

Experimental Evaluation of a Model for Cometabolism: Prediction of Simultaneous Degradation of Trichloroethylene and Methane by a Methanotrophic Mixed Culture

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Abstract: A model for cometabolism is 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 growth substrate oxidation. A systematic method for evaluating model parameters independently 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 transformation and methane utilization for a wide range of concentrations of TCE (0.5 to 9 mg/L) and methane (0.05 to 6 mg/L). © 1997 John Wiley & Sons, Inc. *Biotechnol Bioeng* 56: 492–501, 1997.

Keywords: cometabolism; methanotroph; modeling; trichloroethylene; inhibition

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 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 dam-

age 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 called biomass transformation capacity. This concept has been applied to quantify degradation kinetics for several oxygenase-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., 1993). 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 correcting for the effects 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 transformation products (Alvarez-Cohen and McCarty, 1991c; Henry and Grbić-Galić, 1991; 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 were 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. Ely et al. (1995a, b)

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adopted an alternative approach focusing on enzyme inactivation and recovery. This approach has the advantage that it can account for differences in enzyme activity within a population and does not require assumptions about "active biomass," but it neglects cell death due to product toxicity or reducing power depletion and requires a sophisticated model calibration procedure. Recently, Chang and Alvarez-Cohen (1995a) proposed a general model that includes reducing energy explicitly as a limiting reactant during cometabolism. A simplified case of their model (resting cells) was verified for phenol-oxidizers, propane-oxidizers, and toluene-oxidizers. The resting cell case analyzed by Chang and Alvarez-Cohen neglected endogenous decay as a cause of cell death, assuming instead that product toxicity was the major cause of cell death. They also applied the model to describe growing cell transformation of TCE by methanotrophs, but several parameter estimates were difficult to obtain. For the methanotrophs, the effects of reducing power were separated from toxicity effects by supplying the cells with formate. Formate provides methanotrophs with energy as NADH, but does not support growth, so that formate addition can be used to determine transformation capacity due to product toxicity alone. Unfortunately, formate or a similar substrate could not be identified for phenol-oxidizers, propane-oxidizers, and toluene-oxidizers (Chang and Alvarez-Cohen, 1995b). Thus, the 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 article, the predictive capabilities of a general model of cometabolism are verified using independently obtained parameter estimates. The theoretical basis for this model is described elsewhere (Criddle, 1993). The model 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 model does not require use of energy substrates that do not support growth, such as formate, for model verification. 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). Loss of transformation activity is assumed to result from cell death, either from product toxicity or endogenous decay. In previous experimental work, this model was verified for resting cells (Chang and Criddle, 1995) and for growing cells using best-fit degradation data (Chang et al., 1993), but competitive inhibition between growth and nongrowth substrate was not evaluated, and the model was not subjected to a thorough sensitivity analysis. A systematic method for evaluating model parameters is developed in this work. 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. This choice of experimental system enabled us to evaluate one of the most complex cases of cometabolism (involving competitive inhibition and inactivation due to loss of reducing power and product toxicity), thereby providing a stringent test of the model. In 1985, Wilson and co-workers reported that 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, c). Oldenhuis et al. (1991) 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., 1989, 1991).

MODEL DEVELOPMENT

A cometabolic model was evaluated that included terms for the loss of microbial biomass or enzyme activity caused by auto-oxidation (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:

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

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

$$\mu = Y_m q_s - b - \frac{q_c}{T_c} \quad (3)$$

The endogenous decay term, b , accounts for loss of energy reserves in the absence of nongrowth substrate. This parameter represents the rate of loss of cometabolism activity in a suspension of resting cells that has not previously been exposed to nongrowth substrate. The rate of loss of nongrowth substrate transformation activity in the presence of nongrowth substrate also includes product toxicity. Product toxicity is described exclusively by the transformation capacity term, T_c .

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:

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

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

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

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

$$\mu = -b - \frac{q_c}{T_c} \quad (7)$$

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 :

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

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

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

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 a 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, 2 mol of electrons are consumed for every mole of nongrowth substrate transformed, but this ratio could decrease if the byproducts of transformation are further oxidized by the cometabolizing community or it could increase if the oxygenase oxidizes other substances besides the nongrowth substrate.

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:

$$-\frac{dM_c}{dt} = q_c X V_L \quad (10)$$

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

$$-\frac{dM_s}{dt} = q_s X V_L \quad (12)$$

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

Batch cometabolic transformation in the presence of growth substrate can be described by Eqs. (1)–(5) and (10)–(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 ($C \ll K_c$ in the absence of growth substrate) have been previously verified (Chang and Criddle, 1995).

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 of S. 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 culture was observed by electron-microscope. The methanotroph was a nonmotile irregularly shaped coccobacillus that contained the internal membrane structure characteristics of type II methanotrophs (Henry and Grbić-Galić, 1990). 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 Fe EDTA, 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 ($\sim 21^\circ C$) in a continuously stirred 2-L bottle that 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 L 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 Chemical Co., Milwaukee, WI) to a 250-mL glass bottle capped with a TFE-lined Mininert valve. The bottle was vigorously shaken and allowed to settle at least 24 h. 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, 30 m \times 0.53 mm 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 the 0.5-mL Pressure-Lok Series A-2 gas syringe and injecting the samples onto an HP 5890 series II gas chromatograph equipped with a column (6' \times 1/8" in SS packed with 80/100 washed molecular sieve 13 \times) and a thermal conductivity detector. The GC was operated isothermally at 50°C with helium as carrier. The temperatures 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- μ m filters (Gelman Sciences Inc., Ann Arbor, MI). The filters were prepared by first soaking them in mineral media for 10 min, 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 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 cylinders (99.0% CH₄, Alltech Associates, 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 Verification

The model evaluated in this work was verified using the procedure illustrated in Figure 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. Propagation of errors was used to quantify errors in parameter estimates and in the final prediction (Mandel, 1984). All nonlinear parameter estimates were obtained by nonlinear regression using Systat 5.2.1 (Systat, Inc., Evanston, IL).

Maximum Specific Rate of Transformation for Nongrowth Substrate and Half-Saturation Coefficient of Nongrowth Substrate

K_c and k_c were determined by adding a range of concentrations of TCE to batch cultures of cells harvested in mid-log growth phase from a batch reactor and then incubated in the absence of methane. The initial concentrations C_o were selected so that the corresponding rates ranged from the maximum to half the maximum ($C_o \sim K_c$). 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 . A precaution that must be observed in this case is that the measured initial rates be in the linear range ($r^2 > 0.95$). If inactivation occurs, the rates will slow down, and the initial rates will not be linear. Typically, concentration changes were measured at five or more time points over a 2-h time period. The time-frame of these measurements must be short enough to avoid appreciable loss of activity that would be detected by a decrease in the rate of transformation.

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

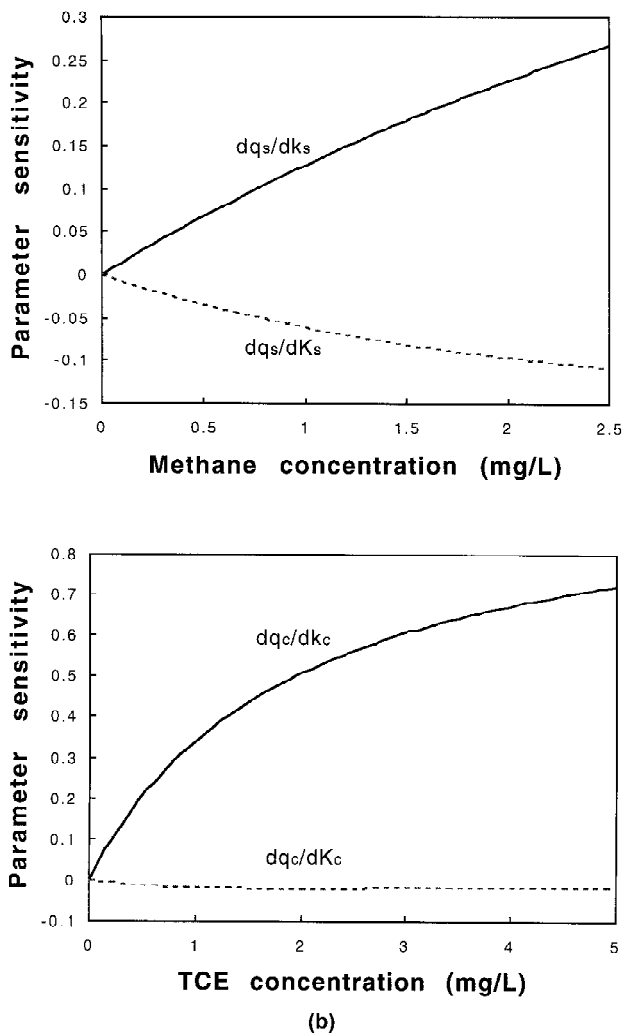
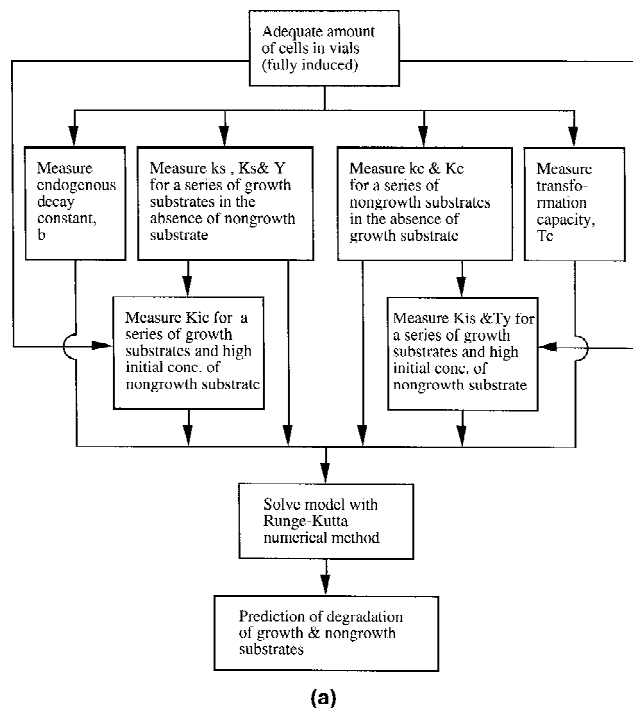


Figure 1. Approach for prediction of degradation of growth and nongrowth substrates by the proposed model.

concentrations S_o were selected so that the corresponding rates ranged from the maximum to half the maximum ($S_o \sim K_s$). 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 specified period of time. The value was obtained during the growth phase, before decay of cell biomass was significant.

Endogenous Decay Coefficient

The endogenous decay coefficient represents loss of TCE transformation activity in the absence of nongrowth substrate. This case is obtained from Eq. (7), where $C = 0$, so $q_c = 0$ and $\mu = -b$. For a batch culture, $dX/dt = -bX$ and $\ln(X/X_0) = -bX$. To measure this value, a sample of mid-log growth phase cells was withdrawn from a batch reactor and aerated over an extended period of time. Subsamples were periodically withdrawn and spiked with high concen-

trations of TCE, so that $C_0 \gg K_c$. 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. Therefore, a semilog plot of active fraction vs. time yields a straight line with slope of $-b$. The endogenous decay coefficient evaluated in this way includes loss of TCE transformation activity caused by cell death and depletion of reducing power. This value is usually greater than the endogenous decay coefficient computed from biomass data.

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 $C_0 \gg K_c$, 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:

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

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 Eqs. (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 .

Sensitivity Analysis

Sensitivity analysis (Robinson, 1985; Robinson and Characklis, 1984) was performed to evaluate the uniqueness of parameter estimates and the relative importance of parameters over the range of substrate concentrations. Three equations derived from the model of Eqs. (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 concentrations. If sensitivity equations for each pair of parameters are not multiples of each other over a wide range of substrate concentrations, 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.

Stoichiometry Evaluation

To further assess the predictive capabilities of the model, parameter estimates were used in the model to predict stoichiometric changes in the ratio of TCE to methane in batch experiments. Observed transformation yield was obtained as the ratio of mass of TCE consumed to the mass of methane consumed. Predicted transformation yield was obtained from the ratio of q_c to q_s [i.e., Eqs. (1) and (2)] with parameter estimates obtained using the methods described above. Other stoichiometric terms were also measured, such

as the oxygen consumed per mole of methane consumed and the observed yield. To obtain appreciable and quantifiable changes, high initial levels of biomass and methane were employed. Low methane concentrations were avoided because changes in biomass levels would have been more difficult to assess.

RESULTS AND DISCUSSION

Cometabolism is a complex phenomenon, especially when both growth and nongrowth substrates are present simultaneously. 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 used 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.

In general, the sensitivity equations for each pair of parameters were not multiples of one another over a wide range of substrate concentrations (Fig. 2). For k_s and K_s , a strong negative correlation was observed (Fig. 2), so that unique values may not have been obtained for these parameters in this system. A weak correlation was observed for k_c and K_c , indicating that these estimates should be unique. Sensitivity equations with respect to parameters in Eqs. (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

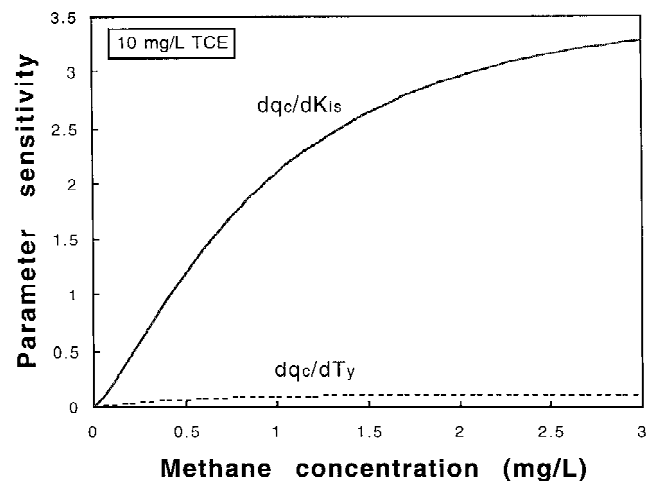


Figure 2. Sensitivity equations for parameters used in model. Concentrations shown on the x-axis span the concentration range used for parameter estimation.

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

Parameters	Value ^a
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
Theoretical transformation yield, T_y	4.01 (± 1.20) mg TCE/mg methane

^aValues represent the 95% confidence interval for triplicate data. Three data sets were evaluated for each nonlinear regression analysis.

equation with respect to K_{is} reached a maximum at the lower concentration of growth substrate.

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 (Table I) suggest that these parameters were different under the present experimental conditions, although the correlation between k_s and K_s precludes a definitive conclusion. A difference between these parameters might be caused by the fact that measurements were performed with whole cells rather than purified enzymes. Factors other than competition for the active site of the enzyme could conceivably influence

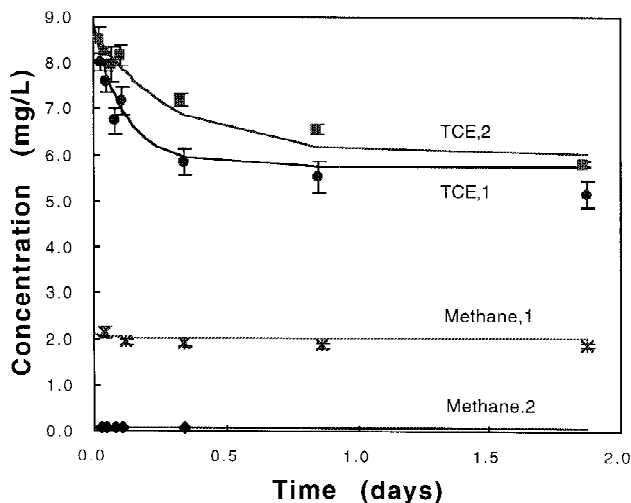


Figure 3. Biotransformation of TCE and methane by methanotrophic mixed culture: comparison with model predictions (for different initial methane concentration). Error bars indicate the 95% confidence interval for triplicate samples.

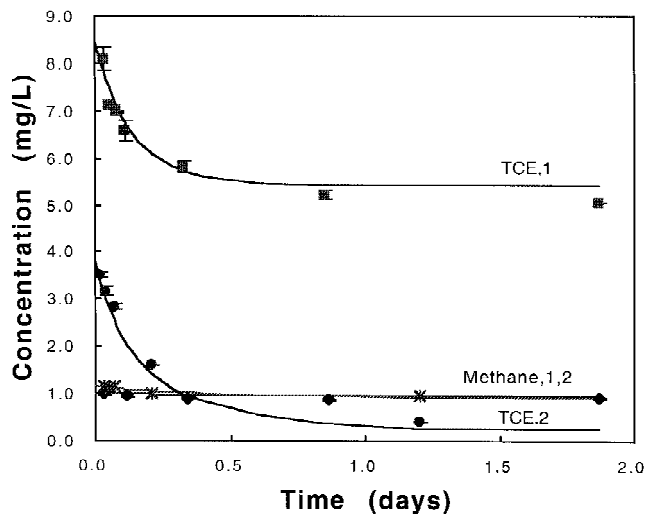


Figure 4. Biotransformation of TCE and methane by methanotrophic mixed culture: comparison with model prediction (for different initial TCE concentration). Error bars indicate the 95% confidence interval for triplicate samples.

the interactions of growth and nongrowth substrates; for instance, reductant supply or substrate transport to the enzyme (Landa et al., 1994). A similar observation was reported for toluene and TCE by Landa et al. (1994).

Independently measured kinetic parameters for TCE degradation are summarized in Table I. Using these parameters, the model was solved numerically to predict methane and TCE degradation under various conditions. Figure 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 (Fig. 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 small amount of methane significantly enhanced TCE degradation, but increases in methane concentration beyond a specific threshold value provided minimal incremental enhancement in TCE transformation (Fig. 5). The threshold value for methane enhancement of TCE transformation was lower at lower TCE concentrations. This pattern can be explained by competitive inhibition between growth and nongrowth substrates. When TCE concentration is low, methane has a greater chance to occupy active sites in the methane monooxygenase, and TCE transformation is inhibited. A similar result was obtained by Chang and Alvarez-Cohen (1995b).

The effect of nongrowth substrate on stoichiometry of cometabolic transformation was also evaluated. The results (Table II) show that an increase in TCE concentration increases the observed transformation yield. It appears that methane is more efficiently used for transformation of TCE

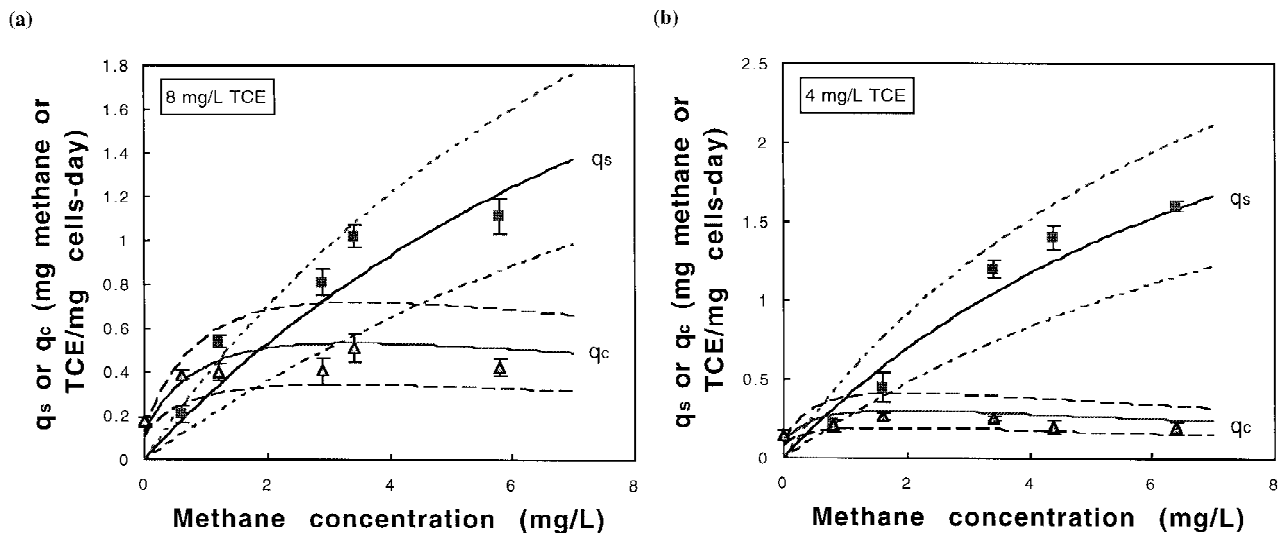


Figure 5. The effect of methane concentration on TCE and methane degradation rate when both substrates are present at same time. (a) Initial concentration of TCE is 8 mg/L and (b) Initial concentration of TCE is 4 mg/L. Error bars indicate 95% confidence interval. Solid lines are predicted values using parameters listed in Table I. Dashed lines indicate propagation of errors in prediction due to errors in parameter estimates.

at high concentrations of TCE. This result is confirmed by the results in Table IV and Figure 6. For higher ratios of TCE to methane, the cultures exhibited net decay after 2 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 cannot 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 III) 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 cannot occur. In Table III, the sum of f_s , f_e , and f_{co} exceed 1. This indicates some error in the electron balance assumptions—perhaps due to errors in the assumed biomass formula.

Inactivation of methane monooxygenase can be caused

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

Initial TCE concentration (mg/L)	Initial CH ₄ concentration (mg/L)	$\Delta\text{TCE}/\Delta\text{CH}_4$ (mol TCE/mol CH ₄)	$\Delta\text{O}_2/\Delta\text{CH}_4$ (mol O ₂ /mol CH ₄)	$\Delta X/\Delta\text{CH}_4^a$ (mol cells/mol CH ₄)
0.0	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

^aThe formula of cells assumed to be C₅H₇O₂N, and yields measured after 2-day incubation.

by the availability of reducing power, endogenous 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 7. ($T_{c,obs}$ is much lower than T_c when growth substrate concentration is low. This is caused by the fact that endogenous decay becomes more significant at low concentrations 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.

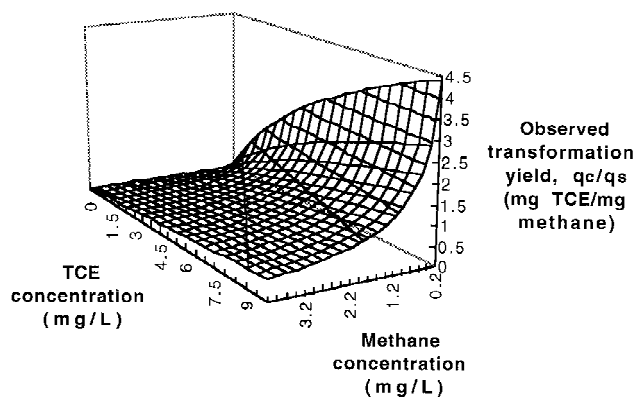


Figure 6. The observed transformation yield (predicted) as a function of the concentration of growth and nongrowth substrates. Prediction is based on parameters in Table I.

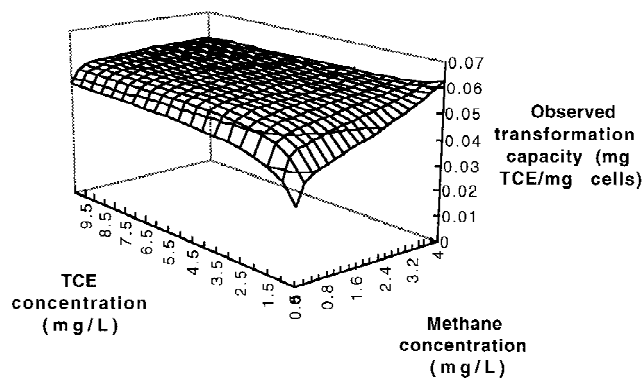


Figure 7. The observed transformation capacity (predicted) as a function of the concentrations of growth and nongrowth substrates. Prediction is based on parameters in Table I.

The observed transformation yields agreed reasonably well with model predictions (Table IV). Of interest is the relative value of these parameters compared to the theoretical value used in the model (T_y). In general, the observed values were less than T_y , although the model predicts that observed values (as obtained from the ratio of q_c to q_s) should exceed T_y at low methane concentrations (Fig. 6). Table IV also shows that observed transformation yield decreases with increasing methane concentration in good agreement with model predictions. Evidently, transformation of TCE was seriously inhibited when high levels of methane were present. Thus, for this culture, the competitive inhibition of methane on TCE transformation can negate the enhancement provided by methane as a source of reducing power and carbon for growth.

In summary, the model presented here can be used to predict simultaneous transformation of growth and nongrowth substrates. In the case of resting cells in the absence of endogenous decay, the model simplifies to a form that is mathematically indistinguishable from the resting cell case verified by Chang and Alvarez-Cohen (1995a) for methane-, propane-, toluene-, and phenol-oxidizers. We have also confirmed the resting cell case, including endogenous

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

Initial TCE concentration (mg/L)	Initial CH ₄ concentration (mg/L)	Fraction of electrons for cometabolism (f_{co}) ^a	Fraction of electron for energy (f_e)	Fraction of electron for synthesis (f_s) ^b
0.0	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

^aAssumes 2 mol of electrons are required per mole of TCE transformation.

^bThe formula of cells assumed to be C₅H₇O₂N, and yields measured after 2-day incubation.

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

Initial TCE concentration (mg/L)	Methane concentration (mg/L)	Observed transformation yield (mg TCE/mg CH ₄)	
		Predicted ^{a,b}	Measured ^b
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

^aErrors calculated from the law of propagation of error.

^bValues represent the 95% confidence interval for triplicate data.

decay, for cometabolism of hydrofluorocarbons and hydrochlorofluorocarbons by the same methanotrophic mixed culture used in this work (Chang and Criddle, 1995). At low concentration of nongrowth substrate, or for cultures with high transformation capacity, endogenous decay is an important mechanism for loss of cell viability and transformation activity. We conclude that the proposed model is suitable for application to a range of growth conditions, organisms, and substrate types. Nevertheless, additional verification using long-term, multigeneration cometabolizing cultures is recommended.

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NOMENCLATURE

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_∞	ultimate concentration of nongrowth substrate at $t = \infty$ (mg nongrowth substrate/L)
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_{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)
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)
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)
μ	specific growth rate of organism (day^{-1})

References

- Alvarez-Cohen, L., McCarty, P. L. 1991a. A cometabolic biotransformation model for halogenated aliphatic compounds exhibiting product toxicity. *Environ. Sci. Technol.* **25**: 1381–1387.
- Alvarez-Cohen, L., McCarty, P. L. 1991b. Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. *Appl. Environ. Microbiol.* **57**: 228–235.
- Alvarez-Cohen, L., McCarty, P. L. 1991c. Product toxicity and cometabolic competitive inhibition modeling of chloroform and trichloroethylene transformation by methanotrophic resting cells. *Appl. Environ. Microbiol.* **57**: 1031–1037.
- Anderson, J. E., McCarty, P. L. 1994. Model for treatment of trichloroethylene by methanotrophic biofilms. *J. Environ. Eng.* **120**: 379–400.
- Broholm, K., Christensen, T. H., Jensen, B. K. 1992. Modelling TCE degradation by a mixed culture of methane-oxidizing bacteria. *Water Res.* **26**: 1177–1185.
- Chang, H.-I., Alvarez-Cohen, L. 1995a. Model for the cometabolic biodegradation of chlorinated organics. *Environ. Sci. Technol.* **29**: 2357–2367.
- Chang, H.-I., Alvarez-Cohen, L. 1995b. Transformation capacities of chlorinated organics by mixed cultures enriched on methane, propane, toluene, or phenol. *Biotechnol. Bioeng.* **45**: 440–449.
- Chang, M.-k., Voice, T. C., Criddle, C. S. 1993. Kinetics of competitive inhibition and cometabolism in the biodegradation of benzene, toluene, and *p*-xylene by two *Pseudomonas* isolates. *Biotechnol. Bioeng.* **41**: 1057–1065.
- Chang, W.-k., Criddle, C. S. 1995. Biotransformation of HCFC-22, HCFC-142b, HCFC-123, HFC-134a by methanotrophic mixed culture MM1. *Biodegradation* **6**: 1–9.
- Criddle, C. S. 1993. The kinetic of cometabolism. *Biotechnol. Bioeng.* **41**: 1048–1056.
- Criddle, C. S., Alvarez, L. A., McCarty, P. L. 1991. Microbial processes in porous media, pp. 639–691. In: J. Bear and M. Y. Corapcioglu (eds.), *Transport processes in porous media*. Kluwer, Dordrecht.
- Ely, R. L., Hyman, M. R., Arp, D. J., Guenther, R. B., Williamson, K. J. 1995a. A cometabolic kinetics model incorporating enzyme inhibition, inactivation, and recovery: II. Trichloroethylene degradation experiments. *Biotechnol. Bioeng.* **46**: 232–245.
- Ely, R. L., Williamson, K. J., Guenther, R. B., Hyman, M. R., Arp, D. J. 1995b. A cometabolic kinetics model incorporating enzyme inhibition, inactivation, and recovery: I. Model development, analysis, and testing. *Biotechnol. Bioeng.* **46**: 218–231.
- Fliermans, C. B., Phelps, T. J., Ringelberg, D., Mikell, A. T., White, D. C. 1988. Mineralization of trichloroethylene by heterotrophic enrichment cultures. *Appl. Environ. Microbiol.* **54**: 1709–1714.
- Fogel, M. M., Taddeo, A. R., Fogel, S. 1986. Biodegradation of chlorinated ethene by a methane-utilizing mixed culture. *Appl. Environ. Microbiol.* **51**: 720–724.
- Fox, B. G., Borneman, J. G., Wackett, L. P., Lipscomb, J. D. 1990. Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. *Biochemistry* **29**: 6419–6427.
- Henry, S. M., Grbić-Galić, D. 1990. Effect of mineral media on trichloroethylene oxidation by aquifer methanotrophs. *Microb. Ecol.* **20**: 151–169.
- Henry, S. M., Grbić-Galić, D. 1991. Influence of endogenous and exogenous electron donors and trichloroethylene oxidation toxicity on trichloroethylene oxidation by methanotrophic cultures from a groundwater aquifer. *Appl. Environ. Microbiol.* **57**: 236–244.
- Hopkins, G. D., Munakata, J., Semprini, L., McCarty, P. L. 1993. Trichloroethylene concentration effects on pilot field-scale in-situ groundwater bioremediation by phenol-oxidizing microorganisms. *Environ. Sci. Technol.* **27**: 2542–2547.
- Horvath, R. S. 1972. Microbial co-metabolism and the degradation of organic compounds in nature. *Bacteriol. Rev.* **36**: 146–155.
- Landa, A. S., Sipkema, E. M., Weijma, J., Beenackers, A. A. C. M., Dolfing, J., Janssen, D. B. 1994. Cometabolic degradation of trichloroethylene by *Pseudomonas cepacia* G4 in a chemostat with toluene as the primary substrate. *Appl. Environ. Microbiol.* **60**: 3368–3374.
- Little, C. D., Palumbo, A. V., Herbes, S. E., Lidstrom, M. E., Tyndall, R. L., Gilmer, P. J. 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. *Appl. Environ. Microbiol.* **54**: 951–956.
- Mandel, J. 1984. *The statistical analysis of experimental data*. Dover Publications, New York.
- Newman, L. M., Wackett, L. P. 1991. Fate of 2,2,2-trichloroacetaldehyde (chloral hydrate) produced during trichloroethylene oxidation by methanotrophs. *Appl. Environ. Microbiol.* **57**: 2399–2402.
- Oldenhuis, R., Oedzes, J. Y., van der Waarde, J. J., Janssen, D. B. 1991. Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. *Appl. Environ. Microbiol.* **57**: 7–14.
- Oldenhuis, R., Vink, R. L. J. M., Janssen, D. B., Witholt, B. 1989. Degradation of chlorinated aliphatic hydrocarbons by *methylosinus trichosporium* OB3b Expressing soluble methane monooxygenase. *Appl. Environ. Microbiol.* **55**: 2819–2826.
- Robinson, J., Characklis, W. G. 1984. Simultaneous estimation of V_{max} , K_m , and the rate of endogenous substrate production (R) from substrate depletion data. *Microb. Ecol.* **10**: 165–178.
- Robinson, J. A. 1985. Determining microbial kinetic parameters using nonlinear regression analysis, pp. 61–114. In: K. C. Marshall (ed.), *Advances in microbial ecology*, vol. 8. Plenum Press, New York.
- Saéz, P. B., Rittmann, B. E. 1991. Biodegradation kinetics of 4-chlorophenol, an inhibitory co-metabolite. *Res. J. Water Pollut. Control Fed.* **63**: 838–847.
- Saéz, P. B., Rittmann, B. E. 1993. Biodegradation kinetics of a mixture containing a primary substrate (phenol) and an inhibitory co-metabolite (4-chlorophenol). *Biodegradation* **4**: 3–21.
- Strand, S. E., Bjelland, M. D., Stensel, H. D. 1990. Kinetics of chlorinated hydrocarbon degradation by suspended cultures of methane-oxidizing bacteria. *Res. J. Water Pollut. Control Fed.* **62**: 124–129.
- Uchiyama, H., Nakajima, T., Yagi, O., Nakahara, T. 1992. Role of heterotrophic bacteria in complete mineralization of trichloroethylene by *Methylocystis* sp. strain M. *Appl. Environ. Microbiol.* **58**: 3067–3071.
- Wackett, L. P., Householder, S. R. 1989. Toxicity of trichloroethylene to *Pseudomonas putida* F1 is mediated by toluene dioxygenase. *Appl. Environ. Microbiol.* **55**: 2723–2725.
- Wilson, J. T., Wilson, B. H. 1985. Biotransformation of trichloroethylene in soil. *Appl. Environ. Microbiol.* **49**: 242–243.