that can be further metabolized, including glyoxylic acid, dichloroacetic acid, carbon monoxide, and formate (Little et al. 1988; Uchiyama et al. 1992) . Some researchers have reported that, in addition to products resulting from epoxide hydrolysis, intramolecular halide or hydride migration can occur, yielding 2,2,2-trichloroacetaldehyde (chloral hydrate). Chloral hydrate can be reduced to trichloroethanol and oxidized to trichloroacetic acid. All these products are potentially toxic and may cause cellular inactivation (Fox et al. 1990; Newman and Wackett 1991). For TCE transformation, formate addition resulted in increased initial specific transformation rates and elevated transformation capacity. Significant declines in methane conversion rates were observed following exposure to TCE for both resting and formate-fed cells, suggesting toxic effects by TCE or its transformation products (Alvarez-Cohen and McCarty 1991b; Alvarez-Cohen and McCarty 1991c). Oldenhuis and co-workers suggested that TCE epoxide can bind covalently to proteins and nucleic acids. Other possible reactive metabolites that might bind irreversibly are chloral, dichloroacetyl chloride, and formyl chloride (Oldenhuis et al. 1991; Oldenhuis et al. 1989).

MATERIALS AND METHODS

Culture and culture conditions

The methanotrophic culture used for these experiments was a mixed culture originally derived from aquifer material at Moffett Field, California (courtesy S. M. Henry). This culture is a stable consortium consisting of one methanotroph, one hyphomicrobium, and several heterotrophs containing Gram-negative thin and fat rods as well as some gram-

positive rods and cocci. The methanotroph in the mixed culture expresses soluble MMO similar to that of *Methylosinus trichosporium* OB3b under similar growth conditions (Henry and Grbić -Galić 1991a). Mixed culture MM1 was grown in Whittenbury Mineral Medium containing (per liter of deionized water): 1.0 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of KNO_3, 276 mg of $CaSO_4 \cdot 2H_2O$, 3.8 mg of FeEDTA, 0.5 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg of $FeSO_4 \cdot 7H_2O$, 0.4 mg of $ZnSO_4 \cdot 7H_2O$, 0.02 mg of $MnCl_2 \cdot 4H_2O$ 0.05 mg of $CoCl_2 \cdot 6H_2O$ 0.01 mg of $NiCl_2 \cdot 6H_2O$, 0.015 mg of H_3BO_3, 0.25 mg of EDTA, 260 mg of KH_2PO_4, and 330 mg of Na_2HPO_4. One liter of culture was grown at room temperature (~21°C) in a continuously stirred 2-liter bottle supplied 30% methane in air at 68 mL/min. Growth curves were monitored and as stationary phase approached, approximately 10 mL of culture was transferred to 1 liter of fresh Whittenbury Medium. Cells were harvested in mid-log growth phase for biotransformation experiments.

Analytical methods

A TCE-saturated water solution was used as the spike solution in all experiments. The spike solution was prepared by adding excess TCE (99+% pure ACS reagent, Aldrich Chemicals Co., Milwankee, WI) to a 250 ml glass bottle capped with TFE-lined Mininert valve. The bottle was vigorously shaken and allowed to settle at least 24 hrs. The upper layer of the solution was transferred to another bottle and capped with a Mininert valve. The spike solution was stored in a refrigerator until needed. One hour before use, it was shaken again and allowed to settle.

TCE was analyzed by withdrawing 0.1 ml of headspace from the test bottles using a 0.5 ml Pressure-Lok Series A-2 gas syringe and injecting the samples onto a Hewlett Packard 5890 gas chromatograph (GC) equipped with a column (DB624, 30m x 0.53mm I.D.) and a flame ionization detector. The GC was operated isothermally at 90°C with helium as carrier. The temperature at the injection port and detector was 250°C.

Methane and oxygen were analyzed by withdrawing 0.1 ml of headspace from the test bottles using a 0.5 ml Pressure-Lok Series A-2 gas syringe and injecting the samples onto a Hewlett Packard 5890 series II gas chromatograph equipped with a column (6 ft x 1/8 in SS packed with 80/100 washed molecular sieve 13X) and a thermal conductivity detector. The GC was operated isothermally at 50°C with helium as carrier. The temperature of the injection port and detector were 50°C and 90°C respectively.

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.

Batch biotransformation experiments

TCE degradation studies were performed using 25 ml glass vials capped with teflon-lined Mininert valves. These vials were incubated with 5 mL of of Whittenbury Mineral Media plus culture. An appropriate amount (measured as dry weight) of mixed culture MM1 was added to each test vial. TCE solutions (dissolved in water) were added to each bottle using Precision gas tight syringes. Methane were withdrawn from Scotty II cyclinders (99.0% CH_4, Alltech Associate, Inc., Deerfield, IL) at fixed exit pressure and injected into batch vials. After adding substrates, the vials were vigorously shaken upside-down on a rotary shaker (250 rpm). Headspace samples were periodically analyzed by GC as described previously.

Model development

A cometabolic model was evaluated that included terms for the loss of microbial biomass or enzyme activity caused by autooxidation (endogenous decay), proteolysis, depletion of cofactors (such as NADH), product toxicity, and suicide inactivation. A theoretical discussion of this model is provided elsewhere (Criddle 1993). For cometabolism in the presence of growth substrate, the following equations provide a complete mathematical description of the specific growth rate and the specific rates of utilization of the growth and the nongrowth substrates throughout the growth and decay periods.

$$q_s = k_s\left(\frac{S}{K_s + S}\right) \tag{1}$$

$$q_c = (T_y q_s + k_c)\left(\frac{C}{K_c + C}\right) \tag{2}$$

$$\mu = Y_m q_s - b - \frac{q_c}{T_c} \tag{3}$$

When there is competitive inhibition between the growth substrate and the nongrowth substrate, $(K_s)_{obs}$ and $(K_c)_{obs}$ replace K_s and K_c, respectively, in Eq. (1) and Eq. (2), where:

$$(K_s)_{obs} = K_s\left(1 + \frac{C}{K_{ic}}\right) \tag{4}$$

$$(K_c)_{obs} = K_c\left(1 + \frac{S}{K_{is}}\right) \tag{5}$$

In the absence of growth substrate, the model simplifies to:

$$q_c = k_c\left(\frac{C}{K_c + C}\right) \tag{6}$$

$$\mu = -b - \frac{q_c}{T_c} \tag{7}$$

Two important stoichiometric parameters are the observed transformation capacity, $(T_c)_{obs}$, and the observed transformation yield, $(T_y)_{obs}$. $(T_c)_{obs}$ is obtained by dividing $-q_c$ by μ:

$$(T_c)_{obs} = \frac{1}{\frac{b - Y_m q_s}{q_c} + \frac{1}{T_c}} \tag{8}$$

For resting cells (q_s=0), Eq. (8) simplifies to:

$$(T_c)_{obs} = \frac{1}{\frac{b}{q_c} + \frac{1}{T_c}} \tag{9}$$

For resting cells, the observed transformation capacity is determined by the theoretical transformation capacity, T_c, and by the ratio of the endogenous decay rate b to the specific rate of TCE transformation, q_c. The observed transformation yield, $(T_y)_{obs}$, is obtained by dividing q_c by q_s.

In the absence of cometabolism, electrons from the growth substrate are used exclusively for cell synthesis and respiration so $f_s + f_e = 1$, where f_s = fraction of electrons for cell synthesis and f_e = fraction of electrons for energy generation. In cometabolic reaction, however, electrons are consumed for growth, respiration and cometabolism. In this case, $f_s + f_e + f_{co} = 1$, where f_{co} = fraction of electrons used for cometabolism (Criddle 1993; Criddle et al. 1991). For oxygenase-mediated reactions, two moles of electrons are consumed for every mole of nongrowth substrate transformed, but this ratio will decrease if the byproducts of transformation are further oxidized by the cometabolizing community.

For batch transformation of a volatile cometabolic substrate in the presence of growth substrate, a mass balance for growth and nongrowth substrates at equilibrium gives:

$$-\frac{dM_c}{dt} = q_c X V_L \tag{10}$$

$$M_c = C(V_L + H_{cc} V_G) \tag{11}$$

$$-\frac{dM_s}{dt} = q_s X V_L \tag{12}$$

$$M_s = S(V_L + H_{cs} V_G) \tag{13}$$

Batch cometabolic transformation in the presence of growth substrate can be described by Eq. (1), (2), (3), (4), (5), (10), (11), (12) and (13). Once the parameters of the model (b, k_c, K_c, T_c, k_s, K_s, Y, K_{ic}, K_{is}, T_y) are determined, these equations can be solved simultaneously using a Runge-Kutta numerical method. Simplified cases of the model ($C << K_c$ in the absence of growth substrate) have been previously verified (Chang and Criddle 1995).

Model verification

The model evaluated in this work was verified using the procedure illustrated in Figure 4.1. Experimental data were compared with predictions from separate measurements of the kinetic parameters. Using the measured parameters, degradation rates for methane and TCE were predicted for specified initial conditions. The predictions were evaluated experimentally. Four independent series of experiments were conducted to measure the maximum specific rate of utilization of substrate (k_c and k_s), the half-saturation coefficient (K_c and K_s), growth yield (Y), endogenous decay constant (b) and the

theoretical transformation capacity (T_c) in the absence of endogenous decay. To measure the remaining parameters (K_{ic}, K_{is} and T_y), an additional set of experiments was conducted over a range of concentrations of growth substrate with high initial TCE concentrations. Details of the experimental evaluation for each of these parameters is described in the following sections. Propogation of errors was used to quantify errors in parameter estimates and in the final prediction (Mandel 1984). All non-linear parameter estimates were obtained by nonlinear regression using Systat 5.2.1 (Systat, Inc., Evanston, IL).

Sensitivity analysis (Robinson and Characklis 1984; Robinson 1985) were performed to evaluate the uniqueness of parameter estimates and the relative importance of parameters over the range of substrate concentration. Three equations derived from model, Eq. (1), (6), and (2) were used to estimate three sets of parameters (k_s and K_s, k_c and K_c, K_{is} and T_y). The derivatives of dependent variable with respect to each set of parameters (dq_s/dk_s and dq_s/dK_s, dq_c/dk_c and dq_c/dK_c, dq_c/dK_{is} and dq_c/dT_y) were evaluated for a range of substrate concentration. If sensitivity equations for each pair of parameters are not multiples of each other over a wide range of substrate concentration, a unique combination of parameters can be estimated from the data set. To determine the relative importance of each parameter on the specific rate of transformation of growth and nongrowth substrates, the derivatives of q_c and q_s with respect to related parameters were also evaluated over a range of substrate concentrations.

Maximum specific rate of transformation for the nongrowth substrate and half-saturation coefficient of the nongrowth substrate

K_c and k_c were determined by adding a range of concentrations of TCE to batch cultures of resting cells that were fully induced for the desired cometabolic activity. The initial concentrations spanned a range that bracketed the concentration above which specific rate of transformation are maximum and the concentration corresponding to the half-

saturation coefficient. The initial slope of the resulting degradation curves for each initial concentration was determined. A nonlinear regression on Eq. (6) was used to estimate k_c and K_c.

Maximum specific rate of utilization of growth substrate, half-saturation coefficient of growth substrate, and observed yield

K_s and k_s were determined by adding a range of concentrations of growth substrate to batch cultures. The initial concentrations spanned a range that bracketed the concentration above which specific rate of transformation are maximum and the concentration corresponding to the half-saturation coefficient. The initial slope of the resulting degradation curves for each initial concentration was determined. A nonlinear regression on Eq. (1) was used to estimate k_s and K_s. Y was determined by measuring the increase of dry weight of biomass with the consumption of growth substrate during a period of time. The value was obtained during the growth phase, before decay of cell biomass was significant.

Endogenous decay coeffcient

Subsamples were withdrawn from the decaying culture and spiked with high concentrations of TCE, so that $C_0 >> K_c$. The initial slope of the resulting degradation curve was proportional to the concentration of cometabolizing cells. The active fraction remaining at any time was computed by dividing the initial slopes for each subsample by the initial slope at the beginning of the decay period. A semilog plot of active fraction vs. time yields a straight line with slope of -*b*.

Theoretical transformation capacity

Once the endogenous decay coefficient *b* and maximum specific rate of utilization k_c

were quantified, T_c was determined by adding a high concentration of TCE to a batch culture of resting cells . For $C_0 >> K_c$, Eq. (6) simplifies to $q_c = k_c$. By combining this result with Eq. (9) and allowing time to become infinite, the actual or observed transformation capacity, $(T_c)_{obs}$ is given by

$$(T_c)_{obs} = \frac{dM_c}{V_L dX} = (\frac{V_L + H_{cc}V_G}{V_L})\frac{dC}{dX} = (\frac{V_L + H_{cc}V_G}{V_L})(\frac{C_0 - C_\infty}{X_o}) = \frac{1}{\frac{b}{k_c} + \frac{1}{T_c}}$$

T_c was calculated from the above relationship.

Theoretical transformation yield, inhibition coefficient of growth substrate on nongrowth substrate utilization, and inhibition coefficient of nongrowth substrate on growth substrate utilization

To evaluate these three parameters, initial specific rates of utilization of growth substrate and nongrowth substrate were measured over a range of concentrations of growth substrate with high initial TCE concentration (10 mg/L). A nonlinear regression on Eq. (1), (2), (4) and (5) with previously determined values for k_s, K_s, k_c and K_c was used to estimate K_{ic}, K_{is} and T_y.

RESULTS AND DISCUSSION

Cometabolism is a complex phenomenon, especially when both growth and nongrowth substrates are simultaneously present. For oxygenase-mediated reactions, nongrowth substrate competitively inhibits utilization of the growth substrate, yet utilization of growth substrate is needed for sustained transformation of nongrowth substrate. The model evaluated in this work attempted to capture this paradox.

In this research, a systematic method was developed to predict simultaneous degradation

of growth and nongrowth substrates. Parameters for growth and nongrowth substrate degradation were first measured alone in the absence of competitive interactions. Thereafter, parameters indicating interaction between growth and nongrowth substrates (K_{ic}, K_{is} and T_y) were measured in the presence of both substrates.

The sensitivity equations for each pair of parameters are not multiples of one another for the wide range of substrate concentration (Figure 4.2). This implies that unique combination of parameters can be estimated from the data set. Sensitivity equations with respect to parameters in Eq. (1), (2), (4), and (5) were also evaluated. The results show much greater sensitivity to maximum specific rate of utilization of growth and nongrowth substrates than to the respective half-saturation coefficients. Of all parameters, K_{is} was the most sensitive parameter affecting the specific utilization rate of nongrowth substrate. K_{ic} was less sensitive. The sensitivity equation with respect to K_{is} reached a maximum at the lower concentration of growth substrate.

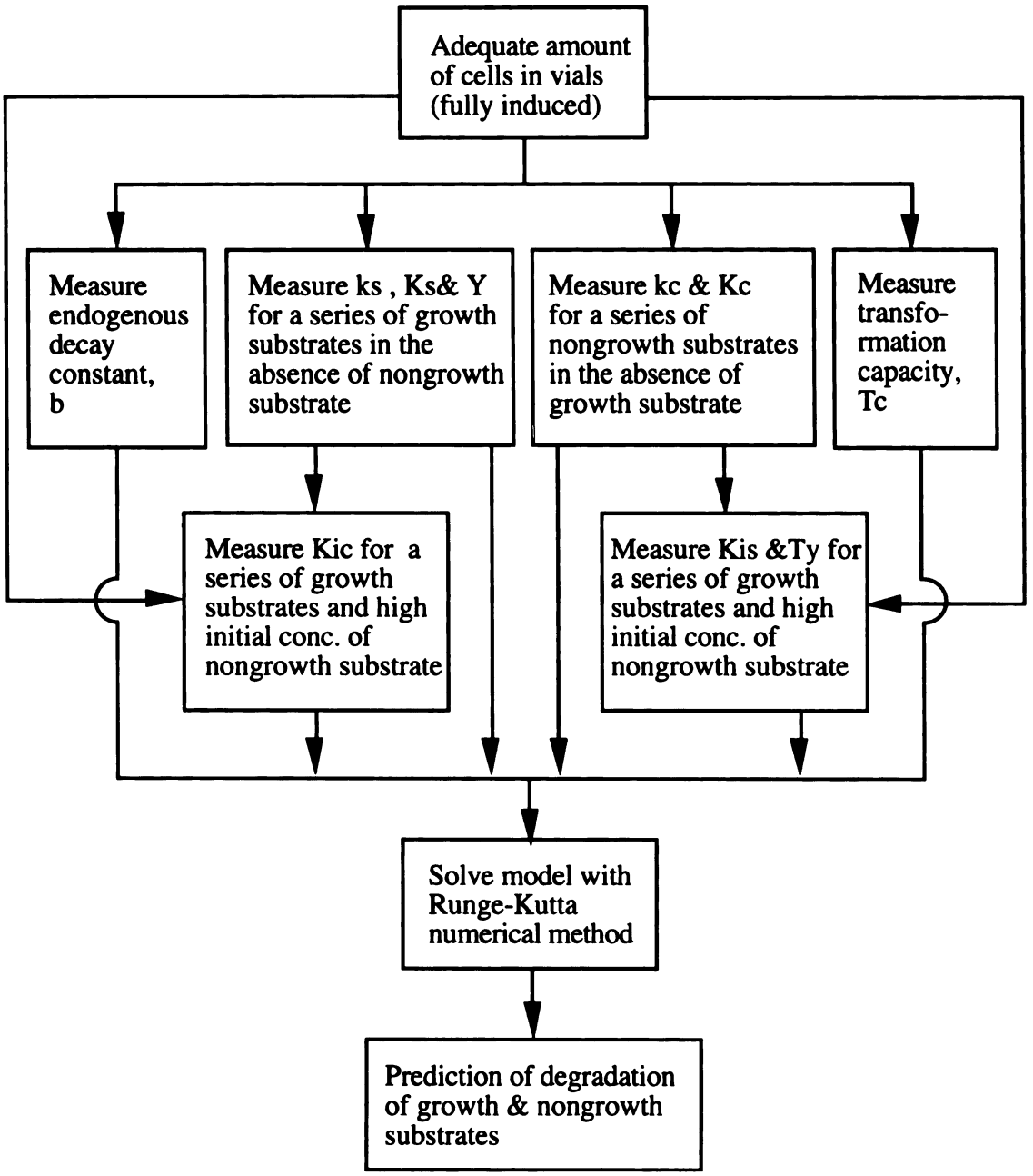
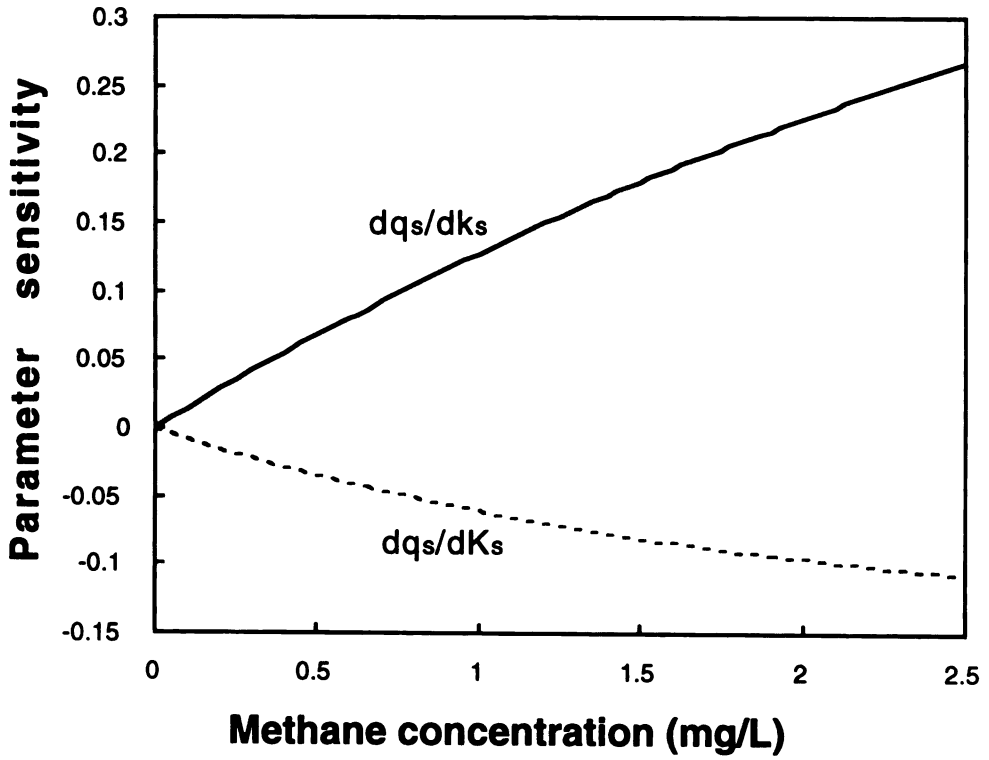
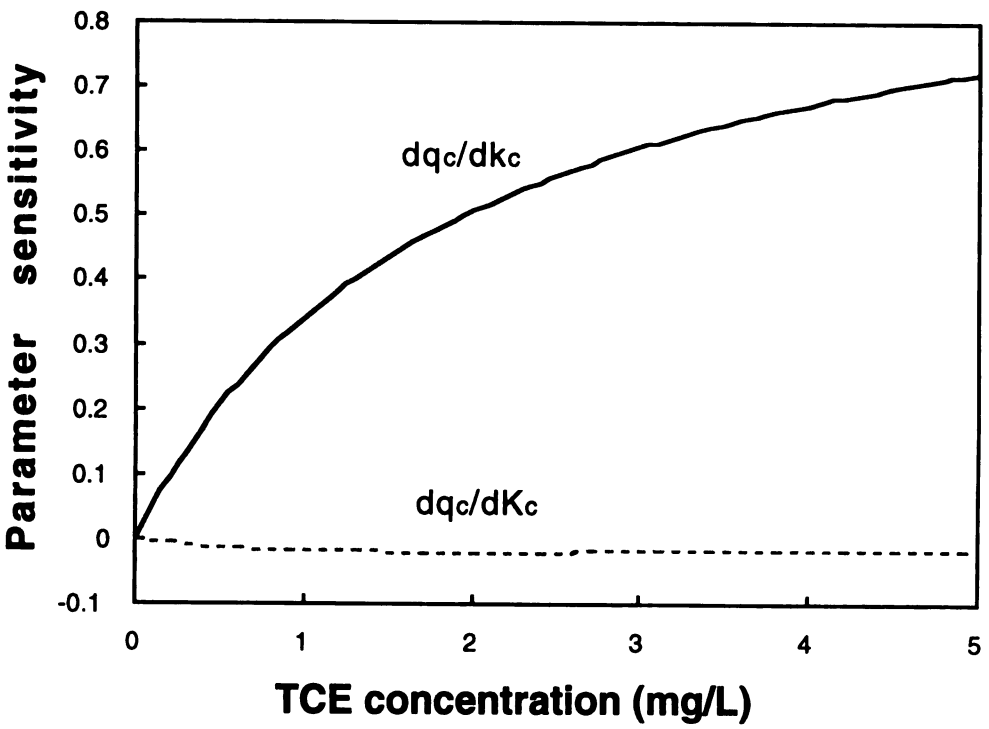


Figure 4.1 Approach for prediction of degradation of growth and nongrowth substrates by the proposed model.

(a)


Parameter sensitivity
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
dqs/dks
dqs/dKs
0
0.5
1
1.5
2
2.5
Methane concentration (mg/L)

(b)


Parameter sensitivity
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
dqc/dkc
dqc/dKc
0
1
2
3
4
5
TCE concentration (mg/L)

(c)

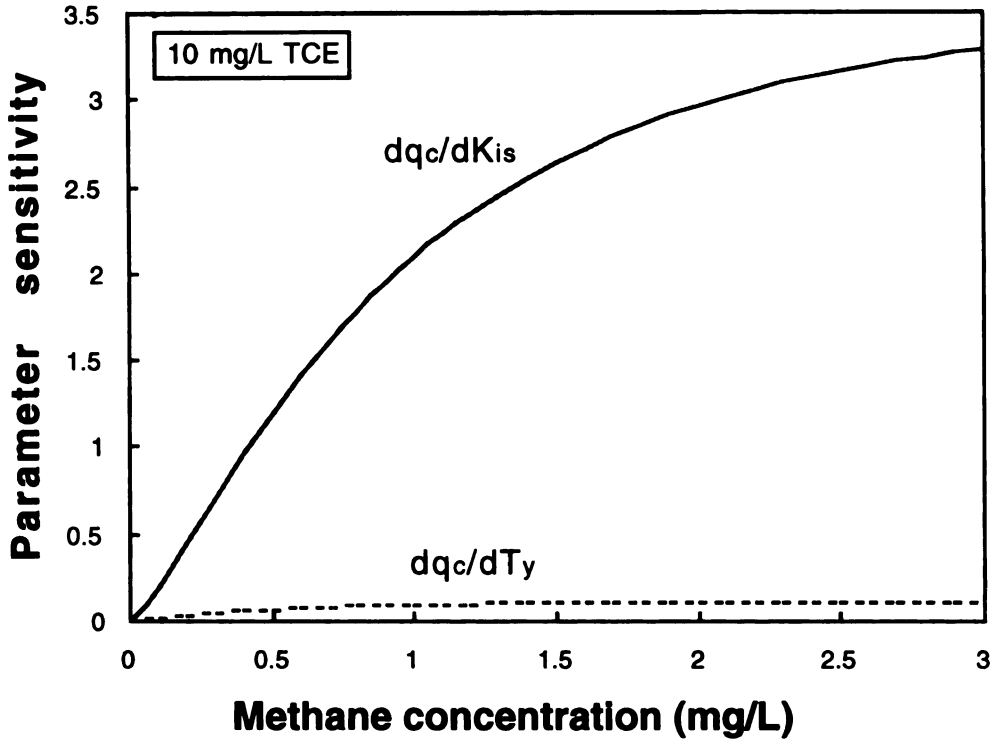


Figure 4.2 The sensitivity equations for parameters estimated nonlinearly from model, concentrations shown here are the ranges for each parameter determination.

The half-saturation coefficients (K_s and K_c) are often assumed to be equal to the inhibition coefficients for the respective substrates (K_{is} and K_{ic}) (Alvarez-Cohen and McCarty 1991c; Anderson and McCarty 1994; Broholm et al. 1992; Chang and Alvarez-Cohen 1995a). Our data indicate that they were significantly different under the present experimental conditions (Table 4.1). This may be caused by the fact that the measurements were performed with whole cells rather than purified enzymes. Factors other than competition for the active site of the enzyme may influence the interactions of growth and nongrowth substrates, e.g., reductant supply or substrate transport to the enzyme (Landa et al. 1994). Thus, the assumption that both values are the same and that

the substrates are substitutable appears to be inappropriate in this case. A similar observation was reported for toluene and TCE by Landa et al. (18).

Independently measured kinetic parameters for TCE degradation are summarized in Table 4.1. Using these parameters, the model was solved numerically to predict methane and TCE degradation under various conditions. Figure 4.3 illustrates model predictions and experimental data for batch transformation of growth substrate in the presence of a high initial TCE concentration. To confirm the consistency of predictions, further independent batch degradation experiments were conducted with lower initial TCE concentrations (Figure 4.4). Methane degraded more slowly in all cases when TCE was present, indicating that methane utilization was strongly inhibited by TCE transformation. The results show that the model can predict methane and TCE degradation with reasonable accuracy.

Addition of a limited level of methane enhanced TCE degradation over a specific range of methane concentrations (Figure 4.5). However, competitive inhibition between growth and nongrowth substrates also played an important role. Thus, further increases in methane concentration eventually decreased TCE degradation. This result is in agreement with the observations of Chang and Alvarez-Cohen (Chang and Alvarez-Cohen 1995b). The effect of methane is less significant for lower initial TCE concentration. Also, maximum degradation rates for TCE are achieved at lower methane concentrations for lower initial TCE concentrations. This phenomenon can be explained by competitive inhibition between growth and nongrowth substrates. When TCE concentration is low, methane has more chance to occupy active sites in the methane monooxygenase, and TCE transformation is inhibited.

Table 4.1 Kinetic and stoichiometric parameters for methane utilization, growth, and TCE degradation for methanotrophic mixed culture MM1.

Parameters	Value[1]
maximum specific rate of utilization of methane, k_s	3.77 (+/- 0.83) mg/mg cell-day
half-saturation coefficient of methane, K_s	6.85 (+/- 1.86) mg/L
maximum specific rate of utilization of TCE, k_c	0.152 (+/- 0.018) mg/mg cell-day
half-saturation coefficient of TCE, K_c	1.94 (+/- 0.46) mg/L
theoretical transformation capacity in the absence of endogenous decay, T_c	0.0602 (+/- 0.0005) mg TCE /mg cell
first-order endogenous decay constant, b	0.549 (+/- 0.044) /day
observed yield, Y	0.426 (+/- 0.023) mg cell/mg methane
inhibition coefficient indicating the effect of methane on TCE utilization rate, K_{is}	0.119 (+/- 0.052) mg/L
inhibition coefficient indicating the effect of TCE on methane utilization rate, K_{ic}	10.8 (+/- 1.45) mg/L
growth substrate transformation capacity, T_y	4.01 (+/- 1.20) mg TCE/mg methane

1. Values represent the 95% confidence interval for triplicate data.

Table 4.2 Stoichiometry of TCE cometabolism by methanotrophic mixed culture MM1 for different initial TCE concentrations.

Initial TCE concentration (mg/L)	Initial CH_4 concentraton (mg/L)	$\Delta TCE/\Delta CH_4$ (mole TCE/ mole CH_4)	$\Delta O_2/\Delta CH_4$ (mole O_2/ mole CH_4)	$\Delta X/\Delta CH_4$[1,2] (mole cells/ mole CH_4)
0	6.42	0	0.88	0.059
0.98	6.19	0.003	1.01	0.054
3.98	6.88	0.232	2.09	0.008
10.2	6.73	0.563	2.35	0
17.2	7.03	0.759	2.34	-0.021

1. The formula of cells was assumed $C_5H_7O_2N$.
2. Yields were measured after two-day incubation.

Table 4.3 Electron flow in methanotrophic mixed culture MM1 for different initial TCE concentrations.

Initial TCE concentration (mg/L)	Initial CH_4 concentraton (mg/L)	Fraction of electrons for cometabolism, fco [1]	Fraction of electron for energy, fe	Fraction of electron for synthesis, fs [2,3]
0	6.42	0	0.44	0.832
0.98	6.19	0.008	0.50	0.760
3.98	6.88	0.058	1.05	0.119
10.2	6.73	0.141	1.18	0
17.2	7.03	0.190	1.17	0

1. Assumes two moles of electrons required per mole of TCE transformation.
2. The formula of cells was assumed $C_5H_7O_2N$.
3. Yields were measured after two-day incubation.

Table 4.4 The effect of methane concentration on observed transformation yield when both substrates are simultaneously present: comparison of measured and predicted values.

Initial TCE concentration	Methane concentration	Observed transformation yield (mg TCE/mg CH_4)	
(mg/L)	(mg/L)	Predicted[1,2]	Measured[2]
4.1	0.8	0.88+/-0.42	0.94+/-0.22
	1.6	0.52+/-0.25	0.60+/-0.13
	3.4	0.28+/-0.13	0.22+/-0.02
	4.4	0.22+/-0.10	0.14+/-0.03
	6.4	0.15+/-0.07	0.12+/-0.03
8.5	0.6	1.99+/-0.89	1.84+/-0.36
	1.2	1.34+/-0.62	0.74+/-0.09
	2.9	0.73+/-0.35	0.52+/-0.08
	3.4	0.65+/-0.30	0.50+/-0.06
	5.8	0.42+/-0.19	0.38+/-0.05

1. Errors calculated from the law of propagation of error.
2. Values represent the 95% confidence interval for triplicate data.

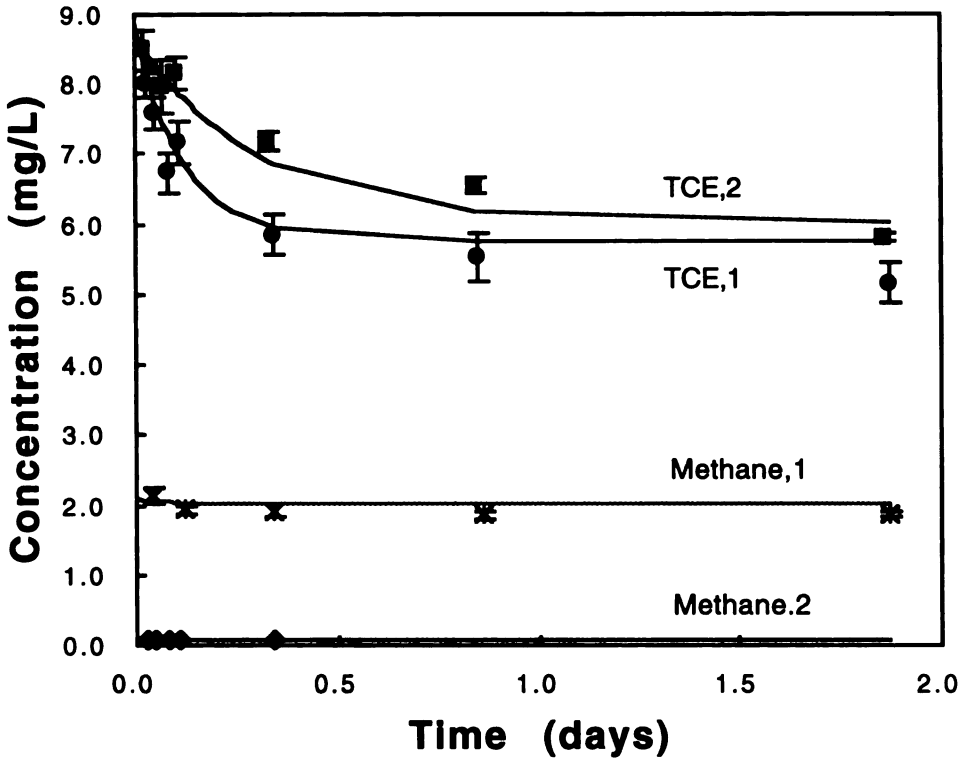


Figure 4.3 Biotransformation of TCE and methane by methanotrophic mixed culture: comparison with model predictions (for different initial methane concentration). Error bars indicate the 95% confidence interval for triplicate samples.

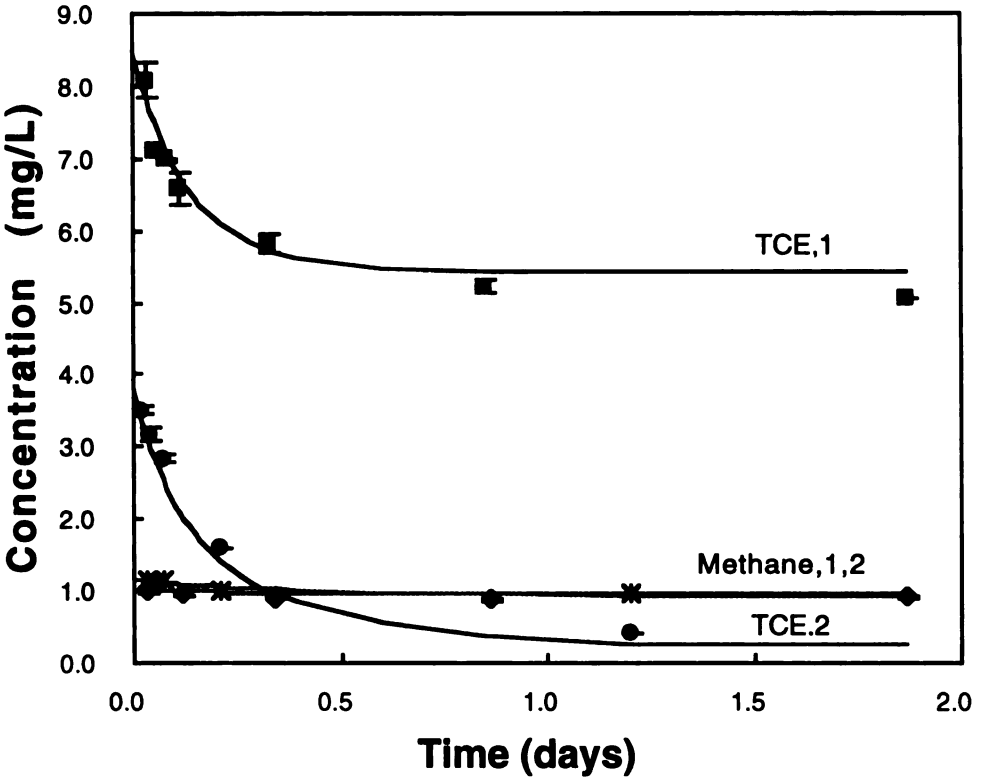


Figure 4.4 Biotransformation of TCE and methane by methanotrophic mixed culture: comparison with model prediction (for different initial TCE concentration). Error bars indicate the 95% confidence interval for triplicate samples.

(a)

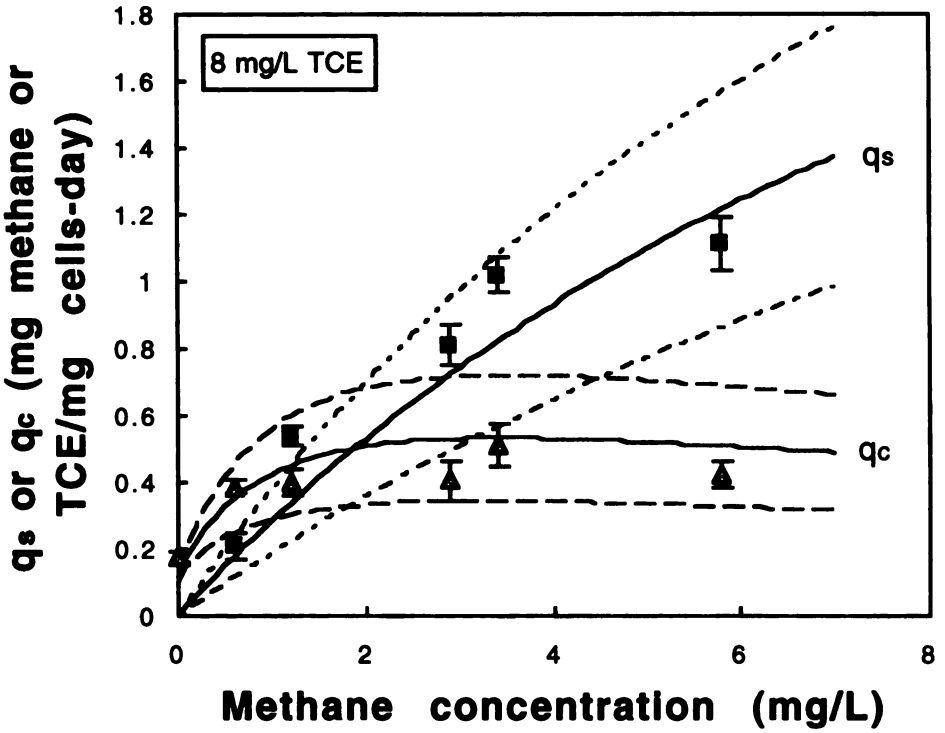


Figure 4.5 The effect of methane concentration on TCE and methane degradation rate when both substrates are present at same time. (a) Initial concentration of TCE is 8 mg/L and (b) Initial concentration of TCE is 4 mg/L. Error bars indicate 95% confidence interval. Dashed lines indicate error range for the prediction.

(b)

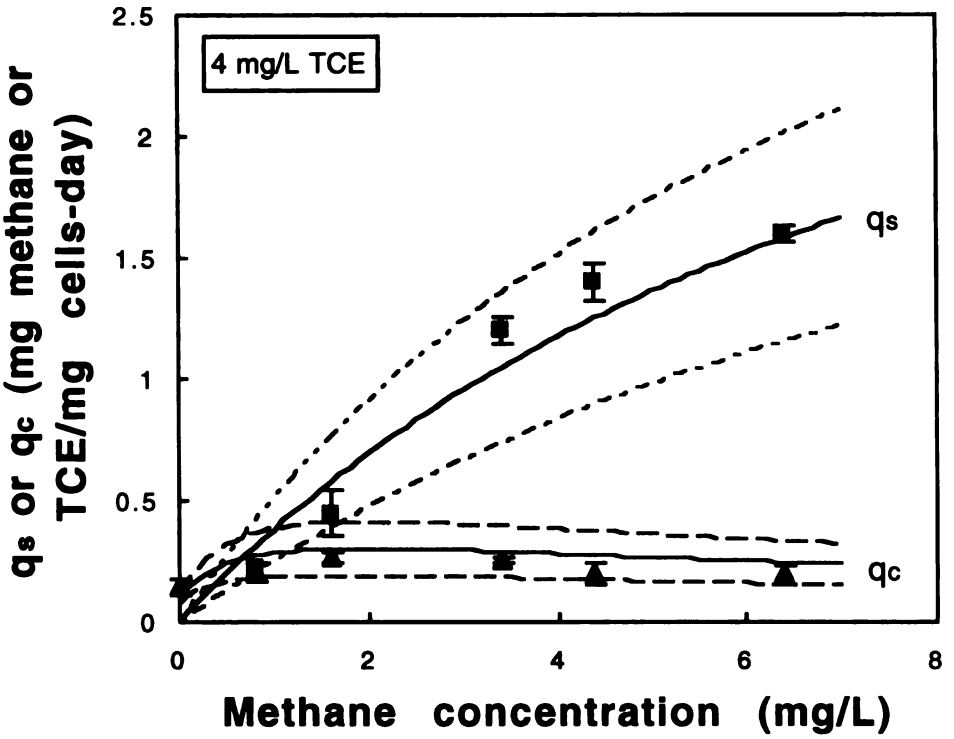


Figure 4.5 The effect of methane concentration on TCE and methane degradation rate when both substrates are present at same time. (a) Initial concentration of TCE is 8 mg/L and (b) Initial concentration of TCE is 4 mg/L. Error bars indicate 95% confidence interval. Dashed lines indicate error range for the prediction.

The effect of nongrowth substrate on stoichiometry of cometabolic transformation was also evaluated. The results (Table 4.2) show that an increase in TCE concentration increases the observed transformation yield. It appears that methane is more efficiently used for transformation of TCE at high concentrations of TCE. This result is confirmed by the results in Table 4.4 and Figure 4.7. For higher ratios of TCE to methane, the cultures exhibited net decay after two days of incubation. Therefore, some minimum level of methane was needed to sustain transformation of TCE. The amount of methane needed to sustain transformation of TCE was related to incubation time in a batch system. Microorganisms can not sustain transformation of TCE if TCE concentration is too high with respect to methane. The stoichiometric ratio for oxygen to methane consumed increases with increasing TCE concentration. Electron flow calculations (Table 4.3) indicate that almost all electrons supplied by methane go to energy generation and TCE transformation with none left for cell synthesis at high TCE concentrations. Under these conditions, utilization of growth substrate is inhibited by transformation of nongrowth substrate, and cell growth can not occur. In Table III the sum of f_s , f_e and f_{co} exceeded 1. This indicates some error in the electron balance assumptions- perhaps due to errors in the assumed biomass formula or, more likely, the assumption that TCE transformation byproducts are not further utilized. Calculation of f_{co} assumed two moles of electrons were required per mole of TCE transformed. In a mixed culture, this assumption is likely to be incorrect.

Inactivation of methane monooxygenase can be caused by the availability of reducing power, endogeneous decay, and transformation of TCE. When there are no external energy sources, a theoretical transformation capacity (T_c) can be computed by correcting for cell inactivation caused by endogenous decay and depletion of reducing power. This is a finite value that is independent of the presence of growth and nongrowth substrate and represents a maximum value that cells can theoretically attain. For this culture, T_c

appeared to be a characteristic value under the specified experimental conditions. Observed transformation capacity values predicted using T_c and b are shown in Figure 4.6. $(T_c)_{obs}$ is much lower than T_c when growth substrate concentration is low. This is caused by the fact that endogeneous decay becomes more significant at low concentration of growth substrate, when toxicity and use of reducing power become less significant. $(T_c)_{obs}$ approaches the theoretical value when the concentration of growth substrate is sufficiently high.

Table IV shows that observed transformation yield decreased with increasing methane concentration. The observed transformation yield is very small compared with the theoretical value (T_y) measured by the model when higher levels of methane are present. Apparently, inhibition of growth substrate plays an important role. Transformation of TCE was seriously inhibited when high levels of methane were present. The effect of inhibition of methane offset its enhancement effects. On the other hand, the observed transformation yield was higher than the theoretical value when trace methane was present. Under this condition, enhanced transformation of nongrowth substrate by growth substrate was dominant and a higher observed transformation yield was attained. Therefore, the theoretical transformation yield represents a theoretical value that the cultures could have and is independent of the effect of growth and nongrowth substrates. The observed transformation yield was a function of concentration of growth and nongrowth substrates, as shown in Figure 4.7: the lower the concentration of TCE, the less significant is the effect of methane on observed transformation yield.

In summary, the model presented here can be used to predict transformation of growth and nongrowth substrate accurately. A systematic method was developed to measure parameters to describe simultaneous degradation of growth and nongrowth substrates. In previous work, a simplified form of the present model (in the absence of growth

substrate) was verified for HFC/HCFC degradation by the same methanotrophic mixed culture (Chang and Criddle 1995). These results suggest that the proposed model can be applied to other cometabolic transformations for a range of concentrations and substrate types.

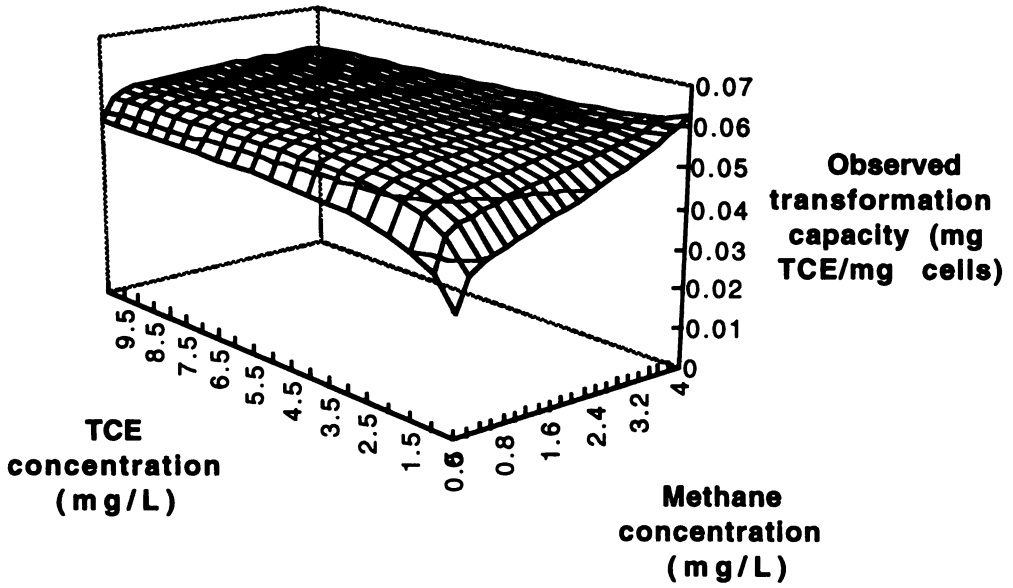


Figure 4.6 The observed transformation capacity as a function of the concentrations of growth and nongrowth substrates. Prediction is based on parameters listed in Table I.

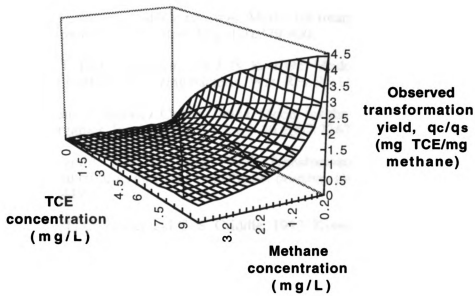


Figure 4.7 The observed transformation yield as a function of the concentration of growth and nongrowth substrates. Prediction is based on parameters listed in Table I.

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CHAPTER 5

THEORY OF ADAPTATION OF COMETABOLIZING COMMUNITIES

INTRODUCTION

Selection in a continuous culture on a limiting carbon source can lead to the development of stable microbial communities. The relative dominance of populations within these communities can be altered by environmental perturbations, such as exposure to nongrowth substrates. Selection among microbial populations usually occurs because of competition between different species or competition between an ancestor and its derived genotypes. As a result of such processes, a new microbial community can develop that is better adapted to its new environment.

For the microbial communities considered in this work, commensal interactions are likely. For such interactions, one species produces compounds which serve as an energy or carbon source for a second species. Such commensal realtionships are often strung together in a chain so that over time a succession of commensal pairs appears. For example, Wilkinson *et al*. (1974) studied the nature of commensal interactions for a microbial community utilizing methane. The community consisted of a methane-utilizing *Pseudomonas* sp., a methanol utilizing *Hyphomicrobium* sp., an *Acinetobacter* sp., and a *Flavobacter* sp. The authors concluded that the *Hyphomicrobium* sp. served to remove the small amounts of methanol which are produced during methane utilization by the *Pseudomonas* sp. and which inhibited its growth. *Acinetobacter* and *Flavobacter* sp. removed the complex products of growth or cell lysis.

The commensal relationship has a twist under cometabolic conditions. The species that are responsible for utilization of growth substrates also transform nongrowth substrates simutaneously. Cometabolizing pure cultures do not mineralize nongrowth substrates and accumulation of transformation products is typical (Henry and Grbić -Galić 1990; Little et al. 1988; Oldenhuis et al. 1989). Not surprisingly, mixed cultures or communities have advantages for mineralization of nongrowth substrates (Uchiyama et al. 1992). Since pure cultures with oxygenase activity typically suffer from product toxicity, the presence of heterotrophs capable of degrading such products may facilitate detoxification, enhancing the growth rate of the community. Thus, selection in cometabolizing environments may favor community structures with higher capabilities for detoxication.

In this chapter, the fitness concept (Lenski et al. 1991) is adapted for evaluation of cometabolism and the changes that occur in a cometabolizing community. The major concept for the model is that changes in a microbial community can be described by a "fitness" parameter which is quantified by changes in the kinetic parameters of the community. The selection gradient for each parameter is defined by the partial derivative of fitness with respect to that parameter. The gradient therefore reflects the direct selection acting on each fitness component, with the other components held constant. The experimental systems selected for investigation in this work is TCE transformation by a methanotrophic mixed culture and by a phenol-degrading community.

MODEL DEVELOPMENT

Adaptive change of microbial communities

In a competitive situation, we are interested in discovering whether a species enjoys a natural advantage. Microorganisms (*r* strategist) with the fastest growth rate should come to dominate when resources are temporarily abundant, since by virtue of their rapid growth, they will be able to utilize more of the limiting factor than the slower-growing organism. *K*

strategists, which reproduce more slowly than *r* strategist, tend to be successful in resource-limited situations (Andrews and Hall 1986). However, considerations should also be given to application of this concept for mixed cultures, consortia, and communities where interactions other than simple competition are important.

For simple communities fed a single substrate, one or a few species are predominant. Intermediates from oxidation of the growth substrate or products of decay of the dominant species sustain other species within the community. Since the whole community is interconnected by amensual or mutualistic relationships, we assume that the whole community can be viewed as a single population with a single phenotype. Under this assumption, phenotypic changes during exposure to nongrowth substrate will be expressed as changes in the phenotype of the whole community. The Malthusian parameter for a specific species over any time period t is given by (Lenski et al. 1991).

$$m = \ln(N / N_0) / t \qquad (1)$$

Where N_0 and N are initial and final cell number during a growth period, respectively. We assume that the above definition can be applied to a whole community derived from a single growth substrate:

$$m = \ln(X / X_0) / t \qquad (2)$$

Where X_0 and X are initial and final cell density, respectively.

The fitness, W of a derived community relative to the ancestor community is expressed as the ratio of their respective Malthusian parameters :

$$W = \frac{m}{m_0} \tag{3}$$

For any short period, the specific growth rate, μ is a constant . Fitness at any instant is then expressed as:

$$W = \frac{m}{m_0} = \frac{\mu}{\mu_0} \tag{4}$$

For a continuous culture, the specific growth rate, μ is the actual Malthusian parameter. Generally, we can measure the concentrations of growth and nongrowth substrate, and kinetic parameters to obtain μ at any instant. The specific growth rate prior to TCE exposure is μ_0. The selection gradient for any trait P is the partial derivative of fitness with respect to that trait, $\partial W / \partial P$. The gradient therefore reflects the direct selection acting on each fitness component, with the other components held constant. To facilitate comparison among selection gradients for the several fitness components, gradients are normalized to create dimensionless quantities that reflect the proportional sensitivity of fitness to each component (Vasi et al. 1994):

$$G_P = \left(\frac{P}{W}\right)\left(\frac{\partial W}{\partial P}\right) \tag{5}$$

Component fitness (fitness contributed by the change of a trait) can be calculated from :

$$\Delta W_n = (\frac{\partial W}{\partial P_n})\Delta P_n \tag{6}$$

Therefore, the change of fitness for a derived community can be expressed as:

$$\Delta W = \sum_{n=1}^{n} \frac{\partial W}{\partial P_n} \Delta P_n \qquad (7)$$

The above equation is assumed to express the development of the community. In principle, changes in community structure can be quantified in this way.

Fitness of cometabolizing cultures

For the case where growth substrate utilization and cometabolic transformation occur in separate stages, growth phase change is described by:

$$\mu = \frac{\mu_m S}{K_s + S} = \frac{Y_m k_s S}{K_s + S} \qquad (8)$$

and

$$W = \frac{m}{m_0} = \frac{\mu}{\mu_0} \qquad (4)$$

Where W=1 for the ancestor community and W=∞ for the theoretical maximum fitness in the community ($\mu_m = \infty$).

During the death phase, cell decay occurs as a result of endogenous decay and transformation of nongrowth substrate (Chapter 3):

$$\mu = -b - \frac{q_c}{T_c} \qquad (9)$$

and

$$q_c = \frac{k_c C}{K_c + C} \qquad (10)$$

In order to have a consistent trend in fitness over periods of net growth and net decay, a

modified definition of fitness and selection gradient is proposed:

$$W = \frac{\mu}{|\mu_0|} \tag{11}$$

$$G_x = \left(\frac{P}{|W|}\right)\left(\frac{\partial W}{\partial P}\right) \tag{12}$$

Where W=-1 for the ancestor community and W=0 for the maximum fitness of the community ($b = 0$ and $q_c / T_c = 0$)

For cometabolism in the presence of growth substrate, the following equations provide a complete mathematical description of the specific growth rate and the specific rates of utilization of growth and nongrowth substrates throughout the growth and the decay periods (Chapter 4).

$$q_s = k_s\left(\frac{S}{K_s\left(1+\frac{C}{K_{ic}}\right)+S}\right) \tag{13}$$

$$q_c = (T_y q_s + k_c)\left(\frac{C}{K_c\left(1+\frac{S}{K_{is}}\right)+C}\right) \tag{14}$$

$$\mu = Y_m q_s - b - \frac{q_c}{T_c} \tag{15}$$

and the definition of fitness given by Eq. (11) can be used for all cases.

$$W = \frac{\mu}{|\mu_0|} \tag{11}$$

Where W=1 or - 1 for the ancestor community and W=∞ for the theoretical maximum fitness in the community ($\mu_m = \infty$, b=0 and $q_c / T_c = 0$).

Fitness in a cometabolizing community can begin with 1 or - 1 for the ancestor community depending upon whether there is sufficient growth substrate to support growth. Subsequently, fitness of the community can evolve throughout the whole 'fitness space'.

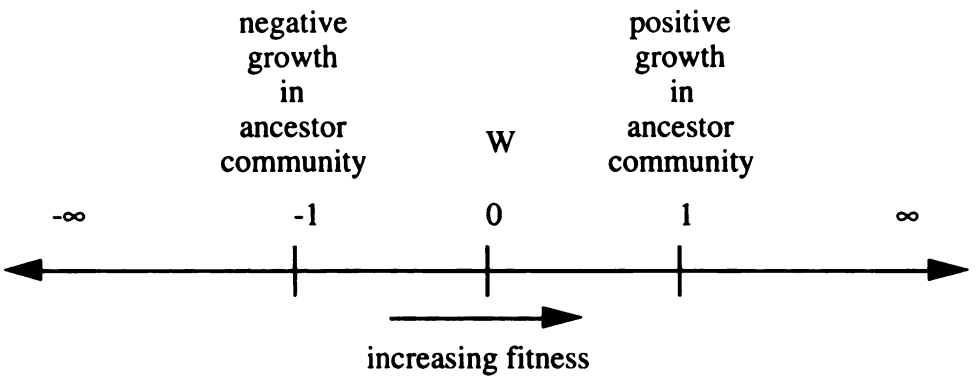


Figure 5.1 Fitness space of cometabolizing community

Selection gradients

The selection gradient is a normalized parameter defined so as to indicate the relative effects of different fitness components on fitness and to enable comparison of selection gradients for several fitness components. The definition of selection gradient has been shown in Eq. (5). Selection gradients with respect to μ_m and K_s can be derived for growth phase using Eq. (8):

$$G_P = \left(\frac{P}{W}\right)\left(\frac{\partial W}{\partial P}\right) \tag{5}$$

$$G_{\mu_m} = \left(\frac{\mu_m}{W}\right)\left(\frac{\partial W}{\partial \mu_m}\right) = 1 \tag{16}$$

$$G_{K_S} = (\frac{K_S}{W})(\frac{\partial W}{\partial K_S}) = \frac{-K_S}{K_S + S} \tag{17}$$

The change of fitness for the derived community can be expressed as:

$$\Delta W = \frac{\partial W}{\partial \mu_m}\Delta\mu_m + \frac{\partial W}{\partial K_S}\Delta K_S \tag{18}$$

Selection gradients for μ_m and K_s are zero when there is no growth substrate. Selection gradients for b, q_c and T_c can then be derived as follows:

$$G_b = (\frac{b}{|W|})(\frac{\partial W}{\partial b}) = \frac{-b}{b + q_c / T_c} \tag{19}$$

$$G_{q_c} = (\frac{q_c}{|W|})(\frac{\partial W}{\partial q_c}) = \frac{-q_c / T_c}{b + q_c / T_c} \tag{20}$$

$$G_{T_c} = (\frac{T_c}{|W|})(\frac{\partial W}{\partial T_c}) = \frac{q_c / T_c}{b + q_c / T_c} \tag{21}$$

The change of fitness for the derived community can be expressed as:

$$\Delta W = \frac{\partial W}{\partial b}\Delta b + \frac{\partial W}{\partial T_c}\Delta T_c + \frac{\partial W}{\partial q_c}\Delta q_c \tag{22}$$

Selection within a community is more complex when both growth and nongrowth substrates are present at the same time. However, referring to Eq. (13), (14), and (15), the change of fitness for the derived community can be expressed as follows:

$$\Delta W = \frac{\partial W}{\partial Y_m}\Delta Y_m + \frac{\partial W}{\partial b}\Delta b + \frac{\partial W}{\partial T_c}\Delta T_c + \frac{\partial W}{\partial T_y}\Delta T_y + \frac{\partial W}{\partial k_s}\Delta k_s + \frac{\partial W}{\partial k_c}\Delta k_c$$
$$+ \frac{\partial W}{\partial K_s}\Delta K_s + \frac{\partial W}{\partial K_c}\Delta K_c + \frac{\partial W}{\partial K_{ic}}\Delta K_{ic} + \frac{\partial W}{\partial K_{is}}\Delta K_{is} \qquad (23)$$

The effect of fitness components on fitness can be deduced from Eq. (16), (17), (19), (20), and (21). Fitness can increase if maximum specific growth rate, μ_m and theoretical biomass transformation capacity, T_c increase. Alternatively, fitness decreases if half-saturation coefficient of growth substrate, K_s, endogenous decay constant, b and specific rate of utilization of nongrowth substrate, q_c increase. These predictions are summarized in Table 5.1.

Table 5.1. The effects of fitness components on fitness

Increase of fitness components	Effects on fitness,W
maximum specific growth rate, μ_m	Increase
half-saturation coefficient of growth substrate, K_S	Decrease
endogenous decay constant, b	Decrease
specific rate of utilization of nongrowth substrate, q_c	Decrease
Theoretical biomass transformation capacity, T_c	Increase

Implications of theory for different model systems

Several model systems have been considered for the evaluation of cometabolism. In most cases, both growth and nongrowth substrates are introduced into the model systems. Thus, the interactions between both substrates are important. The presence of toxic transformation products can be expected to have some impacts on the microbial community structure during long-term TCE exposure. These factors will have different effects in simple mixed

cultures compared to complex communities and in batch reactors compared to continuous reactors. In sequencing batch reactors (SBRs), for example, the microbial community is exposed to a range of growth and nongrowth substrate concentrations. This may select for a more diverse community with "specialist" organisms that occupy variety of niches created by substrate concentration gradients. In contrast, a chemostat favors selection of specific populations at a fixed specific growth rate. This can be expected to result in a less diverse culture. Since nongrowth substrates are not mineralized by cometabolizing species and since heterotrophs are known to play important roles in detoxification, more diverse cultures should have advantages for cometabolism.

In this work, two model communities are evaluated, representing different extremes in the continuum of community. A methanotrophic mixed culture in a chemostat is used to represent one extreme in the continuum. The phenol-degrading SBR community represents a more complex case. The simple methanotroph community is essentially a consortium, with very limited diversity. Methane utilizers, methanol utilizers, and associated heterotrophs are bound together in a commensal relationship, with methane as the sole source of carbon and energy. By contrast, more complex interactions are likely in the batch-fed phenol-degrading community.

As discussed previously, the selection gradient is a normalized parameter that indicates the effects of different parameters on fitness. This definition may be useful in predicting adaptation of specific systems. For example, the selection gradient with respect to K_s could be -1 and 0 for chemostat and SBR respectively because substrate concentration is low for a chemostat ($S << K_s$) and periodically high for a SBR ($S >> K_s$). It may also be possible to use selection gradients (especially more sensitive ones) as criteria for the stability of a community under different environments or different communities in the same environment. Magnitude of selection gradients for different organisms and different

environments can be compared. Lower values of selection gradients indicate a more stable community for a specified environment.

MODEL ANALYSIS

The experimental systems selected for investigation in this work were TCE transformation by a methanotrophic mixed culture and a phenol-degrading community. The model for cometabolism given by Eq.(13), (14), and (15) was verified previously (chapters 3 and 4). Kinetic parameters for the methanotrophs were listed in Table 4-1. The parameters for phenol-degrading community are provided in chapter 7. As discussed previously, the selection gradient is an important indicator of the relative importance of parameters (fitness components) on fitness. These parameters were evaluated over the ranges of growth substrate (methane and phenol) and nongrowth substrate (TCE) concentration. Selection when both substrates are present at the same time is compared to selection when both substrates are supplied at different times for the methanotrophic mixed culture. The phenol-degrading community was also evaluated when both substrates were present separately. For this case, the phenol-degrading community and the methanotroph culture can be compared. To determine the relative importance of each parameter on the specific rate of transformation of growth and nongrowth substrates, the derivatives of q_c and q_s with respect to related parameters were also evaluated over a range of substrate concentrations.

RESULTS AND DISCUSSION

Selection gradients for the methanotrophic mixed culture when growth and nongrowth substrates are supplied separately is shown in Figure 5.2. Apparently, there is no selection with respect to the inhibition constants. The selection gradients of growth and decay phases (including nongrowth substrate transformation) are evaluated separately. The effects of fitness components are in agreement with predictions in Table 1. Similar results were also

observed for the phenol-degrading community when growth and nongrowth substrates are not supplied simultaneously (Figure 5.3).

Comparing Figures 5.2 and 5.3, both the methanotrophic mixed culture and the phenol-degrading community have the same selection gradient with respect to Y_m and k_s (or μ_m). However, there are some differences for selection gradients with respect to T_c, b , k_c, and K_c. For example, a 100% increase in theoretical transformation capacity (T_c) causes 50% increase of fitness at 2 mg/L TCE for phenol-degrading community. The same TCE exposure would cause 70% increase in fitness for the methanotroph culture. There are similar observations for other parameters. Thus, the phenol-degrading community is more stable than methanotrophic mixed culture based on these criteria. This implies that it may be possible to use selection gradients (especially more sensitive ones) as criteria for the stability of a community under a given perturbation.

Figure 5.4 and 5.5 show selection gradients for each fitness component as functions of TCE and methane concentration when both substrates are present at the same time. Over a wide range of concentrations, K_c and K_{is} are the most sensitive gradients with respect to TCE and methane concentration. The selection gradients are higher at higher substrate concentration for these two parameters. This implies that a smaller change in K_c and K_{is} is needed to attain the same level of change in fitness. As a result, the half-saturation coefficient of nongrowth substrate and inhibition coefficient have more significant effects on fitness at high substrate concentrations. Some other differences can also be observed. The selection gradient for k_s shifts to negative values and the selection gradient for K_s shifts to positive values when both substrates are present at the same time. This indicates that improvements in the transformation of TCE (such as an increase of K_{is} and T_c) become more critical for adaptive changes.

Figure 5.6 to 5.9 also provide a sensitivity analysis for q_s and q_c as a function of TCE and methane concentrations, respectively. The results show that an increase in K_{ic} and a decrease in K_s can increase q_c. However, the effect on q_s is less significant. Compared to fitness with respect to several parameters, q_s and q_c are not so sensitive to substrate concentration and change in parameters.

Various model environments also have different effects on selection. The batch growth environment selects strongly for a higher maximum specific growth rate, with much weaker selection for populations that have higher affinity for substrate. The ratio between selection gradients with respect to μ_m and K_s represents a measure of fitness that can be attributed to these two parameters. Dividing Eq. (16) by (17), the proportional selection gradient is obtained:

$$G_{\mu_m} / G_{K_s} = -\frac{K_S + S}{K_S}$$

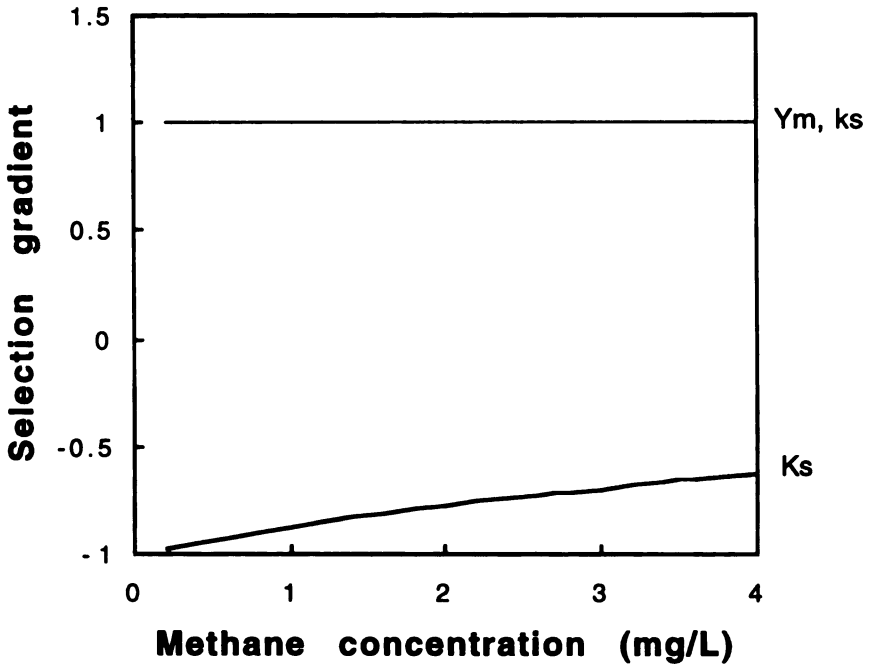
In continuous culture, the dilution rates through the reactor, D, are usually a fraction of the maximum specific growth rate, μ_m. Assuming $\mu = \mu_m S/(K_S + S) = 0.2\mu_m$, the proportional selection gradient for μ_m and K_S is -1.25. By comparison, the proportional selection gradient for μ_m and K_S in a batch reactor can differ by a factor of several hundred(since $S >> K_S$).

In the absence of growth substrate, there is no selection with respect to μ_m and K_S in cultures conducting cometabolic transformations. However, batch cultures are exposed to a higher concentration of nongrowth substrate initially than continuous cultures. Therefore, the selection gradient with respect to q_c is much higher in batch culture since q_c is directly proportional to concentration of nongrowth substrate. However T_c can offset the effect of q_c, so it is difficult to differentiate the selection advantages of batch and continuous

culture. The proportional selection gradient for q_c and T_c is a constant factor ($G_{q_c} / G_{T_c} = -1$). This implies that there is counter selection with respect to q_c and T_c over any range of nongrowth substrate concentration.

In summary, this work extended the fitness concept to describe adaptation of microbial communities. Selection gradient is the key concept underlying a theory for the quantitative description of stability of communities. It appears possible to use selection gradients (especially more sensitive ones) as criteria for the stability of a community under a given perturbation. Communities are likely to exhibit adaptive changes in response to given perturbations (such as, long term nongrowth substrate exposure). A more stable community shows a smaller change in kinetic parameters as a result of a given perturbation. However, a less stable community has a high capacity for change in performance because it is characterized by larger selection gradients. Thus, fitness may be used to track the stability and adaptation of communities under perturbation. An unstable community characterized by high selection gradients and low fitness can be adapted to create a more stable community characterized by low selection gradients and high fitness.

(a)



(b)

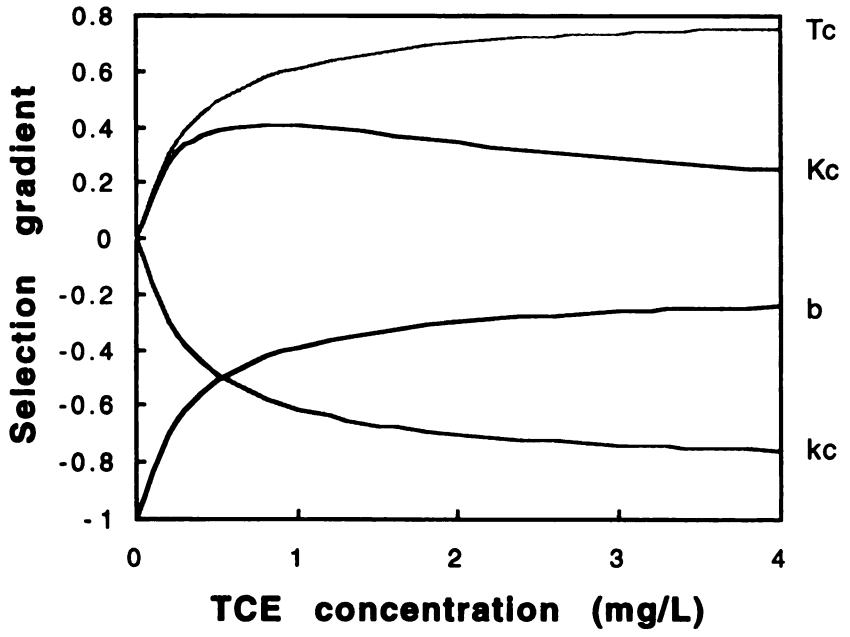
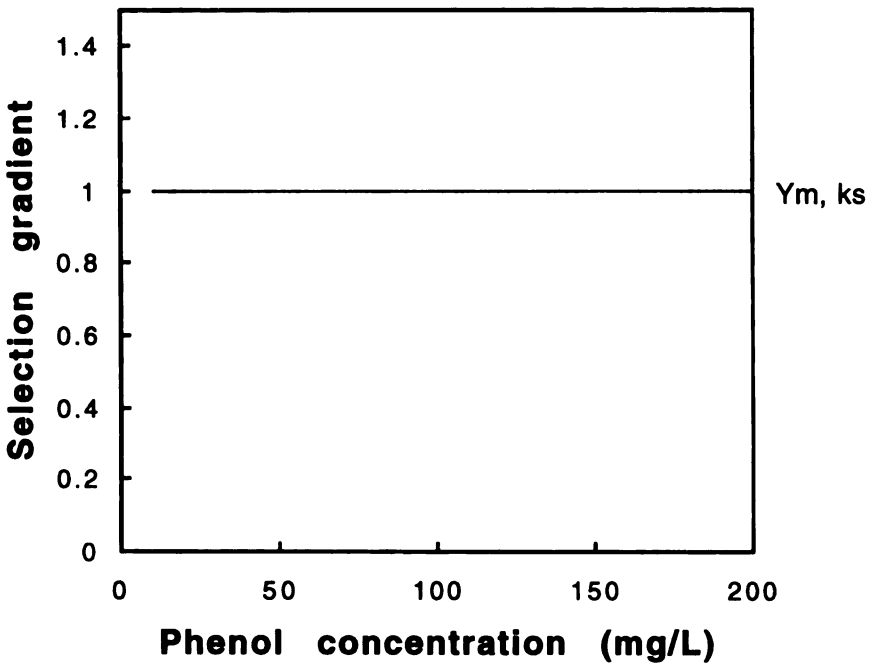


Figure 5.2 Dimensionless selection gradient, (P/W)(∂W/∂P) as a function of methane and TCE concentrations for methanotrophic mixed culture when both substrates are present separately.

(a)



(b)

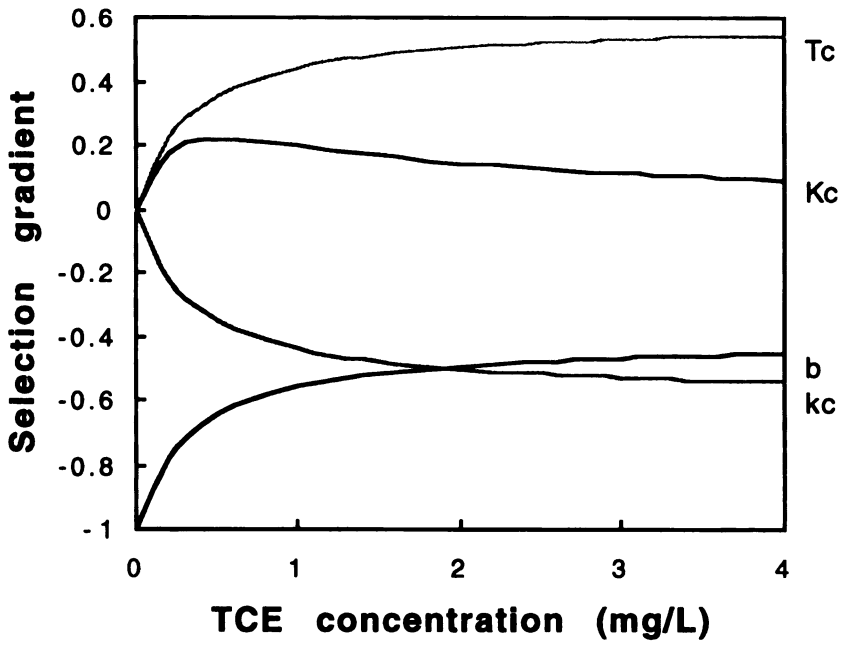
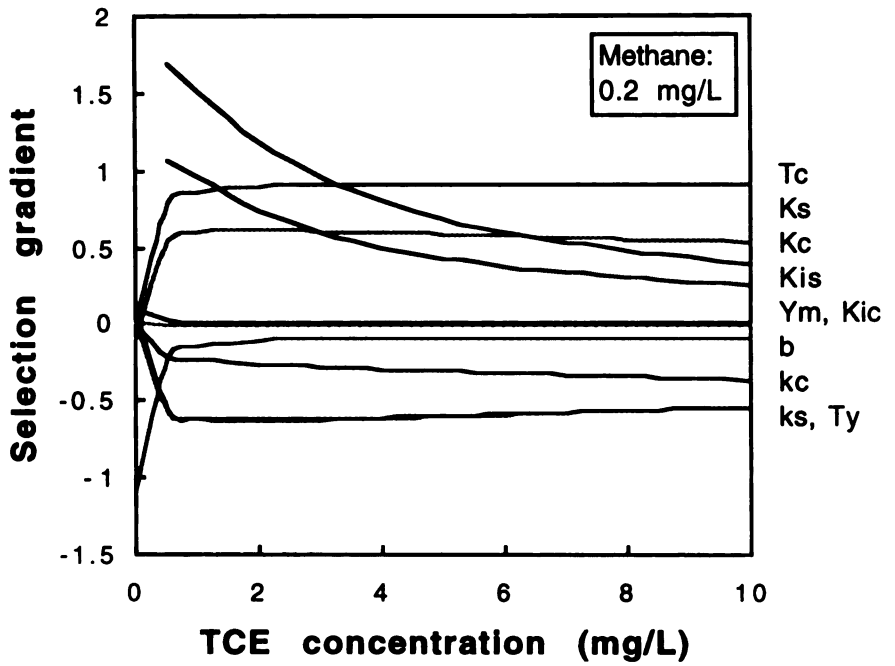


Figure 5.3 Dimensionless selection gradient, (P/W)(∂W/∂P) as a function of phenol and TCE concentrations for phenol degrading culture when both substrates are present separately.

(a)



(b)

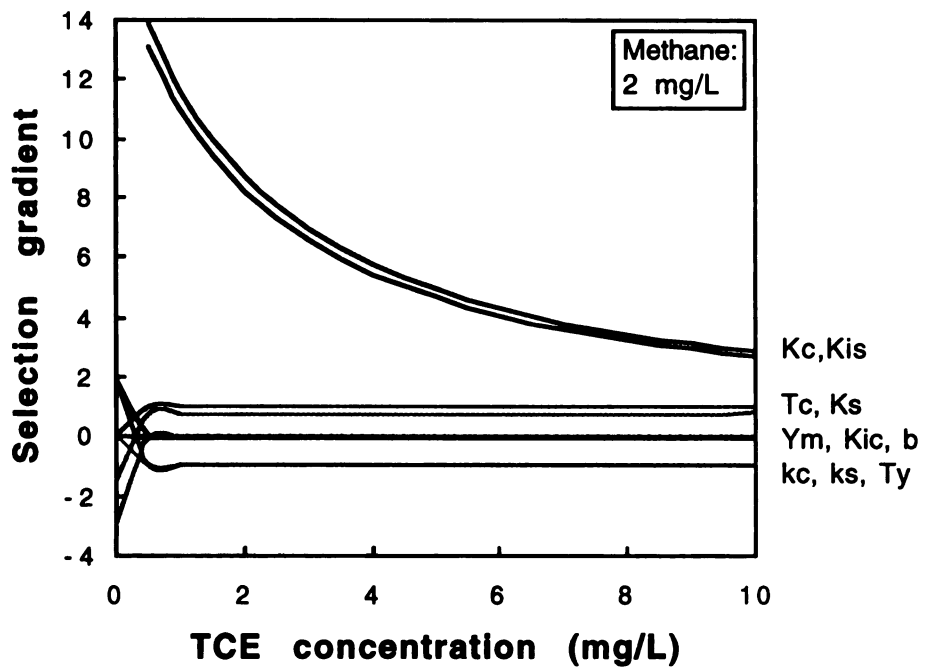
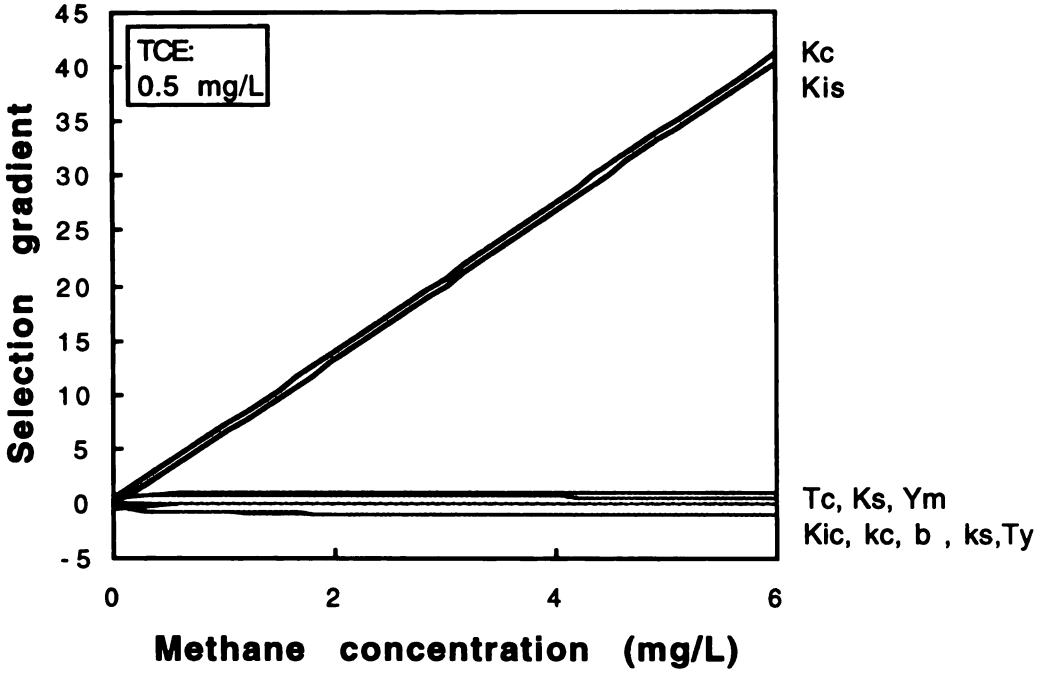


Figure. 5.4 Dimensionless selection gradient, (P/W)(∂W/∂P) as a function of TCE concentration for methanotrophic mixed culture at a specified methane concentration.

(a)



(b)

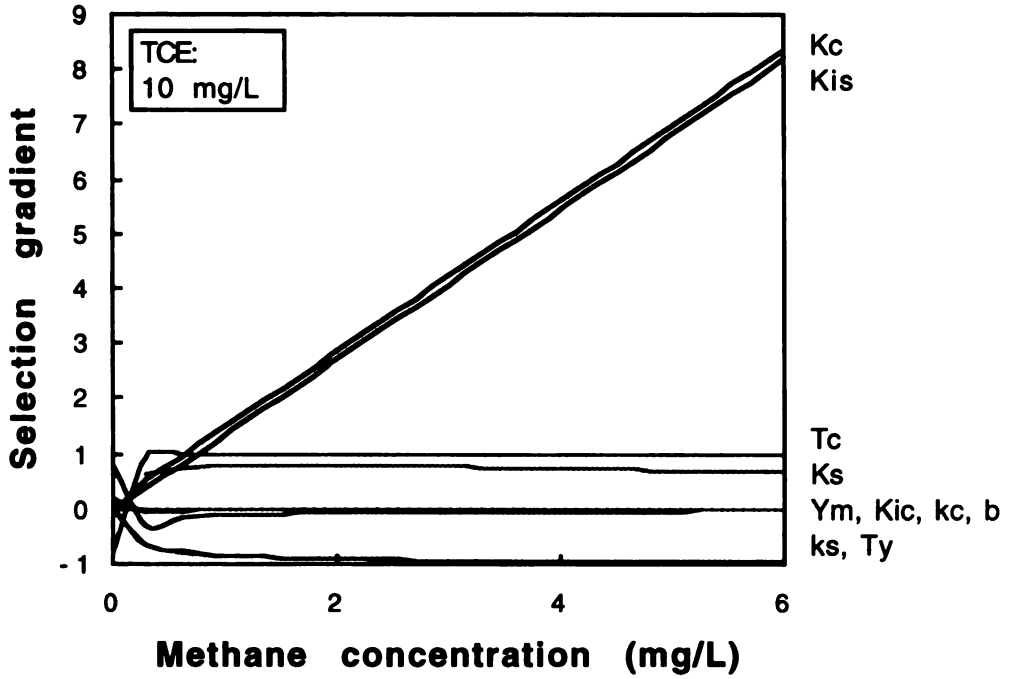


Figure. 5.5 Dimensionless selection gradient, (P/W)(∂W/∂P) as a function of methane concentration for methanotrophic mixed culture at a specified TCE concentration.

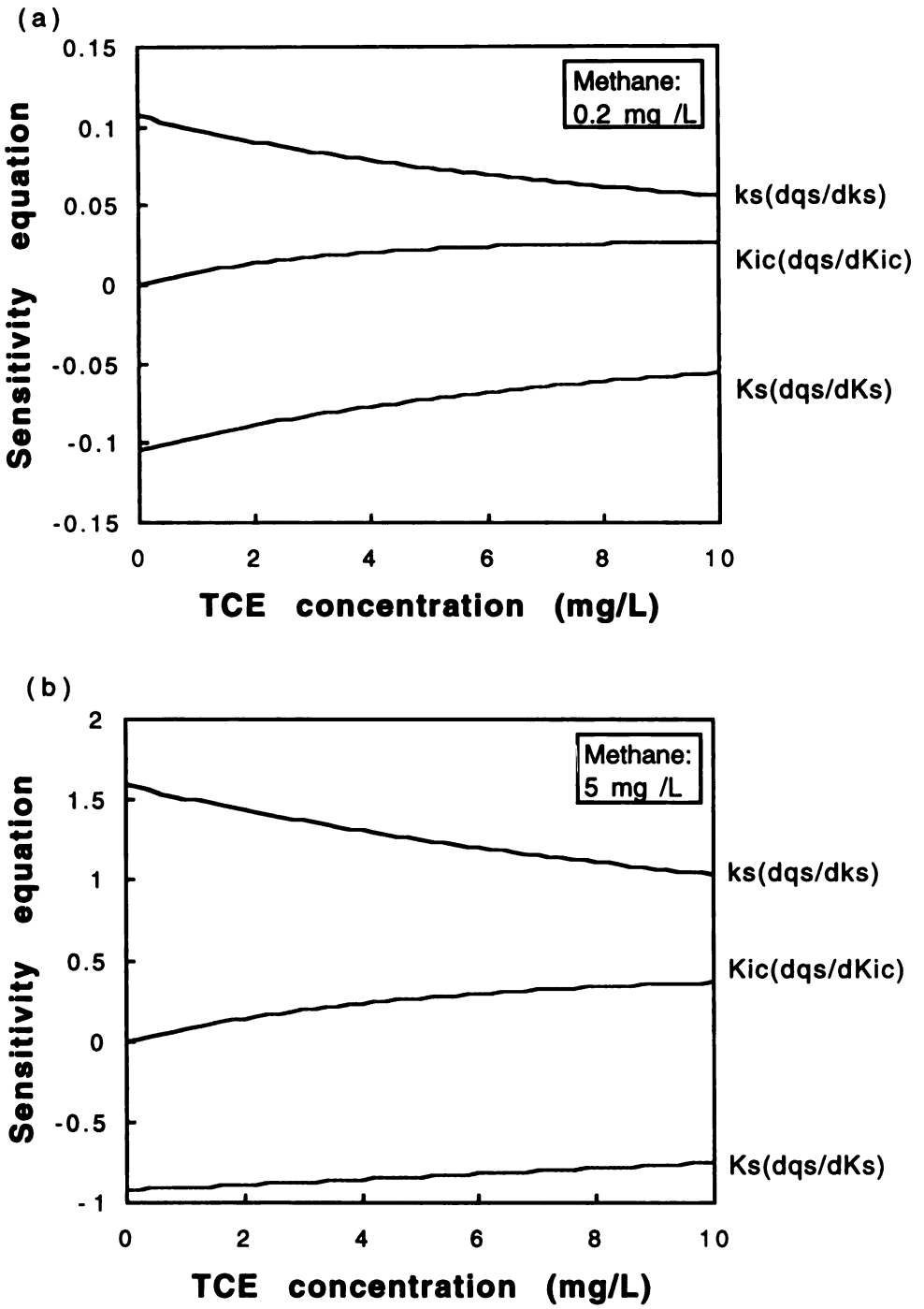


Figure. 5.6 Sensitivity equation for q_s as a function of TCE concentration for methanotrophic mixed culture at a specified methane concentration.

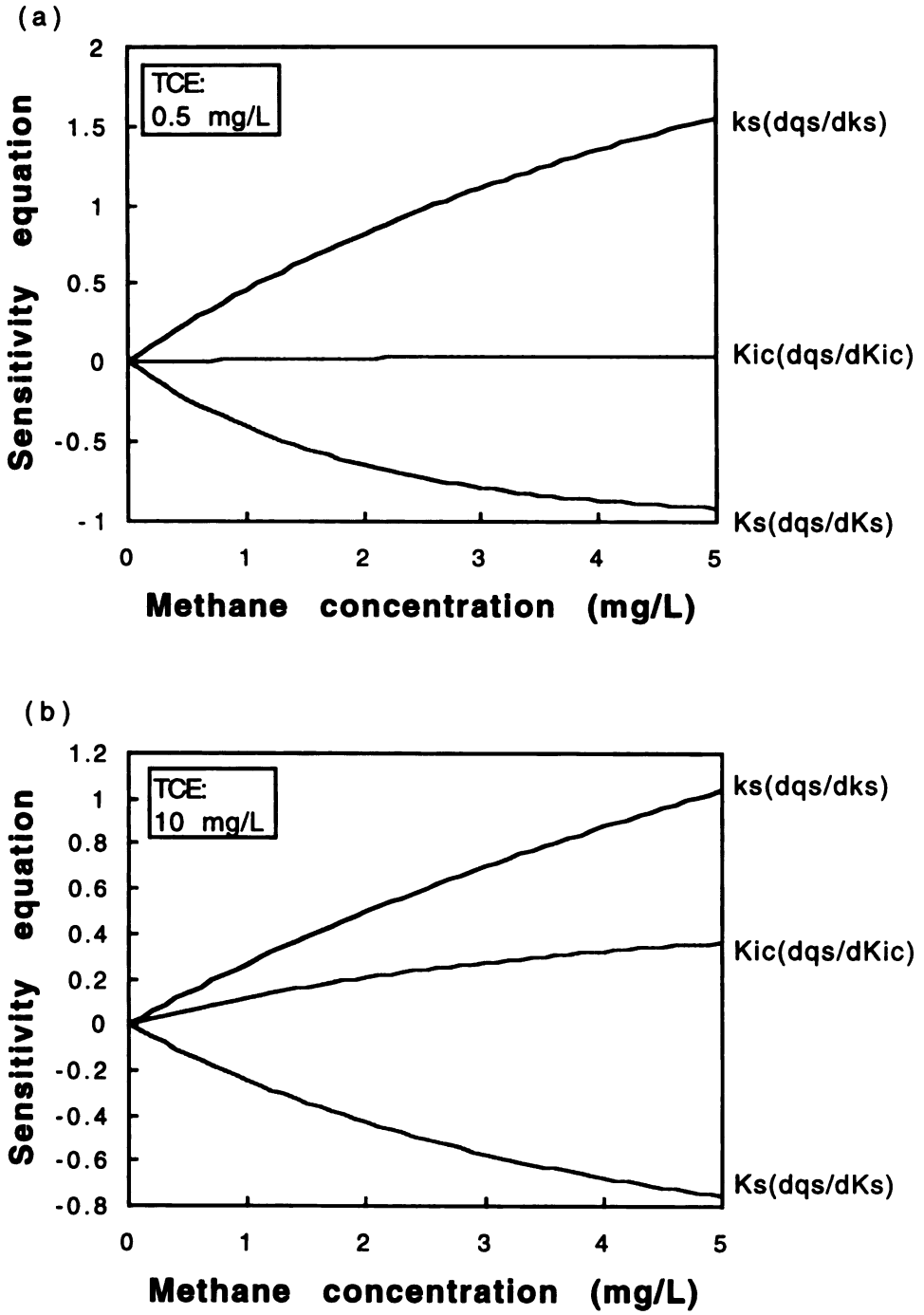


Figure. 5.7 Sensitivity equation for q_s as a function of methane concentration for methanotrophic mixed culture at a specified TCE concentration.

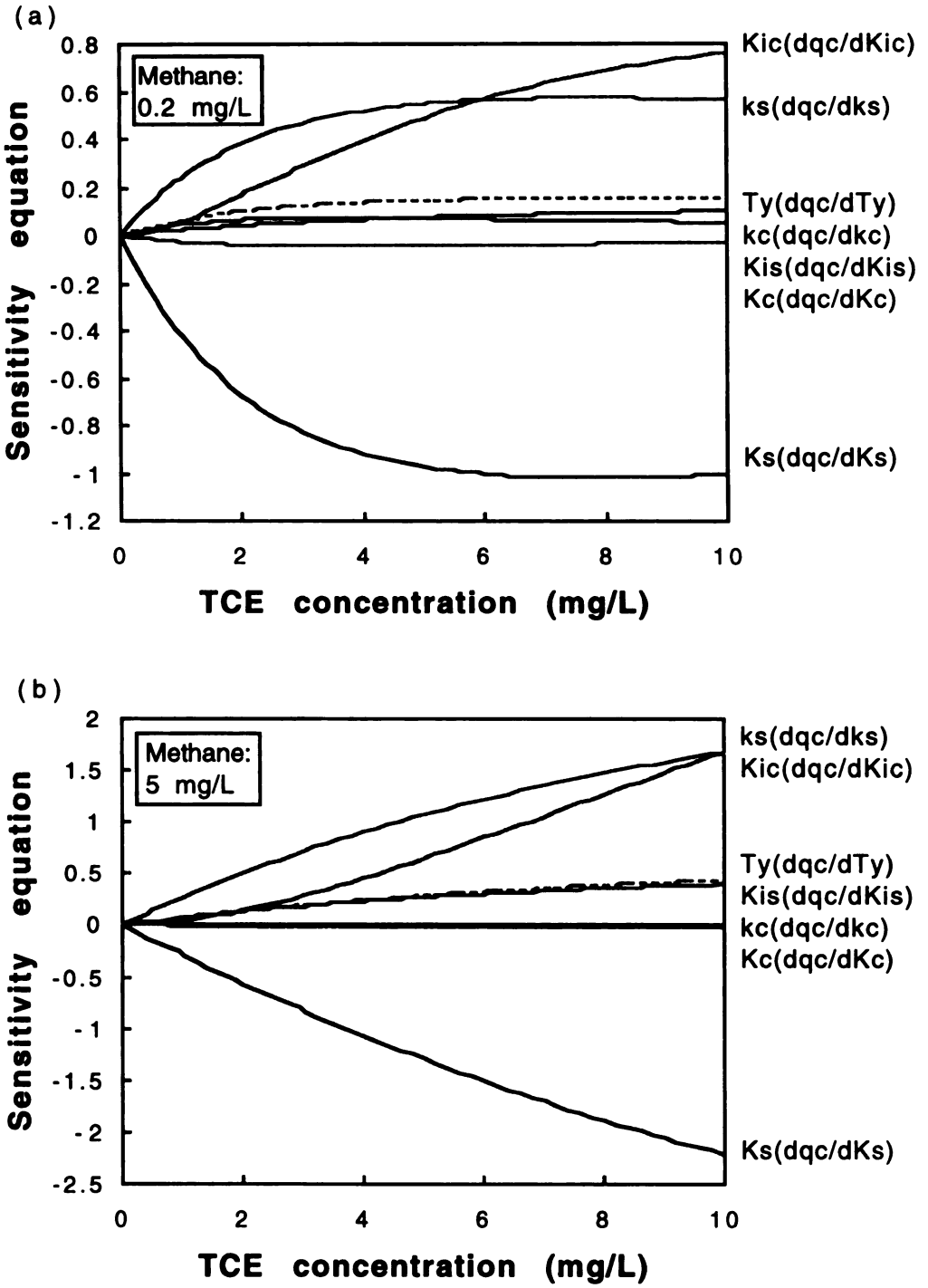


Figure. 5.8 Sensitivity equation for q_c as a function of TCE concentration for methanotrophic mixed culture at a specified methane concentration.

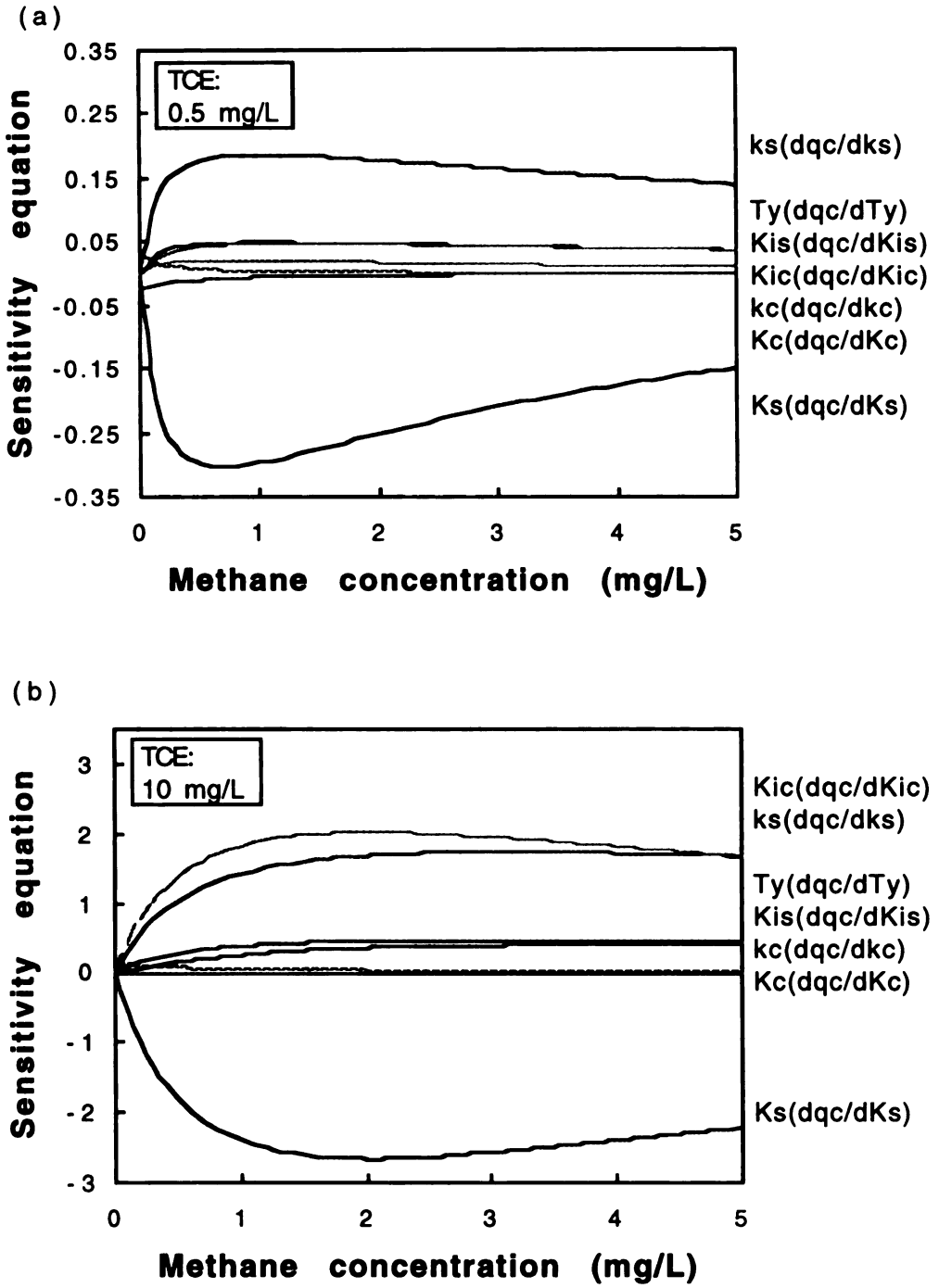


Figure. 5.9 Sensitivity equation for q_c as a function of methane concentration for methanotrophic mixed culture at a specified TCE concentration.

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CHAPTER 6

CHANGE IN COMMUNITY STRUCTURE IN RESPONSE TO LONG TERM TCE EXPOSURE: METHANOTROPHIC MIXED CULTURE IN CHEMOSTAT*

INTRODUCTION

Trichloroethylene (TCE) is widely found in soil and groundwater near industrial sites. In 1985, Wilson and co-workers reported on the possibility of aerobic oxidation of TCE by soil microorganisms with natural gas as the primary energy source (Wilson and Wilson 1985). Since then, the ability of methane-utilizing bacteria to cometabolize TCE has been reported and confirmed by several researchers (Fliermans et al. 1988; Fogel et al. 1986; Little et al. 1988). This work was concentrated on the kinetics (Alvarez-Cohen and McCarty 1991c; Anderson and McCarty 1994; Chang and Alvarez-Cohen 1995a; Criddle 1993; Folsom et al. 1990; Strand et al. 1990) and pathways of degradation (Fliermans et al. 1988; Fogel et al. 1986; Fox et al. 1990; Little et al. 1988; Nakajima et al. 1992; Newman and Wackett 1991; Oldenhuis et al. 1989). To date, few researchers have investigated changes in community structure and performance in response to long-term TCE exposure.

Wilkinson *et al*. (1974) evaluated a stable mixture of four bacterial species in continuous culture with methane as the sole carbon source. The community consisted of a methane-utilizing *Pseudomonas* sp., a methanol utilizing *Hyphomicrobium* sp., and, in addition, an

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* The genetic analyses described in this study performed with the assistance of Dr. Denise Searles

Acinetobacter sp. and a *Flavobacter* sp. The *Pseudomonas* was the only species that could utilize methane as a carbon and energy source, and it constituted the dominant member of the community (ca. 90% of the biomass). The authors concluded that *Hyphomicrobium* sp. removed small amounts of methanol produced during methane utilization by the *Pseudomonas* sp.. Methanol was believed to be inhibitory to the *Pseudomonas* sp.. The *Acinetobacter* and *Flavobacter* sp. apparently removed complex products generated during cell growth or lysis.

Recent studies indicate that TCE transformation capacity is not only a function of the availability of reducing power, but also of the specific cometabolized compound and the toxicity of its transformation products (Alvarez-Cohen and McCarty 1991a; Henry and Grbić -Galić 1991a; Wackett and Householder 1989). Formate addition resulted in increased initial specific TCE transformation rates and elevated transformation capacity. Significant declines in methane conversion rates following exposure to TCE were observed for both resting and formate-fed cells, suggesting toxic effects caused by TCE or its transformation products (Alvarez-Cohen and McCarty 1991a; Alvarez-Cohen and McCarty 1991b). Only a few researchers have examined on the toxicity of transformation products. Oldenhuis and co-workers suggested that TCE epoxide can be expected to bind covalently to proteins and nucleic acids. Other possible reactive metabolites that might bind irreversibly are chloral, dichloroacetyl chloride, and formyl chloride (Oldenhuis et al. 1991). Organisms capable of degrading a large amounts of TCE should possess active detoxification systems for these compounds.

The accumulation of stable TCE breakdown products in methanotroph pure cultures indicates that methanotrophic bacteria alone are not be able to mineralize TCE completely (Henry and Grbić -Galić 1990; Little et al. 1988; Oldenhuis et al. 1989).Some research has shown that methanotrophic mixed cultures are advantageous for mineralization of TCE.

Since methanotrophs suffer from product toxicity when transforming TCE, heterotrophs that degrade the toxic products may play an important role in detoxification. Heterotrophic bacteria in methanotrophic mixed cultures apparently can degrade most of the water-soluble TCE breakdown products, decreasing levels of water-soluble radiolabel and increasing production of $^{14}CO_2$ (Little et al. 1988). Futhermore, Uchiyama and co-workers reported that a heterotrophic bacterium isolated from a methanotrophic mixed culture, *Xanthobacter autotrophicus*, can oxidize dichloroacetic and glyoxylic acid completely and can reduce trichloroacetic acid to lower levels. These results indicate that heterotrophic bacteria play an important role in TCE degradation (Uchiyama et al. 1992).

The presence of toxic transformation products can be expected to have some impacts on the development of microbial communities during long-term TCE exposure. Changes in the populations are likely related to the level of TCE exposure, turnover of transformation products and utilization of growth substrate. Lackey *et al* . (1994) used total-recycle expanded-bed bioreactors to evaluate the degradation potential of TCE by a microbial consortium. Ester-linked phospholipid fatty acid profiles (PLFAME) were used to monitor the change of TCE-affected community during short-term perturbation. The results showed that a propane-utilizing bacterial biomaker increased as TCE was degraded and propane consumed. However, the relationship between community structure and extent of TCE exposure was not clear for these short-term exposures.

Several genetic analysis techniques have been developed for identification of methanotrophic bacteria. Tsuji *et al* . (1990) demonstrated that it is possible to distinguish and classify methanotrophic bacteria using 16S rRNA sequence analysis. Another report described the use of PFGE (pulsed-field gel electrophoresis) -restriction fragment length polymorphisms and the use of a cloned DNA fragment carrying the component B gene to detect soluble MMO genes from methanotrophs on Southern blots prepared from gels on

which large DNA restriction fragments were separated by PFGE. This technique, when combined with fluorescence-labeled oligodeoxynucleotide signature probes and Western blot analysis, enabled characterization of methanotrophs and detection of methanotrophs that synthesize soluble methane monooxygenase (MMO) (Tsien and Hanson 1992). More recently, PCR primers specific for four of the five structural genes in the soluble MMO gene clusters for several methanotrophs were used to amplify specific DNA sequences for direct detection of methanotrophs in natural environments (McDonald et al. 1995).

Molecular biology techniques offer new opportunities for the analysis of the structure and species composition of microbial communities. Some approaches obtain information about microbial communities directly without the need for sequencing. Amplified Ribosomal DNA Restriction Analysis (ARDRA) or Restriction fragment length polymorphism provides a fingerprint of the microbial community under study(Martinez-Murcia et al. 1995; Massol-Deya et al. 1995).If the rDNA fingerprints for individual bacteria in a community are sufficiently different, then one can examine the amplified products for a series of distinct patterns resulting from the different populations that make up the community. Another technique is based on the separation of PCR-amplified fragments of genes coding for 16S rRNA, all the same length, by denaturing gradient gel electrophoresis (DGGE). DGGE analysis of different microbial communities demonstrated the presence of up to 10 distinguishable bands in the separation pattern, which were likely derived from 10 different species constituting these populations. It is possible to identify constitutes which represent only 1% of the total community with this technique (Muyzer et al. 1993). These methods could be used for a quick assessment of genotypic changes over time or between different location reflecting different environmental conditions.

In this study, a methanotrophic mixed culture was exposed to different levels of TCE for extended time periods. A chemostat was chosen as the model environment for the

methanotrophic mixed culture. Changes in the consortia were monitored and the effects of TCE exposure on community structure were evaluated. Various phenotypic parameters were also monitored. Community analyses were also conducted to monitor shifts in microbial community structure. Changes in the community were analyzed using the fitness theory presented in chapter 5.

MATERIALS AND METHODS

Culture conditions

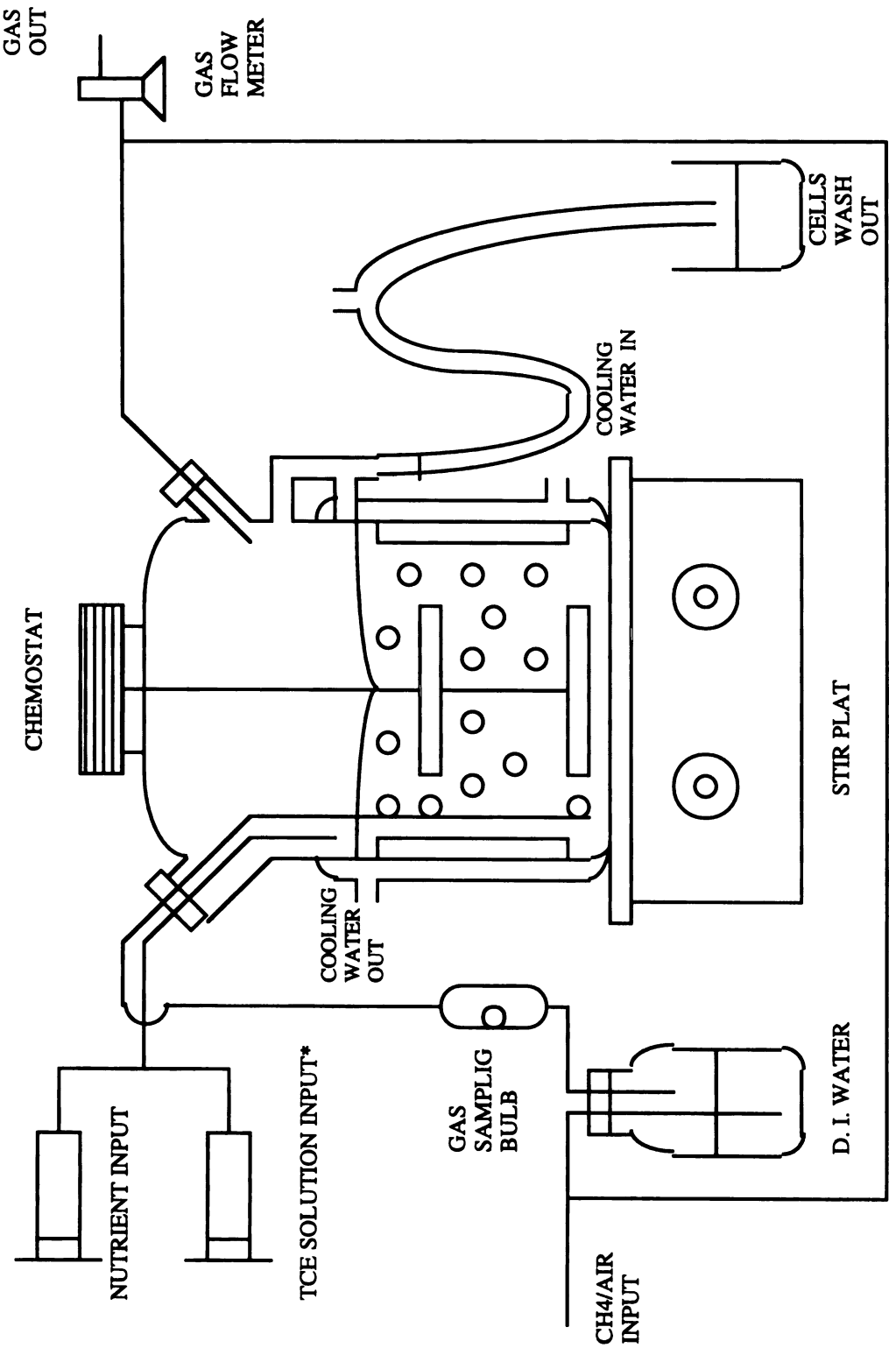
A methanotrophic enrichment obtained from aquifer material at Moffett Field, California, was used for these experiments. This culture is a stable consortium consisting of one methanotroph, one hyphomicrobium, and several heterotrophs containing Gram-negative thin and fat rods as well as some Gram-positive rods and cocci. The methanotroph in the mixed culture expresses soluble MMO similar to that of *Methylosinus trichosporium* OB3b under similar growth conditions (Henry and Grbić -Galić 1991a).

Mixed culture MM1 was grown in Whittenbury Mineral Medium containing (per liter of deionized water): 1.0 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of KNO_3, 276 mg of $CaSO_4 \cdot 2H_2O$, 3.8 mg of FeEDTA, 0.5 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg of $FeSO_4 \cdot 7H_2O$, 0.4 mg of $ZnSO_4 \cdot 7H_2O$, 0.02 mg of $MnCl_2 \cdot 4H_2O$, 0.05 mg of $CoCl_2 \cdot 6H_2O$, 0.01 mg of $NiCl_2 \cdot 6H_2O$, 0.015 mg of H_3BO_3, 0.25 mg of EDTA, 260 mg of KH_2PO_4, and 330 mg of Na_2HPO_4. One liter of culture was grown at room temperature (~21°C) in a continuously stirred 2-liter bottle supplied 30% methane in air at 68 mL/min. Growth curves were monitored and as stationary phase approached, approximately 10 mL of culture was transferred to a 1 liter of fresh Whittenbury Medium. Cells were harvested in mid-log growth phase for inoculation of two chemostats.

Perturbation of TCE

Possible feed modes for TCE peturbation are pulse input and step input. Pulse input should be able to adapt consortia for enhanced transformation of TCE. After exposure to TCE for a period of time, the consortia is allowed to recover and grow back. Alternatively, a step input can be used to evaluate the impact of toxicity of TCE transformation on the community. In this study, an input mode combining the two basic modes was used. TCE was injected in an impulse way but with step increases of the TCE level for more extended periods of time .

Duplicate chemostats were seeded with the methanotrophic mixed culture taken from the batch reactor. The set-up of chemostats is shown in Figure 6.1. After the reactors had grown to steady state, one was injected with TCE solution. TCE exposure was continuous over a finite period, halted to allow recovery of the consortia, and then reintroduced for another period at a higher concentration with another recovery period. This pattern was repeated ten times (Figure 6.2). The operating conditions for the chemostats are summarized in Table 6.1.



* w/o TCE input for control chemostat

Figure 6.1. Chemostat for TCE Transformation by a Methanotrophic Mixed Culture

Table 6.1. Operation conditions for chemostat

Conditions in chemostats	Values
pH	6.8(input) 8.2(output)
Liquid volume	1650 mL
Gas feed,	
methane	30 mL/min
air	270 mL/min
Liquid feed	
Nutrient feed	120 mL/day
TCE loading.*	Impluse, 6 - 18 mg/day @ 50 - 150 mg/L in feed
Hydraulic retension time	13.75 days
Solid retension time	13.75 days

* w/o TCE input for control chemostat

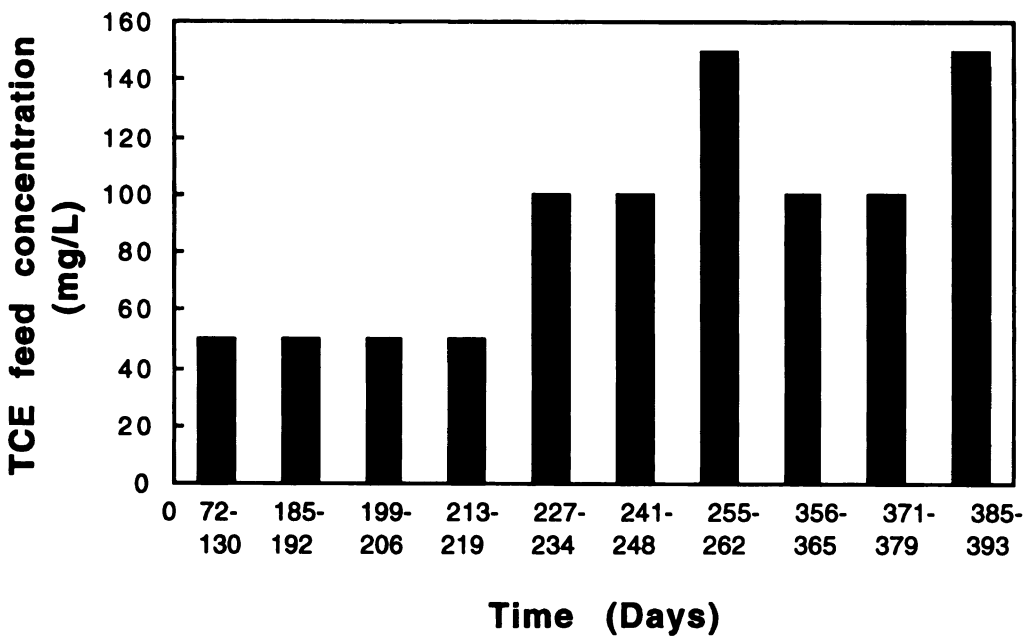


Figure 6.2. TCE feed concentration for long-term TCE exposure experiment in chemostate

To maintain a constant cell density in the chemostat, the higher TCE exposure should correspond to a longer period of cell recovery. During TCE exposure, the following relationship was assumed to be valid:

$$(T_c)_{obs} = \frac{\Delta M_c}{V_L \Delta X} = \frac{\frac{\Delta M_c}{t_c} t_c}{V_L \Delta X} = \frac{F_L C_0 t_c}{V_L \Delta X} = \frac{F_L C_0 t_c}{\Delta M_b}$$

Where $(T_c)_{obs}$ is the observed transformation capacity (mgTCE/mg cells), M_c is the mass of TCE (mg), t_c is the length of period of TCE exposure in impulse input (days), M_b is the mass of cells (mg), X is the cell density in chemostat (mg/L), V_L is the liquid volume in chemostat (L), F_L is the liquid feed rate (mL/day), C_0 is TCE concentration in liquid feed solution (mg TCE/L).

When TCE is absent, the continuing input of methane contributes to the growth of cells. Under steady state, the specific growth rate is equal to the dilution rate, μ=D. The net rate of cell mass growth is μX, then the amount of cell growth within this period t_g is

$$\Delta M_b = \mu X V t_g$$

At steady state, the cells consumed in the exposure period are equal to those produced during the growth period:

$$\frac{F_L C_0 t_c}{(T_c)_{obs}} = \mu X V t_g$$

Rearranging the above equation, the following expression is derived:

$$\frac{t_g}{t_c} = \frac{F_L C_0}{(T_c)_{obs} \mu X V}$$

The above equation relates TCE exposure period, t_c to cell growth period, t_g. This derivation supplies a theoretical basis for pulse perturbations in a chemostat.

Monitoring of consortia

From the method described above, TCE was introduced into the reactor, to encourage the change of consortia that resist toxic products or transform TCE by a different transformation pathway. Since heterotrophic bacteria play an important role in complete TCE degradation, long term TCE exposure should have some impacts on community structure. To monitor the change in the consortia, the following parameters were measured periodically (methods for measuring these parameter are described in chapter 4) :

(a). Theoretical transformation capacity in the absence of endogenous decay, T_c

(b). Endogenous decay rate, b

(c). Maximum specific rate of utilization of TCE in the absence of methane, k_c

(d). Half saturation coefficient of TCE, K_c

(e). Maximum specific rate of utilization of methane in the absence of TCE, k_s

(f). Observed yield of cells, Y

Cell samples were also taken periodically and the change in morphology and community structure were observed. Genetic analyses were conducted to show the change on community structure. The history of different parameter values was compared to the results from morphological or genetic analysis.

Adaptation of cometabolizing community in chemostat

The methanotrophic mixed culture chosen in this work is a consortium with closely commensal interaction and limited diversity. Furthermore, as discussed previously (Chapter 5), the chemostat selects specific populations by maintaining a minimum specific growth rate to avoid wash out. Thus, the experimental system may represent a less stable system when a long-term nongrowth substrate exposure is introduced repeatedly. In this work, both growth and nongrowth substrates were fed into chemostats simultaneously. Methane was the only growth and energy substrate, and the intermediates from oxidation of the growth substrate or products of decay of the dominant species are utilized by other populations in the community. It is assumed that the community can be treated as a single population (Chapter 5). With this assumption, the fitness, W of a community relative to an ancestor community is expressed as the ratio of their respected specific growth rate:

$$W = \frac{\mu}{\mu_0}$$

To facilitate comparison among the selection gradients for several fitness components, the gradients are normalized to create dimensionless quantities that reflect the proportional sensitivity of fitness to each component (Vasi et al. 1994):

$$G_P = \left(\frac{P}{W}\right)\left(\frac{\partial W}{\partial P}\right)$$

where P is the parameters (fitness components) that characterize the microbial system. The change of fitness for a derived community can be expressed as follows:

$$\Delta W = \sum_{n=1}^{n} \frac{\partial W}{\partial P_n} \Delta P_n$$

The above equation was used to quantity adaptive changes within the community.

In a chemostat, non-steady state conditions occur during nongrowth substrate exposure. During this period, a mass balance can be applied to organisms using the stoichiometric and kinetic relationships given earlier in this work:

General material balance

Accumulation = Influent - Effluent + Source - Sink

Organism balance

$$V_L \Delta X = 0 - QX\Delta t + \mu X V_L \Delta t - bXV\Delta t$$

It has been assumed that there are no organisms in the reactor influent. The above equation may be rearranged as follows:

$$\frac{dX}{dt} = -\frac{Q}{V}X + \mu X - bX$$
$$= -DX + \mu_{net} X$$

The above expression represent a mathematical description of the dynamic nature of a completely mixed reactor. μ_{net} is determined when cell density over a period and dilution rate are measured. This derivation offers a way to measure fitness, W, under conditions of TCE exposure, Where :

$$W = \frac{\mu_{net}}{\mu_0}$$

General procedures for biotransformation measurement

Batch biotransformation experiments

Biotransformation studies were performed using 20 ml glass vials sealed with teflon coated butyl rubber stoppers and aluminum crimp caps. These vials were incubated with 5 mL of of Whittenbury Mineral Media plus culture. An appropriate amount (measured as dry weight) of mixed culture MM1 was added to each test vial. TCE solutions (dissolved in water) were added to each bottle using Precision gas tight syringes. Methane was withdrawn from Scotty II cyclinders (99.0% CH_4, Alltech Associate, Inc., Deerfield, IL) at a fixed exit pressure and injected into batch vials. After adding substrates, the vials were vigorously shaken upside-down on a rotary shaker (250 rpm). Headspace samples of TCE and methane were periodically analyzed by GC .

Analytical methods

A TCE-saturated water solution was used as the spike solution in all experiments. The spike solution was prepared by adding excess TCE (99+% pure ACS reagent, Aldrich Chemicals Co., Milwankee, WI) to a 250 ml glass bottle capped with TFE-lined Mininert valve. The bottle was vigorously shaken and allowed to settle at least 24 hrs. The upper layer of the solution was transferred to another bottle and capped with a Mininert valve. The spike solution was stored in a refrigerator until needed. One hour before use, it was shaken again and allowed to settle.

TCE was analyzed by withdrawing 0.1 ml of headspace from the test bottles using a 0.5 ml Pressure-Lok Series A-2 gas syringe and injecting the samples onto a Hewlett Packard

5890 gas chromatograph (GC) equipped with a capillary column (DB624, 30m x 0.53mm I.D.), a flame ionization detector (FID) and a Electron capture detector (ECD). The GC was operated isothermally at 90°C with helium as carrier (12 mL/min). The injection port was set at 250°C. The temperature of FID and ECD were 250°C and 350°C respectively.

Methane and oxygen were analyzed by withdrawing 0.1 ml of headspace from the test bottles using a 0.5 ml Pressure-Lok Series A-2 gas syringe and injecting the samples onto a Hewlett Packard 5890 series II gas chromatograph equipped with a column (6 ft x 1/8 in SS packed with 80/100 washed molecular sieve 13X) and a thermal conductivity detector. The GC was operated isothermally at 50°C with helium as carrier. The temperature of the injection port and detector were 50°C and 90°C respectively.

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.

Community Analysis

DNA extraction and purification

Samples (15 ml) were collected from reactors at intervals, and cells were harvested by centrifugation and frozen. Pellets were later resuspended in a buffer consisting of 100 mM Tris, 100 mM EDTA (pH 8.0), 1.5 M NaCl, 1% CTAB. 2 ml SDS (10%) was added and the cells were incubated at 65°C for 30 minutes in a rotary water bath (150 rpm). Samples were cooled to 37°C, at which time 50 µl proteinase K was added (10 µg/µl) and the lysates were incubated at 30°C for 2 hours, with shaking. DNA was then purified by

extraction with chloroform/isoamyl alcohol (24:1) and phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with cold isopropanol.

Electropheresis

Changes in the communities were monitored by ARDRA (amplified ribosomal DNA restriction analysis). Thirty microliters PCR reactions contained approximately 10 ng template DNA, 0.75U *Taq* DNA Polymerase (GibcoBRL), 10X buffer supplied by GibcoBRL, 2 mM Mg^{+2}, 0.3 μg BSA, and 200 mM dNTPs, and 0.5 μM of each primer described by Martinez-Murcia, et al.(Martinez-Murcia et al. 1995). An initial denaturing step of 92°C for 6 minutes was followed by 30 cycles of 92°C (70 seconds), 55°C (30 seconds) and 72°C (2 minutes 10 seconds), and a final extension of 72°C for 6 minutes. Fifteen microliters of product were digested with 2.5 U each of *Hin*fI and *Mbo*I, at 37 °C for 3 hours and analyzed by electrophoresis on a 10% polyacrylamide gel. Bands were visualized by silverstaining.

RESULTS

For the long-term TCE exposure experiment with the methanotrophic mixed culture, TCE was fed continuously over a finite period, halted to allow recovery of the consortia, and then reintroduced for another period at a higher concentration with another recovery period. This pattern was repeated ten times (Figure 6.2). Changes of cell density in response to long-term TCE exposure are shown in Figure 6.3. To monitor the changes in community structure and function, the following parameters were measured periodically: biomass transformation capacity, endogenous decay rate, and observed yield. The history of these parameters along the long-term TCE exposure are shown in Table 6.2 and Figure 6.4 to 6.5. The fitness of the consortia in the TCE exposed and unexposed chemostats are shown in Figure 6.6.

Amplified ribosomal DNA analysis (ARDRA) was used to observe changes in community structure. ARDRA gives a unique "fingerprint" for each species or community through amplification and digestion of 16S rDNA. Changes in community structure may be detected by this technique through the appearance and/or disappearance of bands. Such changes may indicate the gain or loss of populations within a community, or merely a shift in dominance in the community structure. Results of this analysis are shown in Figure 6.7.

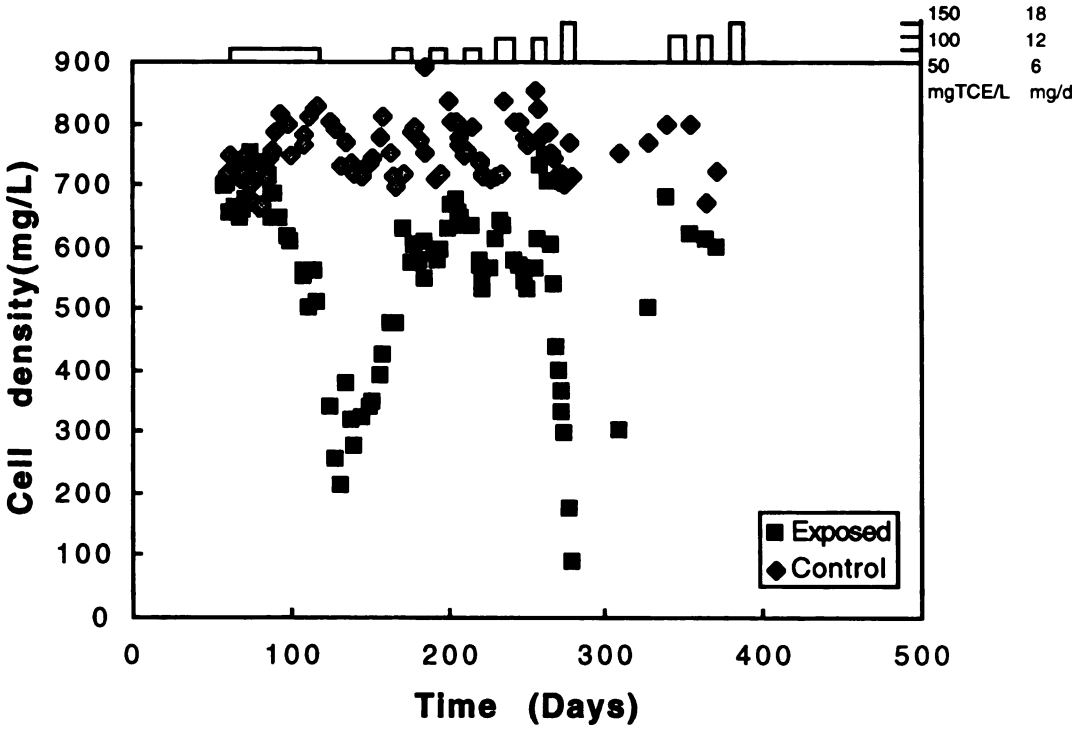


Figure 6.3 Changes of Cell density of methanotrophic mixed cultures in respond to long-term TCE exposure

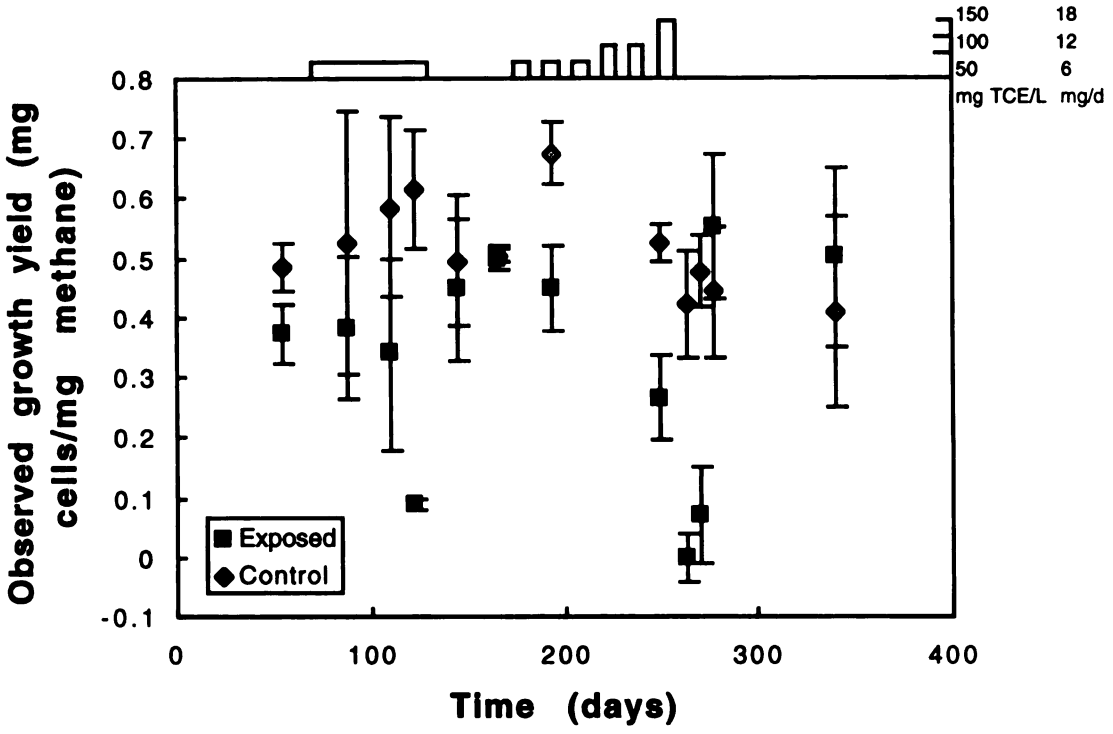


Figure 6.4 Observed growth yield for methanotrophic mixed cultures during long-term TCE exposure

Table 6.2 Endogeneous decay constant for methanotrophic mixed cultures during long-term TCE exposure

Time (Days)	Endogenous decay constant, 1/day		r^2 for regression	
	Exposed	Control	Exposed	Control
25	0.881	0.804	0.824	0.878
54	0.657	0.921	0.999	0.953
94	0.631	0.988	0.718	0.811
115	0.438	0.786	0.954	0.899
124	0.62	0.758	0.586	0.663
136	0.261	0.616	0.843	0.857
164	0.654	0.459	0.777	0.807
193	0.549	0.225	0.945	0.955

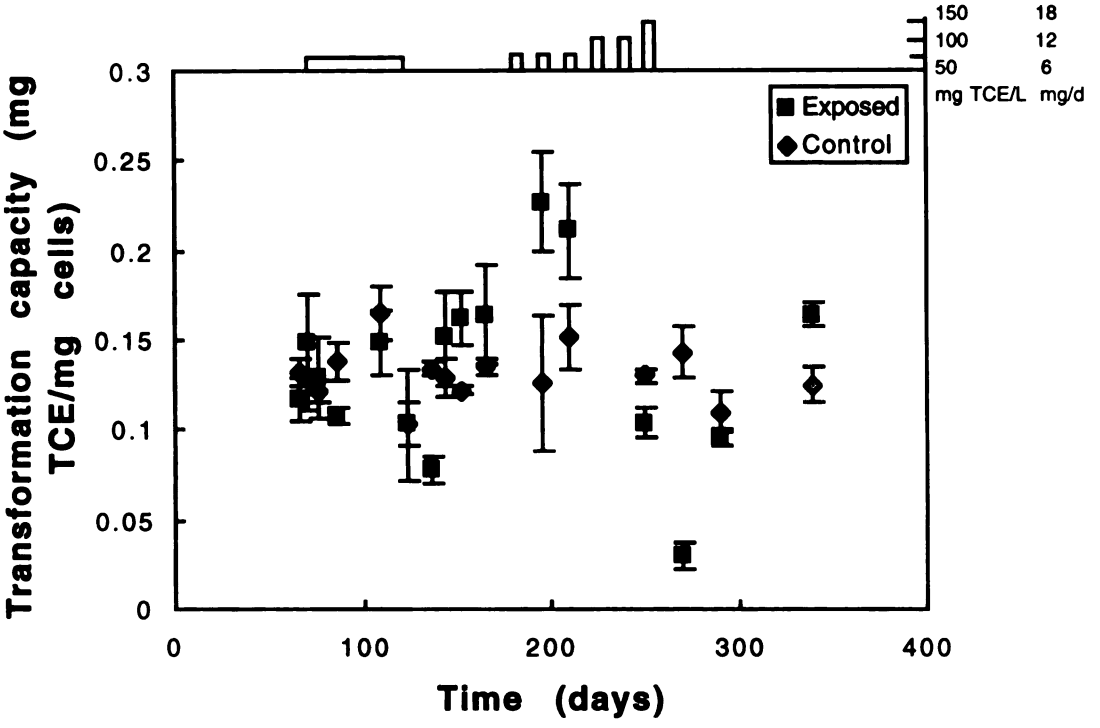


Figure 6.5 Transformation capacity for methanotrophic mixed cultures during long-term TCE exposure

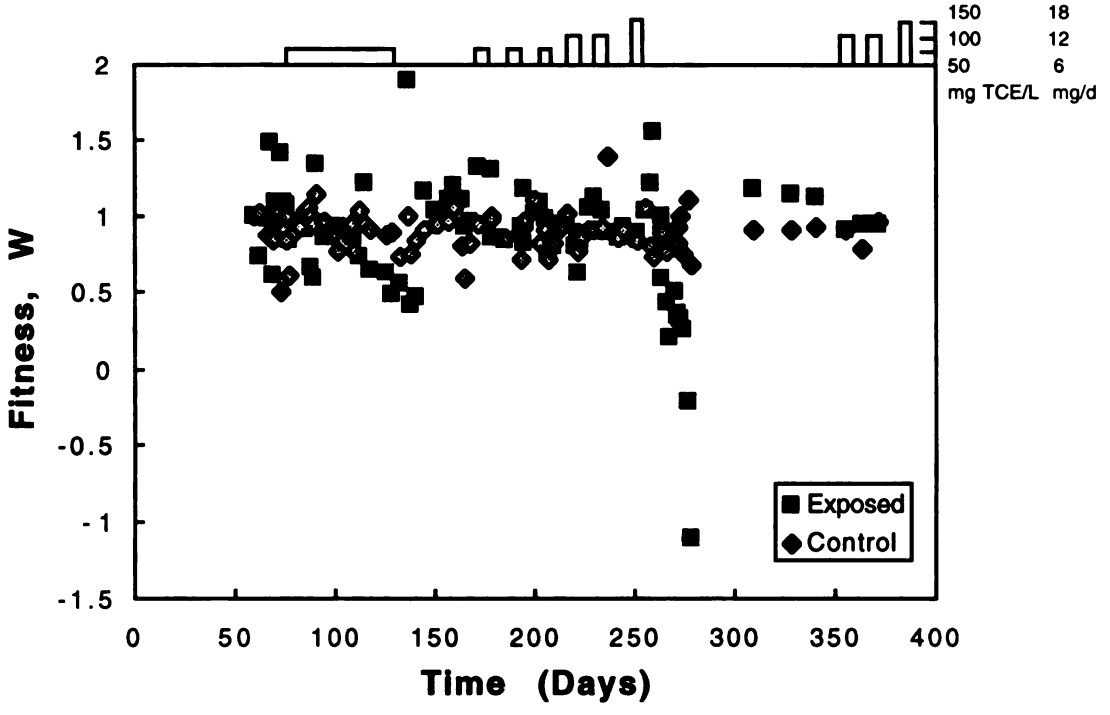


Figure 6.6 Fitness of methanotrophic mixed cultures in respond to long-term TCE exposure in chemostats

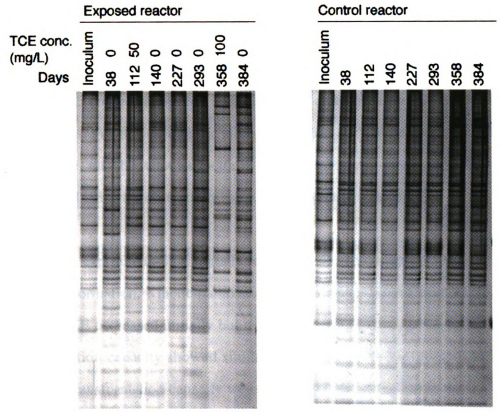


Figure 6.7 ARDRA fingerprints of control and TCE-exposed methanotrophic communities.

DISCUSSION

Changes in cell density for methanotrophic mixed culture exposed to periods of continuous loading of TCE are shown in Figure 6.3. Continuous exposure to TCE initially resulted in loss of capability to utilize methane and degrade TCE . Cell densities dropped drastically and increased slowly after TCE addition stopped. TCE concentration in aqueous phase within the exposed reactor changed from 0.01 to 0.1 mg/L approximately when cell densities dropped. This suggests that product toxicity impacted the microbial community. Greater TCE concentrations had a similar effect, and more time was needed for recovery of the biomass. For this culture the strong inhibition of TCE on methane utilization (K_{ic} = 10.8 mg/L) may be another factor in the decline of cell density. Clearly, simultaneous feeding of TCE to the community had a great impact on growth.

Continuous exposure to TCE initially resulted in decreased rates of methane utilization and TCE transformation. Kinetic parameter measurements showed that maximum utilization rates of TCE decreased initially but returned as cell density increased. Growth yield and transformation capacity showed similar trends during TCE exposure. However, greater values of transformation capacity were observed between cell recovery and the next TCE exposure. This result suggests that the community adapted to TCE exposure by increasing transformation capacity. The relationship between the improvement in transformation capacity and diversity of community is still not clear from the data obtained.

Specific growth rates of methanotrophic mixed culture in the chemostat were calculated from cell density data by mass balance of biomass. Fitness was calculated as the ratio of specific growth rate of the culture at any time to the respected value before the initiation of TCE exposure. The results indicate some decline in fitness just after initiation of TCE exposure (50 mg/L TCE in feeding), with subsequent recovery to the original value. After 150 mg/L TCE feeding was initiated, a significant drop in fitness was observed(from 1 to

-1). The fitness recovered to 1 after TCE feeding was halted. This result indicates that the microbial community was not sustainable when TCE was fed continuously. A possible explanation is the limited diversity of this methanotrophic mixed culture, perhaps contributing to the low tolerance to TCE exposure. Another explanation is the sensitivity of this particular culture to competitive inhibition of methane utilization by TCE. As discussed previously, the relatively high selection gradient for this culture indicates a greater instability for the culture (chapter 5).

ARDRA results indicated changes in both the chemostat cultures from the original batch culture inoculum. However, there were no further changes in the control reactor and only minor changes could be seen in the TCE-exposed community, even after TCE feed concentration was raised to 150 mg/L (Figure 6.7). These results indicate that the community structure of the methanotrophic mixed culture was stable despite exposure to TCE. While transitory phenotypic changes occurred, the populations present within the community remained unchanged. Greater changes in community structure occurred when TCE feed concentration was subsequently reduced to 100 mg/L. Less diversity was observed at this exposure. The more diverse community again appeared when influent levels were restored to 150 mg/L. The results suggest that higher diversity corresponded to higher TCE exposure.

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CHAPTER 7

CHANGE IN COMMUNITY STRUCTURE IN RESPONSE TO LONG TERM TCE EXPOSURE: PHENOL-DEGRADING COMMUNITY IN SEQUENCING BATCH REACTOR*

INTRODUCTION

Trichloroethylene (TCE) is widely found in soil and groundwater. Considerable research has established that certain phenol- and toluene-degrading *Pseudomonas* species can rapidly cometabolize TCE (Folsom et al. 1990; Nelson et al. 1987; Wackett and Gibson 1988). To date, this research has concentrated on kinetics (Alvarez-Cohen and McCarty 1991c; Chang and Alvarez-Cohen 1995a; Criddle 1993; Folsom et al. 1990; Strand et al. 1990) and pathways (Harker and Kim 1990; Nelson et al. 1988; Shields et al. 1989; Wackett and Gibson 1988) with relatively little research on adaptive changes in microbial community structure.

TCE transformation capacity is a function of available reducing power and toxicity of transformation products (Alvarez-Cohen and McCarty 1991a; Henry and Grbić -Galić 1991a; Wackett and Householder 1989). Reducing power is required for monooxygenase activity and can be derived from either the growth substrate or supplemental electron donors, such as formate. For example, formate addition to a phenol-degrading community resulted in increased transformation yield and elevated transformation capacity (Hopkins et

* The genetic analyses described in this study were performed with the assistance of Dr. Denise Searles

al. 1993b). Other reports indicate that the toxicity of certain transformation products can limit the extent of transformation. Oldenhuis and co-workers suggested that TCE epoxide can bind covalently to proteins and nucleic acids. Other possible reactive metabolites that might bind irreversibly are chloral, dichloroacetyl chloride, and formyl chloride (Oldenhuis et al. 1991). Communities and populations capable of degrading a large amounts of TCE should possess active detoxification systems for these compounds.

Several reports indicate that TCE cometabolism by pure cultures does not result in the mineralization of TCE (Henry and Grbić -Galić 1990; Little et al. 1988; Oldenhuis et al. 1989). Other research suggests that microbial consortia or community have advantages for mineralization of TCE. Because the TCE-oxidizers suffer from product toxicity, associated heterotrophs may play an important role in detoxification (Little et al. 1988). Uchiyama and co-workers (1992) reported that a heterotrophic bacterium in a methanotrophic mixed culture, *Xanthobacter autotrophicus*, can oxidize dichloroacetic and glyoxylic acid completely and can reduce trichloroacetic acid to lower levels. These results indicate that heterotrophic bacteria play an important role in TCE detoxification.

The presence of toxic transformation products can be expected to impact the development of microbial communities during long-term TCE exposure. Changes in the populations are likely related to the level of TCE exposure, turnover of transformation products and utilization of growth substrate. Lackey *et al* . (1994) used total-recycle expanded-bed bioreactors to evaluate the degradation potential of TCE by a microbial consortium. Ester-linked phospholipid fatty acid profiles (PLFAME) were used to monitor changes in a propane-fed community during short-term perturbation with TCE. A propane-utilizing bacterial biomaker increased as TCE was degraded and as propane was consumed. However, the relationship between community structure and extent of TCE exposure was not clear for these short-term exposures.

The effect of growth substrate feeding pattern on the structure and potential cometabolic activity of a phenol-degrading community was evaluated by Shih et al. (1996). The results indicated that the manner of growth substrate addition can have a pronounced effect on community structure and cometabolic activity. Communities enriched with continuous or protracted feeding intervals exhibited limited long-term capacity for TCE transformation. Communities enriched with short feeding intervals maintained higher TCE transformation rates. Pulse feeding also resulted in more stable and diverse communities. However, this research did not investigate community changes during long-term exposure to TCE.

Adaptation of communities to changing environments may proceed in several ways. Genetic recombination is one mechanism that can lead to new genotypes in the absence of mutation. Genetic elements brought together may enable microorganisms to carry out new functions, and can result in adaptative changes. Selection among species is another mechanism of adaptation. The impact of the selection in evidenced by the characteristic of the community. In chapter 5, a theory of community "fitness" was presented. In this chapter, the cometabolism model verified previously (Chapter 5) is used to analyze adaptive changes in terms of the " fitness " concept. In this manner, fitness was quantified in terms of measurable kinetic parameters.

Sequencing batch reactors (SBRs) have several advantages for cometabolic transformations. An SBR can alternate between periods of growth on growth substrates and periods of cometabolism of nongrowth substrate, eliminating the possibility of competitive inhibition for enzymes between growth and nongrowth substrates. SBRs also have a great ability to periodically change environmental condition, creating temporal concentration gradients, selecting or enriching specific microbial populations. Thus, SBRs offer a good model environment to study the dynamic changes of microbial communities.

In this study, sequencing batch reactors were chosen as model environments. A phenol-degrading community was exposed to increasing levels of TCE over time. Changes in the community were monitored and the effects of TCE exposure on community structure were evaluated. Various phenotypic parameters were measured. Community analyses were also conducted to monitor shifts in microbial community structure. Changes in the community were analyzed using the fitness theory presented in chapter 5.

MATERIALS AND METHODS

Culture conditions

A stable phenol-degrading microbial community was obtained by seeding a chemostat with activated sludge from a municipal wastewater treatment plant (East Lansing, Michigan) and providing a phenol-containing medium for two months at a dilution rate of 0.1 day^{-1}. The enrichment was matained at 21.5 ± 1.0°C. This culture was inoculated into a 2-liter stirred reactor supplied continuously with air. Two hundred milliliters of medium (2000 mg/L phenol) was provided as a single daily pulse immediately after removing the same volume of biomass from the reactor (Shih et al. 1996). This fed-batch system was operated for over 400 days, yielding a stable community with good TCE transformation properties. Microscopic examination revealed a community with distinctive floc structures of spherical and rod-shaped bacteria. Some fungi were also observed. The community was then used to inoculate duplicate SBRs.

Phenol feed medium contained the following (per liter of deionized water): 2 g of phenol, 2.13 g of Na_2HPO_4, 2.04 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.067 g $CaCl_2 \cdot 2H_2O$, 0.248 g of $MgCl_2 \cdot 6H_2O$, 0.5 mg of $FeSO_4 \cdot 7H_2O$, 0.4 mg of $ZnSO_4 \cdot 7H_2O$, 0.02 mg of $MnCl_2 \cdot 4H_2O$, 0.05 mg of $CoCl_2 \cdot 6H_2O$, 0.01 mg of $NiCl_2 \cdot 6H_2O$, 0.015 mg of H_3BO_3, 0.25 mg of EDTA. The pH of the medium was 6.8.

Perturbation of TCE

The set-up of SBRs is shown in Figure 7.1. After both reactors had stabilized, one was injected with TCE over a one-hour interval during the filling period of cycle operation. Phenol was provided in a separate period for both reactors. Influent TCE levels were increased gradually from 0.5 to 25 mg/L over 4 months (Table 7.1). The operating mode of the SBR during a cycle is shown in Figure 7.2. The operating conditions for the SBRs are summarized in Table 7.2.

Monitoring of consortia

To monitor changes in the community, the following parameters were measured periodically (the methods for measuring these parameter are described in chapter 4) :

(a). Theoretical transformation capacity in the absence of endogenous decay, T_c

(b). Endogenous decay rate, b

(c). Second order rate coeficient of transfornation of TCE in the absence of methane, $k_c^{'}$

(d). Maximum specific rate of utilization of phenol in the absence of TCE, k_s

(e). Observed yield of cells, Y

Specific growth rate of the community was obtained by multiplying observed yield of cells by maximum specific rate of utilization of phenol. Also, cell samples were taken periodically and the change in community structure were observed. Microscopic examination was also performed periodically. Genetic analysis was used to evaluate changes in community structure. The change in kinetic parameter values was compared to the results from morphological observation or genetic analysis.

Adaptation of cometabolizing community in SBR

In this work, phenol was the sole growth and energy substrate, so that tha actual substrate available to members of the community is phenol, intermediates generated by oxidation of phenol or products of cell decay. Under these conditions, it is assumed that the community can be treated as a single population (chapter 5). The fitness, W of such a community relative to ancestor community is expressed as the ratio of the respective specific growth rates:

$$W = \frac{\mu}{\mu_0} \tag{1}$$

The phenol-degrading community in SBR utilized phenol according to zero order kinetic, thus $q_c = k_s$, and the specific growth rate of the cells is $\mu = Y_m k_s$. Selection gradients with respect to Y_m and k_s can be derived as follows:

$$G_{Y_m} = \left(\frac{Y_m}{W}\right)\left(\frac{\partial W}{\partial Y_m}\right) = 1 \tag{2}$$

$$G_{k_s} = \left(\frac{k_s}{W}\right)\left(\frac{\partial W}{\partial k_s}\right) = 1 \tag{3}$$

The change of fitness for the derived community can be expressed as:

$$\Delta W = \frac{\partial W}{\partial Y_m}\Delta Y_m + \frac{\partial W}{\partial k_s}\Delta k_s \tag{4}$$

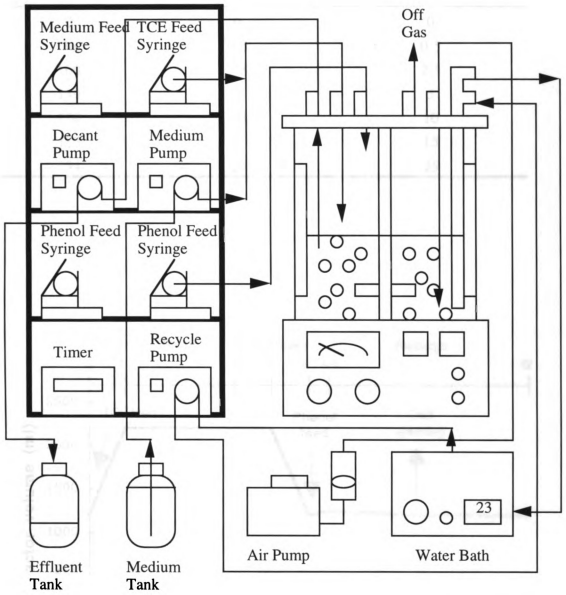

Figure 7.1. Experimental setup for bench-scale sequencing batch reactors

Table 7.1. TCE feed concentration for long-term TCE exposure experiment

Start days (days)	TCE feed concentration (mg/L)	TCE loading per cycle (mg)
0	0	0
76	0.5	0.5
100	2.5	2.5
140	5	5
150	10	10
183	15	15
191	25	25

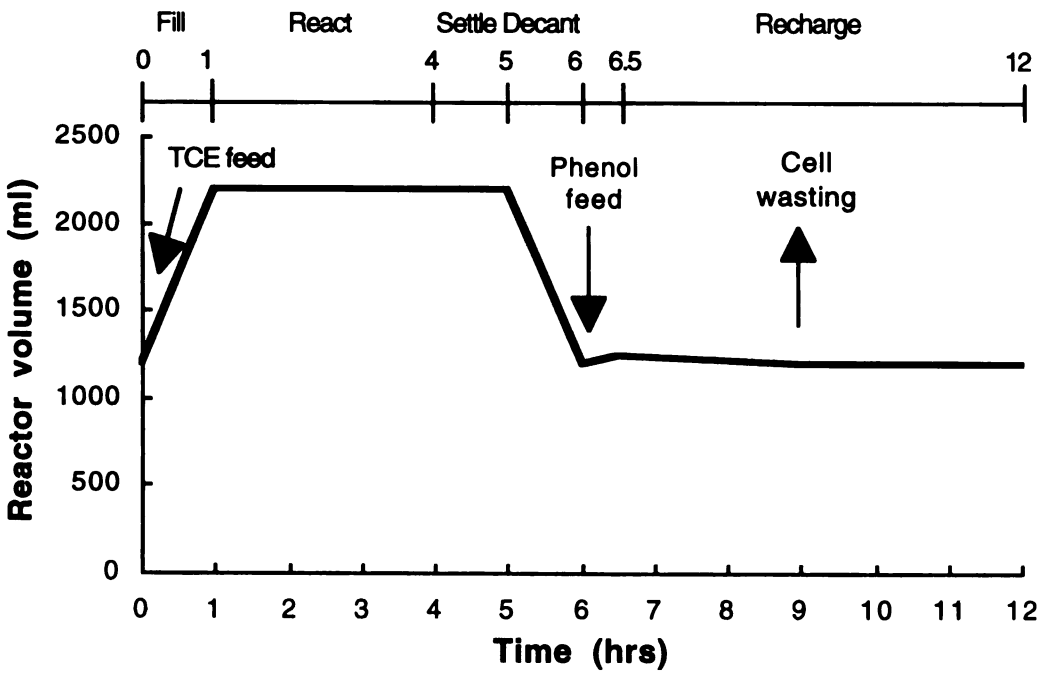

Figure 7.2. The operating mode of SBR in a cycle

Table 7.2 Summary of bench-scale SBR operating condition

Parameter	Value
Reactor volume	
Total volume	2500 mL
Liquid volume	1100 - 2200 mL
Headspace volume	300 - 1400 mL
Initial volume	1100 mL
Flow rate	
Influent flow rate	16.7 mL/min
TCE concentration	0.5-25 mg/L
Recharge flow rate	1.2 mL/min
Phenol concentration	5000 mg/L
Air flow	220-280 mL/min
Minimum oxygen concentration in reactor	2 mg/L
Operating mode	
Fill time	1 hr
Reaction time	3 hrs
Settle time	1 hr
Decant time	1 hr
Recharge time	6 hr
- phenol feed mode	0.5 hr
- phenol react mode	5.5 hr
Operating cycle time	12 hrs/cycle
Sludge age, SRT	10 day

General procedures for biotransformation measurement

Batch biotransformation experiments

Biotransformation studies were performed using 20-ml glass vials sealed with teflon-coated butyl rubber stoppers and aluminum crimp caps. These vials were incubated with 5 mL of of Whittenbury Mineral Media plus culture. An appropriate amount (measured as dry

weight) of mixed culture MM1 was added to each test vial. TCE solutions (dissolved in water) were added to each bottle using Precision gas tight syringes. Methane were withdrawn from Scotty II cyclinders (99.0% CH_4, Alltech Associate, Inc., Deerfield, IL) at a fixed exit pressure and injected into batch vials. Phenol was added to vials from a 40 g/L stock solution. After adding substrates, the vials were vigorously shaken upside-down on a rotary shaker (250 rpm). Headspace samples of TCE and methane were periodically analyzed by GC. Cell solution was filtered with 0.2 μm syringe filter. Phenol in the filtrate was analyzed by HPLC.

Analytical methods

A TCE-saturated water solution was used as the spike solution in all experiments. The spike solution was prepared by adding excess TCE (99+% pure ACS reagent, Aldrich Chemicals Co., Milwankee, WI) to a 250 ml glass bottle capped with TFE-lined Mininert valve. The bottle was vigorously shaken and allowed to settle at least 24 hrs. The upper layer of the solution was transferred to another bottle and capped with a Mininert valve. The spike solution was stored in a refrigerator until needed. One hour before use, it was shaken again and allowed to settle.

TCE was analyzed by withdrawing 0.1 ml of headspace from the test bottles using a 0.5 ml Pressure-Lok Series A-2 gas syringe and injecting the samples onto a Hewlett Packard 5890 gas chromatograph (GC) equipped with a capillary column (DB624, 30m x 0.53mm I.D.), a flame ionization detector (FID) and a Electron capture detector (ECD). The GC was operated isothermally at 90°C with helium as carrier (12 mL/min). The injection port was set at 250°C. The temperature of FID and ECD were 250°C and 350°C, respectively.

Phenol was analyzed by HPLC. Cell samples were collected by syringes and injected through 0.2 μm NYLON syringe filters. Filtrate (2 ml) was collected for analysis. Water

HPLC (WISP 710B+ Model 510 pump) equipped with a column (Econosil C18, 10 micron, 250 mm, Alltech Cat No. 288138) and a UV detector (Lambda-Max Model 481 LC spectrophotometer) was operated isocratically (60% acetonitrile + 40% water) at a total flow rate 1 mL/min. The wavelength of UV detector was 235 nm and the injection amount of samples were 30 µL. The limit of detection for phenol was approximately 1 mg/L.

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.

Community Analysis

DNA extraction and purification

Fifteen milliliter samples were collected from reactors at intervals, and cells were harvested by centrifugation and frozen. Pellets were later resuspended in a buffer consisting of 100 mM Tris, 100 mM EDTA (pH 8.0), 1.5 M NaCl, 1% CTAB. Two milliliters SDS (10%) was added and the cells were incubated at 65°C for 30 minutes in a rotary water bath (150 rpm). Samples were cooled to 37°C, then supplemented with 50 µl proteinase K (10 µg/µl). The lysates were incubated at 30°C for 2 hours with shaking. DNA was purified by extraction with chloroform/isoamyl alcohol (24:1) and phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with cold isopropanol.

Electropheresis

Changes in the communities were monitored using DGGE (denaturing gradient gel electrophoresis). Thirty microliter PCR reactions contained approximately 10 ng template DNA, 1U *Taq* DNA polymerase, 10X buffer, 10% DMSO (v/v), 2mM Mg^{+2}, 200 mM dNTPs, and 1 μM of primers GM5F and 907R described by Muyzer, et al. (Muyzer et al. 1995). A CG clamp was added to the 5' end of GM5F. Products were analyzed on an 8% polyacrylamide gel (37.5 :1 acrylamide/bisacrylamide) containing a 40 -60% gradient of urea and formamide. The separationwas achieved at 200 V and a temperature of 60°C.

RESULTS

For the TCE exposure experiment, TCE was injected over a one-hour interval during the fill period. Phenol was provided in a separate period, following the decant step. Influent TCE levels were increased gradually from 0.5 to 25 mg/L over 6 months (Table 7.1). Changes of cell density in response to long-term TCE exposure are shown in Figure. 7.3. To monitor changes in community structure and function, the following parameters were measured periodically: second order rate coefficient for TCE transformation, maximum specific rate of utilization of phenol, observed yield, biomass transformation capacity, and endogenous decay rate. The history of these parameters during long-term TCE exposure is shown in Figures 7.4 to 7.6 and Table 7.3 to 7.4. Figure 7.7 illustrates fitness of the two communities during the period when the TCE-exposed reactor was exposed to 25 mg/L of TCE feed solution.

Typical changes in the apparent second order rate coefficient for TCE transformation during a SBR operating cycle are illustrated in Figure 7.8. A decline in TCE transformation was observed during the fill, react, settle and decant periods. However, recovery of activity was observed during the recharge period. From a mass balance on TCE, more than 95% of the

added TCE was removed by microbial degradation and only 3% was removed by air stripping. TCE concentration in gas and liquid phase within the reactor during the fill period are shown in Figure 7.9. Gas and liquid phase approach equilibrium except during the initial fill.

One molecular technique - denaturing gradient gel electrophoresis (DGGE) - was used to detect changes in community structure. A small fragment (~400 bp) of the 16S gene is amplified and the products are resolved on an acrylamide gel containing a gradient of urea and formamide. The amplified PCR products from different species are of the same size and do not resolve on agarose or acrylamide. However, the different GC content of each fragment allows resolution on the gradient gel as fragments with higher GC content are transported further into the gel before denaturing. Most species will yield one band so a simplified community fingerprint is obtained. Changes in community structure can be detected through the appearance and/or disappearance of bands. DGGE fingerprints from control and TCE-exposed communities are shown in Figure 7.10.

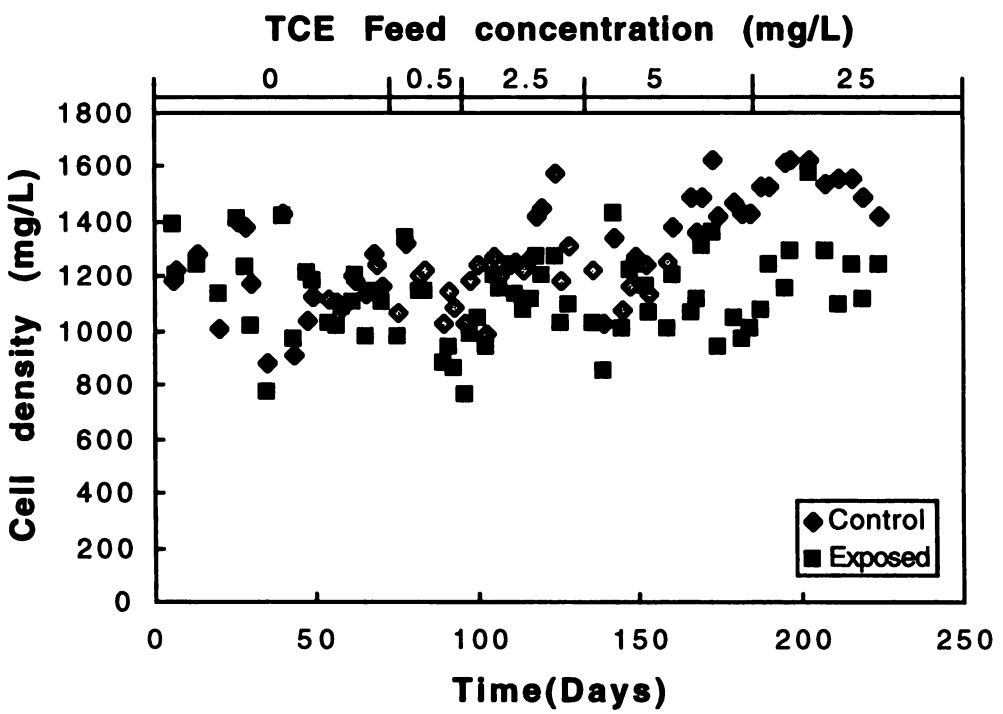

Figure 7.3 Changes in cell density of the phenol-degrading reactor communities.

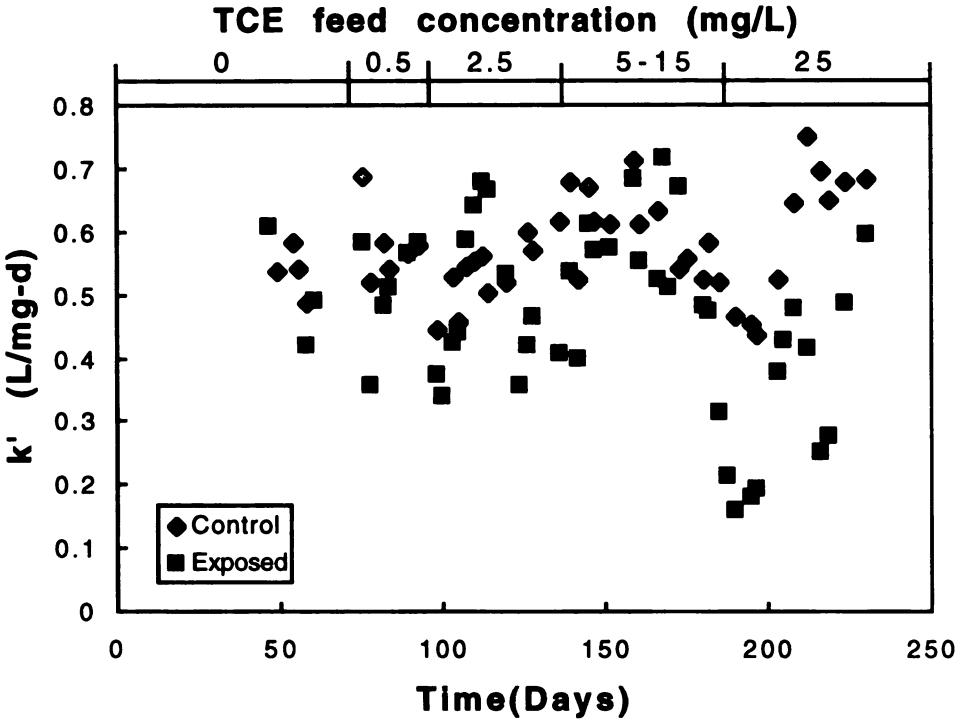

Figure 7.4 Second order rate coefficients of TCE transformation by phenol-degrading reactor communities.

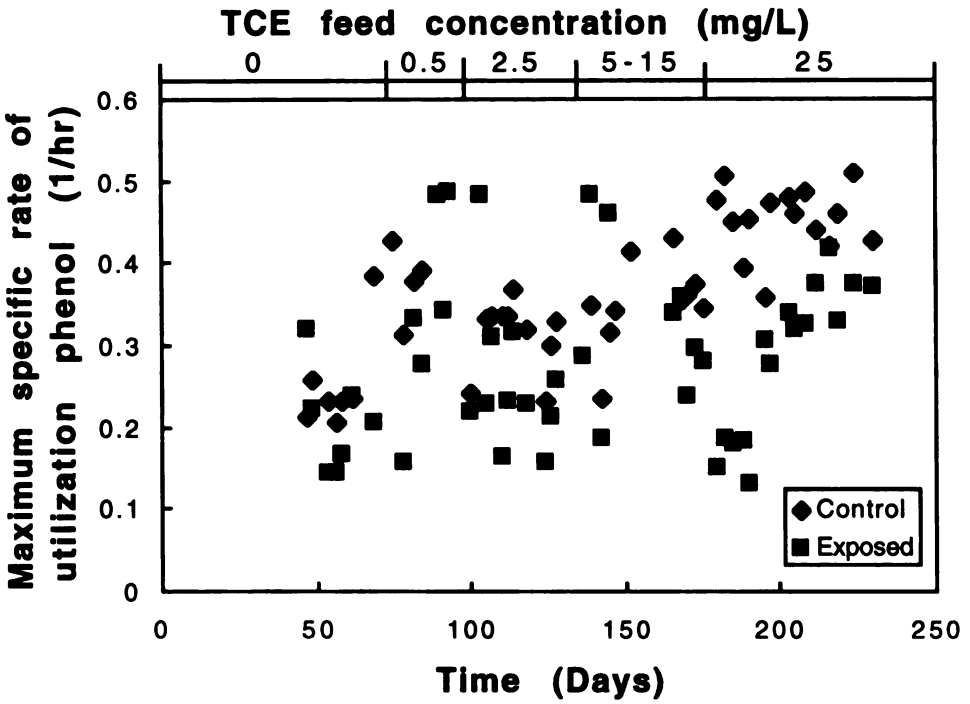

Figure 7.5 Maximum specific rate of phenol utilization by phenol-degrading reactor communities.

Table 7.3. Theoretical transformation capacity for phenol-degrading reactor communities.

Time (Days)	Theoretical transformationcapacity, mg TCE/mg cells		T,exposure/ T,control
	Exposed	Control	
68	0.366±0.016	0.345±0.045	1.06
96	0.333±0.023	0.324±0.043	1.03
174	0.342±0.055	0.355±0.044	0.96
189	0.222±0.031	0.344±0.005	0.65
210	0.332±0.017	0.34±0.022	0.98
222	0.345±0.041	0.351±0.055	0.98

Table 7.4. Endogeneous decay constant for phenol-degrading communities during long-term TCE exposure in SBRs.

Time (Days)	Endogenous decay constant, 1/day		b,exposure/ b,control	r^2 for regression	
	Exposed	Control		Exposed	Control
68	0.398	0.349	1.14	0.875	0.771
96	0.177	0.202	0.88	0.735	0.668
174	0.369	0.318	1.16	0.948	0.685
189	0.267	0.248	1.08	0.744	0.791
210	0.152	0.367	0.41	0.704	0.762
222	0.314	0.718	0.44	0.998	0.923

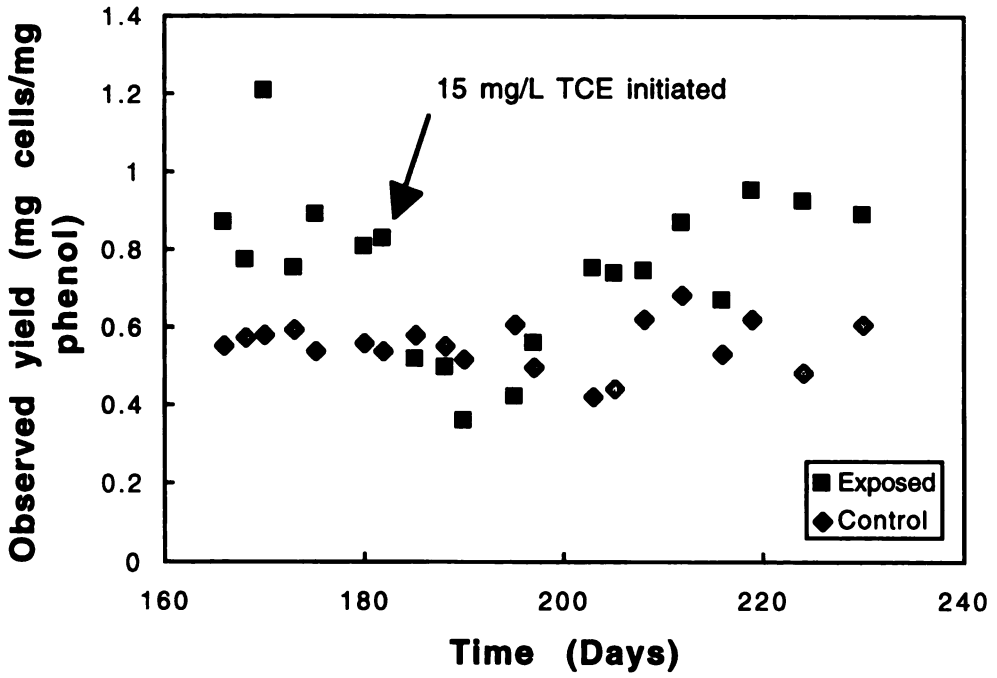

Figure 7.6 Long-term changes in observed yield for the phenol-degrading reactor communities.

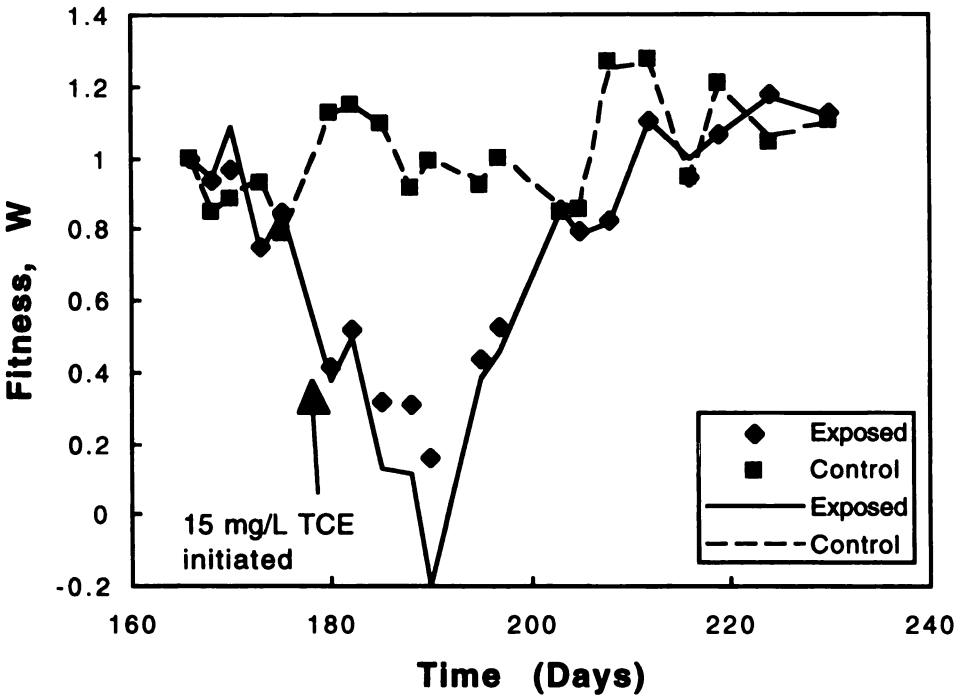

Figure 7.7 Fitness of phenol-degrading communities during period when the exposed reactor received an influent concentration 25 mg/L TCE. Fitness calculations are based on μ measurements (♦, •) and fitness components (—, ---), respectively.

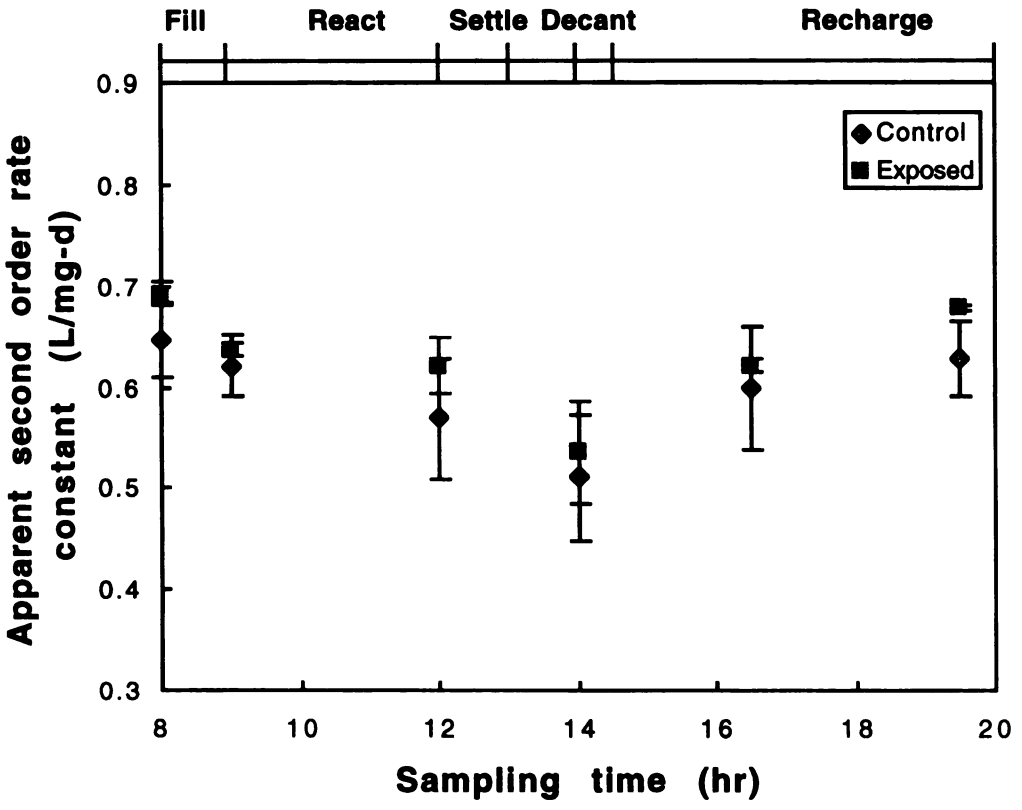

Figure 7.8 Change in the second order rate coefficient for TCE transformation in a typical SBR operating cycle.

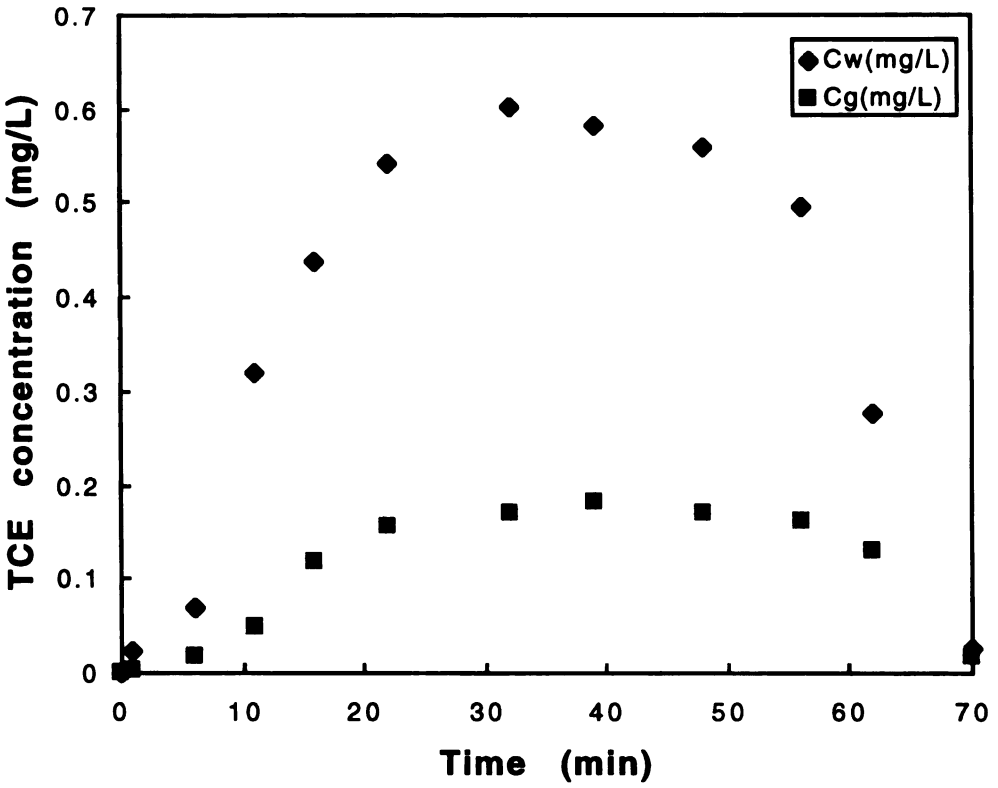

Figure 7.9 TCE concentration in gas (Cg) and liquid (Cw) phase during the fill and react periods for a feed solution of 25 mg/L TCE.

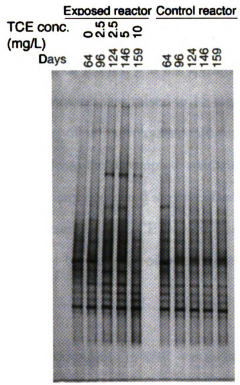

Figure 7.10 DGGE fingerprints from control and TCE-exposed communities in sequencing batch reactors.

DISCUSSION

The SBR microbial community cometabolized TCE for extended periods without loss of transformation activity. No significant changes in phenotypic parameters occurred when the TCE concentration was less than 10 mg/L. With increased influent TCE concentrations, declines in biomass, observed yield, specific rates of phenol utilization and specific rates of TCE transformation were observed. Theoretical transformation capacity (defined as the mass of nongrowth substrate transformed per unit mass of cells in the absence of endogenous decay) decreased to 60% of the control value (from 0.344+/-0.005 to 0.222+/-0.031). After one more month of perturbation, theoretical transformation capacity recovered to the original value. Of interest was the appearance of a persistent yellow color after the influent TCE concentration was raised to 15 mg/L. The yellow color may have been due to the accumulation of α-hydroxymuconic semialdehyde, an intermediate of phenol degradation resulting from the incomplete oxidation of phenol (Shih et al. 1996). The endogenous decay coefficient decreased slightly after prolonged TCE exposure. This suggests that the enzymes present in the TCE exposure culture became more resistant to endogenous decay. This may be because of adaptation to TCE or its transformation products.

Fitness of phenol-degrading community in SBR was calculated from the ratio of specific rate of phenol utilization at any time to the value just before initiating the 25 mg/L TCE feed. The results show that a decline in fitness occurred just after the initiation of 25 mg/L TCE feed, with subsequently recovery to the original value. The changes in fitness during TCE exposure closely match those of the observed yield, specific rates of phenol utilization, specific rates of TCE transformation and theoretical transformation capacity. These observations suggest that microbial community can adapt to TCE exposure, and that this adaptation can be quantitatively described by fitness. To further demonstrate the fitness theory, selection gradients with respect to fitness components (Y_m, k_s) and history of these

fitness components were used to calculated fitness (Eq.(1) to Eq. (4)). The results indicate fitness can be calculated accurately without knowledge of fitness itself (Figure 7.7).

Microscopic examination revealed that the community has a distinctive floc structure of spherical and rod-shaped bacteria. This characteristic is similar to that of the inoculum from the fed-batch pulse reactor (Shih et al. 1996). During the settle period, the TCE-exposed reactor community had a finer floc structure than the control reactor. This difference appeared soon after TCE exposure and persisted after higher TCE exposures were implemented. A possible explanation for lower cell density in the TCE-exposed reactor may be loss of biomass in the decant period because of poor settling properties.

The apparent second order rate coefficients declined during the fill, react, settle and decant periods and recovered during the recharge period. For specific growth rate, similar trends were observed. The apparent first order rate coefficient is equal to the true second order coefficient ($k_c^{'}$) multiplied by the concentration of organisms (X) that can degrade TCE (X). Therefore, changes in the apparent second order rate coefficient during a operating cycle may correspond to the changes in the concentration of TCE degrading organisms (lower X) or to loss of reducing power during TCE transformation (lower $k_c^{'}$). The organisms that were deactivated or killed during TCE transformation were reactivated by regrowth on the phenol. The fact that there was no significant difference in endogenous decay rate between the exposed and control reactor communities suggests that toxicity did not play an important role for this level of TCE exposure (25 mg/L in feed).

Changes in the structure of the microbial communities were monitored using denaturing gradient gel electrophoresis (DGGE). Total community DNA was isolated from samples taken from the reactor and regions of the 16S rDNA genes were amplified by PCR using universal primers, and resolved by electrophoresis in an acrylamide gel with a urea and

formamide gradient. The control community showed no change over time. The TCE-exposed community was also largely unchanged, with the exception of the appearance of a distinct new band after TCE concentration was increased to 2.5 mg/L (Figure 7.10). This change suggests the gain of a population within a community, or perhaps a shift in dominance in the community structure.

Previous research established that communities fed phenol over short intervals were more stable and diverse. This research establishes that such a community is capable of long-term TCE transformation. SBR reactor environments are well suited to cometabolism because they offer a wide variety niches for metabolism of phenol and cometabolism. In this work, the methanotrophic mixed culture chemostat experiment (Chapter 6) and the phenol-degrading SBR community represent two extreme cases for adaptation of cultures. Higher diversity within the SBR system offers a more stable community structure and higher potential for TCE transformation than the chemostat under the ranges tested (1-50 mg TCE/day and 6-18 mg TCE/day, respectively). As discussed previously, it may be possible to use selection gradients (especially more sensitive ones) as criteria for the stability of a community under a given perturbation. The selection gradients with respect to T_c, b , k_c, and K_c for methanotrophic mixed culture are higher than those of phenol-degrading community (Chapter 5). Thus, the phenol-degrading community was expected to be more stable than methanotrophic mixed culture. This was confirmed by experimental results.

Mass balance data show that over 95% of TCE was removed by microbial degradation and only 3% of TCE was stripped by aeration. TCE concentration in gas and liquid phase within the SBR approached equilibrium. From a process engineering point of view, these data verify that the current SBR design can treat 25 mg/L TCE with satisfactory results.

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CHAPTER 8

ENGINEERING SIGNIFICANCE

Cometabolism is a complicated phenomenon. Several factors interact simultaneously. This presents a challenge for engineering application since rational design requires use of appropriate kinetic expressions. A generally accepted model is needed with model parameters that can be evaluated without difficulty. In this research, a model for the most general case was verified. The model also covers a simplied case in which growth and nongrowth substrate transformation occurs separately. Different microbial systems can therefore be evaluated under this same framework. Furthermore, the model can serve as the basis for design of treatment processes and for prediction of treatment efficiency.

Cometabolic transformations often generate toxic products. The extent to which these products affect the microorganisms depends upon the type of organisms and the compounds transformed. Toxicity can affect microbial communities at both the population level and the community level. At the population level, specific populations may experience mutation and selection or changes in gene expression. At the community level, the community can be expected to undergo dynamic changes in composition. All of these factors contribute to changes in the phenotype of the community. By analyzing selection gradients, the sensitivity of different parameters on growth can be evaluated. This may suggest means whereby microbial populations or communities can be "designed" or improved. For example, improvements in transformation capacity in cultures that are sensitive to that parameter would enhance the sustainability of cometabolic transformation.

The patterns of addition of growth and nongrowth substrate can have a great impact on the performance of a system. In this work, two extreme cases were examined. A continuous system with both substrates fed simultaneously, and a SBR with both substrates fed at separate stages. When both substrates are provided simultaneously, growth and nongrowth substrates compete for the same enzymes, growth substrate is used inefficiently, and growth of cells declines. Futhermore, product toxicity accelerates cell decay. All these factors contribute to the instability of continuous systems fed both substrates simultaneously. On the other hand, sequencing batch reactors have certain advantages. An SBR can alternate between periods of growth on growth substrates and periods of cometabolism of nongrowth substrate, eliminating the possibility of competitive inhibition for enzymes between growth and nongrowth substrates. Since microorganisms are reactivated after the transformation of nongrowth substrate, a steady and sustainable community can be maintained. Thus, SBRs offer a good treatment technology for cometabolic transformation of substance that do not support growth of microorganisms.

Stability of microbial communities is an important factor for engineering application. Communities usually undergo dynamic changes in structures and performance in response to perturbation. In this research, nongrowth substrate exposure is the sole perturbation that the communities experience. A stable community should be able to maintain its structure and performance under nongrowth substrate exposure. Based on the results of this study, a diverse community seems to have better stability. It is feasible to use sensitive selection gradients as the criteria of stability of community. The parameter can be used as a standard for comparison between different communities in the same environment or the same communities in different environments.

CHAPTER 9

SUMMARY AND CONCLUSIONS

CONCLUSIONS

1. An unstructured model for cometabolism is presented and verified experimentally for a defined methanotrophic mixed culture. The model includes the effects of cell growth, endogenous cell decay, product toxicity, and competitive inhibition with the assumption that cometabolic transformation rates are enhanced by reducing power obtained from oxidation of growth substrates. A theoretical transformation yield is used to quantify the enhancement resulting from oxidation. A systematic method for evaluating model parameters is described. The applicability of the model is evaluated by comparing experimental data for methanotrophic cometabolism of TCE with model predictions from independently measured model parameters. Propagation of errors is used to quantify errors in parameter estimates and in the final prediction. The model successfully predicts TCE and methane transformation for a wide range of concentrations of TCE (0.5 - 9 mg/L) and methane (0.05 - 6 mg/L).

2. This research investigated the potential for methanotrophic biotransformation of three HCFCs -- chlorodifluoromethane (HCFC-22); 1-chloro-1,1-difluoroethane (HCFC-142b); and 1,1-dichloro-2,2,2-trifluoroethane (HCFC-123); and one HFC -- 1,2,2,2-tetrafluoroethane (HFC-134a). All of these compounds were biotransformed to differing degrees by methanotrophic mixed culture MM1. Intrinsic rates of transformation were obtained by combining a second order rate expression with an expression describing loss of transformation activity due to either endogenous decay or product toxicity. For

HCFC-123 and HFC-134a, the independently measured endogenous decay rate for mixed culture MM1 (0.594/day) was sufficient to account for the observed loss of transformation activity with time for the one case examined. However, the endogenous decay rate did not account for the loss of transformation activity for HCFC-22 and HCFC-142b. A model based on product toxicity provided a reasonable representation of the loss of transformation activity for all these compounds. The order of reactivity was HCFC-22 > HCFC-142b > HFC-134a > HCFC-123, with second order rate coefficients of 0.014, 0.0096, 0.00091, and 0.00054 L/mg-day, respectively. Transformation capacities for HCFC-22 and HCFC-142b were 2.47 and 1.11 μg substrate/mg biomass, respectively.

3. Theoretical transformation capacity is a function of organisms and target compounds. For two groups of compounds and two types of organisms studied here, the order of theoretical transformation capacity is phenol degrader/TCE > methanotrophic mixed culture/ TCE > methanotrophic mixed culture/ HCFC/HFC, with typical values of 0.35, 0.06, and 0.002 mg TCE or HCFC/mg cells. This indicates that product toxicity plays a much more important effect on methanotrophic mixed culture with HCFC/HFC transformation, but less important for phenol degrader with TCE transformation.

4. A fitness concept was developed for communities and combined with the cometabolism model to describe adaptation of cometabolizing communities. A major concept of the model is that gross phenotype changes within a microbial community can be quantitified by a " fitness" parameter which can be calculated using kinetic parameters that describe the community. The selection gradient for each parameter is defined by the partial derivative of fitness with respect to that parameter. The gradient, therefore, reflects the direct selection acting on each fitness component, with the other components held

constant. It appears possible to use selection gradients as criteria for the stability of a community under a given perturbation.

5. A methanotrophic mixed culture and a phenol degrading culture were exposed to different levels of TCE over extended periods of time. The changes of community were monitored and the effects of TCE exposure on community structure were evaluated. Various phenotypic parameters were measured. Genetic community analysis (ARDRA and DGGE) was also used to monitor shifts in microbial community structure. The results indicate that phenotypic and genetic changes occurred during TCE exposure. Both microbial communities adapted to TCE exposure with improvement in the observed transformation capacities and endogenous decay constants.

6. This research establishes that a phenol-degrading community is capable of long-term TCE transformation. SBR reactor environments are well suited to cometabolism because they offer a wide variety niches for metabolism of phenol and cometabolism. In this work, the methanotrophic mixed culture chemostat experiment and the phenol-degrading SBR community represent two extreme cases for adaptation of cultures. Based on an analysis of the selection gradients for these two communities, the phenol fed SBR community was expected to be more stable than methanotrophic chemostat mixed culture. This was confirmed by experimental results for the ranges tested.

FUTURE RESEARCH

1. Hyphomicrobia in methanotrophic mixed culture were thought to be possible indicators of MMO activity. However, in this research, no significant change in this population was detectable by image analysis. This may be because an insufficient number of images were taken. Further research is needed to determine the number of images required to accurately quantify a given morphotype.

2. SBRs provide flexible operation for cometabolism. Several operating parameters (length of periods, exposure level, growth substrate concentration and extent of aeration) can affect performance. Optimization of SBR systems will assist the engineering application of SBRs.

3. Genetic analyses (ARDRA and DGGE) were used in this research for characterization of community structure. Other methods of community analysis, such as analysis of fatty acid methyl esters (FAME), might also be employed for analysis of cometabolizing communities.

4. Key populations responsible for phenol and TCE transformations in the phenol-degrading community should be isolated and characterized.

5. Use of selection gradients to quantify community stability should be evaluated further. More extensive studies are needed for different communities within the same reactor type and for the same community within the different reactor types.

6. For the range of TCE exposures studied in this work, the phenol-degrading community was always able to adapt to TCE exposure. Further research should be conducted to determine the upper limit of exposure and to assess whether TCE could conceivably be used as a growth substrate.