A Combined Method for Determining Inhibition Type, Kinetic Parameters, and Inhibition Coefficients for Aerobic Cometabolism of 1,1,1-Trichloroethane by a Butane-Grown Mixed Culture

Young Kim,1 Daniel J. Arp,2 Lewis Semprini1

1Department of Civil, Construction, and Environmental Engineering, Oregon State University, Corvallis, Oregon 97331-2302; e-mail: lewis.semprini@orst.edu
2Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331

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Abstract: A combined method for determining inhibition type, kinetic parameters, and inhibition coefficients is developed and presented. The method was validated by applying it to data obtained from batch kinetics of the aerobic cometabolism of 1,1,1-trichloroethane (1,1,1-TCA) by a butane-grown mixed culture. The maximum degradation rates (kmax) and half-saturation coefficients (Ks) were independently determined in single compound tests, and compared with those obtained from inhibition tests. The inhibition type was determined using direct linear plots at various substrate and inhibitor concentrations. Kinetic parameters (kmax and Ks) and inhibition coefficients (Kic and Ki) were determined by nonlinear least squares regression (NLSR) fits of the inhibition model determined from the direct linear plots. Initial guesses of the kinetic parameters for NLSR were determined from linearized inhibition equations that were derived from the correlations between apparent maximum degradation rates (kapp) and/or the apparent half-saturation coefficient (Kapp) and the kmax, Ks, and inhibitor concentration (I) for each inhibition equation. Two different inhibition types were indicated from the direct linear plots: competitive inhibition of 1,1,1-TCA on butane degradation, and mixed inhibition of 1,1,1-TCA transformation by butane. Good agreement was achieved between independently measured kmax and Ks values and those obtained from both NLSR and the linearized inhibition equations. The initial guesses of all the kinetic parameters determined from linear plots were in the range of the values estimated from NLSR analysis. Overall the results show that use of the direct linear plot method to identify the inhibition type, coupled with initial guesses from linearized plots for NLSR analysis, results in an accurate method for determining inhibition types and coefficients. Detailed studies with pure cultures and purified enzymes are needed to further demonstrate the utility of this method. © 2002 John Wiley & Sons, Inc. Biotechnol Bioeng 77: 564-576, 2002; DOI 10.1002/bit.10145

Keywords: direct linear plot; linearized inhibition equations; nonlinear least squares regression; inhibition type; kinetic parameters; aerobic cometabolism of 1,1,1-trichloroethane

INTRODUCTION

Competitive inhibition is an important process to consider in the aerobic cometabolism of chlorinated aliphatic hydrocarbons (CAHs), because there is competition between CAH and growth substrate for enzyme active sites due to lack of enzyme specificity. Competitive inhibition during aerobic cometabolism has been most widely proposed and successfully modeled with methane oxidizers, Nitrosomonas europaea, and Pseudomonas cepacia G4 (Anderson and McCarty, 1994, 1996; Chang and Alvarez-Cohen, 1995; Chang and Criddle, 1997; Ely et al., 1997; Landa et al., 1994). However, different inhibition types have also been observed. Keener and Arp (1993) reported noncompetitive inhibition of chloromethane and chloroethane and competitive inhibition of methane and ethylene on NH4+-dependent NO2- production by N. europaea. Keenan and co-workers (1994) reported that propane inhibition of trichloroethylene (TCE) transformation by a propane-oxidizing enrichment culture was best fit by a noncompetitive inhibition model. However, studies to determine the inhibition type between the growth substrate and the CAH are limited.

Convenient linearized plots, especially Lineweaver-Burk plot, have been widely used to determine the inhibition type and to estimate kinetic parameters for the aerobic cometabolism of CAHs (Alvarez-Cohen and McCarty, 1991; Chang and Alvarez-Cohen, 1995, 1996, 1997; Keener and Arp, 1993). Visual determination of inhibition type and the estimation of kinetic parameters from classical linear plotting are not straightforward as

Correspondence to: L. Semprini
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discussed by many researchers (Dowd and Riggs, 1965; Eisenthal and Cornish-Bowden, 1974; Robinson, 1985; Robinson and Charaklis, 1984).

Here we present a combined method for determining the inhibition type, the kinetic parameters, and the inhibition coefficients. The direct linear plot method of Eisenthal and Cornish-Bowden (1974) was used to determine the inhibition type. This method has several advantages over other convenient linearized plots, such as Lineweaver-Burk and Hanse plot. The main advantage is that data inversion is not required. With linear regression methods, outliers can dramatically affect kinetic parameter estimates. However, the direct linear plot method is less sensitive to outliers, because the best estimate is the median rather than the mean. Nonlinear least squares regression (NLSR) analysis has been shown to be a better method for estimating the kinetic parameters than the convenient linearized plots (Robinson, 1985; Robinson and Charaklis, 1984). However, distinguishing between inhibition models can be problematic when using NLSR, and fairly accurate initial guesses of parameters are required. This study presents a combined method, both direct linear plots to identify the inhibition type and NLSR analysis to estimate the kinetic parameters, using graphically estimated kinetic parameters as initial guesses, for evaluating kinetic parameters, inhibition types, and inhibition coefficients.

Laboratory and field studies have shown that 1,1,1-trichloroethane (1,1,1-TCA), which is one of major CAH-contaminants in groundwater is difficult to treat through aerobic cometabolism. Only slow rates of 1,1,1-TCA transformation were observed in the laboratory with Methylosinus trichosporium OB3b and its mutant (Aziz et al., 1999; Oldenhuis et al., 1991). During in situ studies with methane-utilizing microorganisms (Semprini et al., 1990) or with phenol utilizing microorganisms (Hopkins et al., 1993; Hopkins and McCarty, 1995), 1,1,1-TCA was not transformed, despite effective transformation of chlorinated ethenes. In our previous studies with resting cells, a butane-grown mixed culture effectively transformed 1,1,1-TCA (Kim et al., 2000). For example, the culture had a greater ability to transform 1,1,1-TCA than a methane-grown mixed culture on the basis of amount transformed per unit mass cells (Chang and Alvarez-Cohen, 1996). Thus, the method was applied to inhibition kinetic studies of 1,1,1-TCA transformations with this butane-grown culture.

**MATERIALS AND METHODS**

**A Butane-Utilizing Mixed Culture**

The butane-utilizing enrichment was obtained from Hanford soil microcosms and grown in batch incubations described by Kim et al. (1997, 2000). To obtain a reproducible inoculum for the kinetic tests, the cells grown in media were washed, and cell suspensions (1 mL) were transferred to autoclaved 2-mL cryogenic vials (Nalgene Company, Rochester, NY), containing 70 µL of dimethyl sulfoxide (DMSO). The vials were stored in liquid nitrogen. Over the course of the kinetic tests the stored cells were used as the inoculum for batch-grown cells. This method of storing cells showed stable activity in methanotrophic studies (Anderson and McCarty, 1996, 1997) and in our studies.

To grow cells for the batch kinetic tests, the frozen cells were thawed, washed, and resuspended three times to remove DMSO and grown in batch growth reactors on 10% butane (vol/vol) in air. The cells (250 mL) were incubated in the dark at 30°C on a rotary table at 210 rpm, and harvested at OD_{600} of 0.6. The cells were concentrated by three cycles of centrifuging (6,000 x g for 15 minutes), washing, and resuspending in media. Based on butane uptake activity, resting cell activity was stable for 10 hours after harvesting. Thus, all kinetic tests were performed within 10 hours of harvesting the cells.

**Chemicals**

Butane (≥99%) was purchased from AIRCO (Vancouver, WA). 1,1,1-TCA (99.5% anhydrous) was purchased from Aldrich Chemical Co. (Milwaukee, WI). A saturated aqueous stock solution of 1,1,1-TCA was prepared at 20°C by adding specific amounts of the pure liquid compound to 125-mL serum bottles containing autoclaved deionized water. This procedure eliminated the use of carrier solvents, such as methanol. The bottles were shaken for 6 hours prior to use to ensure saturation, and then allowed to settle for 6 hours before use. Butane was volumetrically transferred to the batch bottles using gastight syringes (Precision Sampling Corp., Baton Rouge, LA).

**Batch Experiments**

Batch kinetic studies were performed at 20°C in 26-mL glass serum vials. Mineral medium (4.5 mL) described by Kim et al. (2000) was added to vials, and the remaining volume (21.5 mL) was filled with air. The vials were then crimp sealed with Teflon-lined rubber septa (Kimble, Vineland, NJ). No external energy source was added. The tests, therefore, relied on the internal energy reserves to drive 1,1,1-TCA transformation as described by Kim et al. (2000).

A volumetric amount of saturated aqueous stock solution of 1,1,1-TCA or butane or both was added into the vials to achieve desired initial aqueous concentrations. The vials were shaken on a rotary shaker at 260 rpm to equilibrate to 20°C. The initial headspace concentration was measured prior to adding the cells to initiate the transformation reaction. After cell addition, the bottles were vigorously hand-shaken for 10 seconds, and then placed on a rotary shaker operated at 260 rpm.
Mass transfer experiments were performed to assure that the equilibrium assumption was valid over the time scale of the kinetic experiments at this shaking speed (Kim, 2000). Headspace concentrations were measured at five equally spaced time intervals over a period of approximately 20 min. The headspace concentrations were converted into total mass in the reaction vial based on mass balance, and then initial transformation/degradation rates were determined by applying linear regression on the data. An example set of data and corresponding linear regression for 1,1,1-TCA inhibition of butane utilization are shown in Figure 1.

Measuring an accurate initial rate of CAH transformation is difficult, because CAHs are transformed with finite capacity due to transformation product toxicity and energy limitations. Therefore, maintaining a low ratio of the amount of CAH transformed to initial cell mass compared to the finite capacity is important to obtain accurate initial rates of CAH transformation. Based on our previous results (Kim et al., 2000), the amount of 1,1,1-TCA transformed per cell mass over 30 hours was 0.33 μmol/mg total suspended solids (TSS), and the initial transformation rate of 1,1,1-TCA was not greatly affected at less than 30% of this value. For the kinetic studies reported here, the amount of 1,1,1-TCA ultimately transformed per milligram TSS over the test period ranged from 5 to 21% of the value. Thus, the ratio of the amount of 1,1,1-TCA transformed to mass of cells added was low enough so the loss in cell activity resulting from 1,1,1-TCA transformation was minimal, and thus did not affect the transformation rate.

Procedures for performing inhibition studies followed the batch kinetic test method described by Cornish-Bowden (1994). For single substrate kinetic tests to determine $k_{\text{max}}$ and $K_v$ values, triplicate vials were prepared at each of 10 different substrate concentrations. Preliminary inhibition experiments were performed to determine the range of concentrations of an inhibitor (a substance that decreases the rate of substrate degradation when present in the reaction mixture) and a substrate (a substance the reaction rate of which is decreased when an inhibitor is present in the reaction mixture) to be used in the studies.

Acetylene Inactivation Experiment

To investigate the kinetic diversity of the butane monooxygenase enzyme in the mixed culture, enzyme inactivation studies as described by Silverman (1988) were conducted. An experiment to evaluate the loss of butane uptake activity on exposure to acetylene, a mechanism-based inactivator (suicide substrate) of methane, ammonia, and butane monooxygenases (Hamamura et al., 1999; Keener et al., 1998; Prior and Daltan, 1985), was performed. The loss in activity as a function of time was used to determine the monooxygenase diversity of the butane-grown mixed culture.

To measure a time-dependent loss of butane uptake activity on exposure to acetylene, 100 μL-acetylene (0.33% vol/vol in headspace) was added into the 50-mL gas tight syringe (Unimetrics No 7450, Folsom, CA) containing cell suspension in media (20 mL) and air (30 mL). The syringe was sealed and hand-shaken throughout the experiment. Samples of the acetylene-treated cell suspension (1.5 mL) were taken as a function of time from the 50-mL gas tight syringe using 5-mL syringes. The acetylene was removed from the samples by equilibrating with air and removing air. The 1-mL cell suspensions were added into 26-mL batch bottles containing growth media (3 mL), butane (0.5 mL pure butane), and headspace air (22 mL). Initial butane degradation rates were measured as described above.

Analysis

The gaseous concentrations of butane and 1,1,1-TCA were determined by analysis of the serum vials headspace. The compound’s masses in the test bottle were calculated, using the headspace and aqueous volumes and published Henry’s constants (Gossett, 1987; Mackay and Shiu, 1981). Calibration curves for the compounds were developed using external standards. Headspace concentrations of the compounds were determined by injecting 100 μL of the headspace sample into a HP5890 series gas chromatograph (GC) connected to a photoionization detector (PID), followed by a flame ionization detector (FID) at 250°C. The GC was operated at the following conditions: oven temperature, 190°C; carrier
gas (He) flow, 15 mL/min; H₂ flow to detectors, 35 mL/min; airflow to detectors, 165 mL/min; and FID detector makeup gas (He) flow, 15 mL/min. Chromatographic separation was performed with a 30-m megabore GSQ-PLOT column from J&W Scientific (Folsom, CA).

Culture density was determined as TSS (American Public Health Association, 1985), using 0.1-μm membrane filter (Micro Separation Inc., Westboro, MA). The OD₆₀₀ of cultures was measured using an HP8453 UV-visible spectrophotometer.

A COMBINED METHOD FOR DETERMINING INHIBITION TYPE AND KINETIC PARAMETERS

Inhibition Types

For batch kinetic and inhibition studies the Michaelis-Menten equation can be modified to include a mass balance between the air and aqueous phase, assuming equilibrium partitioning. Mass transfer experiments were performed to assure that the equilibrium assumption was valid over the time scale of the kinetic experiments (Kim, 2000). The modified Michaelis-Menten equation for a substrate and the substrate concentration in the liquid phase are provided in Eqs. (1), (2), and (3),

\[
v = \frac{-k_{\text{app}}^{\max} S_L}{K_s^{\text{app}} + S_L} \quad (1)
\]

\[
S_L = \left( \frac{M_i}{V_L + V_G H_i} \right) \quad (2)
\]

\[
v = \frac{-k_{\text{app}}^{\max} \left( \frac{M_i}{V_L + V_G H_i} \right)}{K_i^{\text{app}} + \left( \frac{M_i}{V_L + V_G H_i} \right)} \quad (3)
\]

where \( v \) is specific substrate degradation rate (μmol/mg TSS/h), and \( k_{\text{app}}^{\max} \) and \( K_i^{\text{app}} \) are the apparent values of \( k_{\text{max}} \) and \( K_s \) in the presence of inhibitor, respectively. Thus, \( k_{\text{app}}^{\max} \) and \( K_i^{\text{app}} \) are equal to \( k_{\text{max}} \) and \( K_s \), respectively, in the absence of inhibitor. \( S_L \) is the substrate concentration in liquid phase (μM), \( M_i \) is the total substrate mass in bottle (μmol), \( H_i \) is the dimensionless Henry’s constant of substrate, \( V_L \) is the volume of liquid phase (L), and \( V_G \) is the volume of gas phase (L).

For each of the four inhibition types—competitive, uncompetitive, mixed, and noncompetitive inhibition (Cornish-Bowden, 1994)—the inhibition model equation can be written by substituting \( k_{\text{app}}^{\max} \) and \( K_i^{\text{app}} \) as presented in Table I into Eq. (1). In Table I, \( K_i \) and \( K_i^{\text{app}} \) are inhibition coefficients (μM), and \( I_L \) is an inhibitor concentration in liquid phase (μM) expressed by Eq. (4),

\[
I_L = \left( \frac{M_i}{V_L + V_G H_i} \right) \quad (4)
\]

where \( M_i \) is total mass of inhibitor in bottle (μmol) and \( H_i \) is a Henry’s constant of an inhibitor. For purified enzymes with one substrate and an unreactive inhibitor, \( K_i \) and \( K_i^{\text{app}} \) are the equilibrium constants for inhibitor binding to the free enzyme \( E \) and the enzyme-substrate complex \( ES \), respectively (Cornish-Bowden, 1994). We assume that the kinetics for purified enzymes can be applied to whole cells.

Table I: The effects of inhibitors on the parameters of the Michaelis-Menten equation and linearized inhibition equations.

<table>
<thead>
<tr>
<th>Type of inhibition</th>
<th>( k_{\text{app}}^{\max} )</th>
<th>( K_i^{\text{app}} )</th>
<th>Linearized inhibition equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed (Mix)</td>
<td>( \frac{k_{\text{app}}^{\max}}{1 + \frac{I_L}{k_s}} )</td>
<td>( \frac{K_i^{\text{app}}}{K_s} )</td>
<td>( I_L = \frac{K_i^{\text{app}}}{k_{\text{app}}^{\max} + \frac{K_i^{\text{app}}}{K_s}} )</td>
</tr>
<tr>
<td>Noncompetitive (NC)</td>
<td>( \frac{k_{\text{app}}^{\max}}{1 + \frac{I_L}{k_s}} )</td>
<td>( K_s )</td>
<td>( I_L = \frac{K_i^{\text{app}}}{k_{\text{app}}^{\max} + \frac{K_i^{\text{app}}}{K_s}} )</td>
</tr>
<tr>
<td>Competitive (Com)</td>
<td>( k_{\text{app}}^{\max} )</td>
<td>( k_{\text{app}}^{\max} )</td>
<td>( K_i^{\text{app}} = K_s + \frac{K_i^{\text{app}}}{K_s} I_L )</td>
</tr>
<tr>
<td>Uncompetitive (UC)</td>
<td>( \frac{k_{\text{app}}^{\max}}{1 + \frac{I_L}{k_s}} )</td>
<td>0</td>
<td>( I_L = \frac{K_i^{\text{app}}}{k_{\text{app}}^{\max} + \frac{K_i^{\text{app}}}{K_s}} )</td>
</tr>
</tbody>
</table>

Note: \( I_L \) – inhibitor concentration in liquid phase (μM); \( K_i \) = competitive inhibition coefficient (μM); \( K_i^{\text{app}} \) = uncompetitive inhibition coefficient (μM). Adapted from Cornish-Bowden, 1994.

Direct Linear Plot

The direct linear plot of Eisenthal and Cornish-Bowden (1974) and Cornish-Bowden and Eisenthal (1978) was...
used to determine the inhibition types. Eq. (1) can be rearranged to show the dependence of $k_{\text{max}}^{\text{opp}}$ on $K_{\text{s}}^{\text{opp}}$:

$$
\frac{k_{\text{max}}^{\text{opp}}}{v} = \frac{v}{S_L} K_{s}^{\text{opp}}
$$

(5)

If $k_{\text{max}}^{\text{opp}}$ and $K_{s}^{\text{opp}}$ are treated as variables, and $v$ and $S_L$ as constants, this equation defines a straight line of slope $v/S_L$, y-axis intercept $v$ and x-axis intercept $-S_L$. The straight line drawn according to Eq. (5) relates all pairs of $k_{\text{max}}^{\text{opp}}$ and $K_{s}^{\text{opp}}$ values that satisfy one observation exactly. A second line drawn for a second observation will relate all pairs of $k_{\text{max}}^{\text{opp}}$ and $K_{s}^{\text{opp}}$ values that satisfy the second observation exactly. An intersection point of the two lines defines the unique pair of $k_{\text{max}}^{\text{opp}}$ and $K_{s}^{\text{opp}}$ values that satisfy both observations exactly. Ideally, there should be one intersection point that satisfies several observations. However, several intersection points are typically obtained from the plot. The medians of each set of $k_{\text{max}}^{\text{opp}}$ and $K_{s}^{\text{opp}}$ (rather than averages) provide best estimates. Although the median values can be obtained from graphic analyses, external calculation using each set of $k_{\text{max}}^{\text{opp}}$ and $K_{s}^{\text{opp}}$ was performed to provide more accurate values.

An example of a direct linear plot in the case of 1,1,1-TCA transformation in the absence of butane is shown in Figure 2A. Values of $v$ are plotted on the vertical axis and the corresponding negative $S_L$ values are plotted on the horizontal axis. The corresponding points are then joined and extrapolated, with intersections (small open circle) defining a unique pair of $k_{\text{max}}^{\text{opp}}$ and $K_{s}^{\text{opp}}$ values.

![Diagram](https://via.placeholder.com/150)

**Figure 2.** Direct linear plot of $v$ against $-S_L$ in the case of 1,1,1-TCA transformation in the absence of butane (A) and a plot of $k_{\text{max}}^{\text{opp}}$ against $K_{s}^{\text{opp}}$ (B). In panel A the symbols on the vertical axis represent $v$ values, and the same symbols on the horizontal axis represent the corresponding negative $S_L$ values. A small open circle at an intersection of two lines represents a unique pair of $k_{\text{max}}^{\text{opp}}$ and $K_{s}^{\text{opp}}$ values that satisfy two sets of observations, and a large open square symbol represents the best estimates of $k_{\text{max}}^{\text{opp}}$ and $K_{s}^{\text{opp}}$. 

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that satisfy two sets of observations. The medians of each set of \( k_{\text{max}}^{\text{app}} \) and \( K_{\text{app}}^{\text{opp}} \) provide best estimates (large open square symbol). As shown in Figure 2A, the \( k_{\text{max}} \) and \( K_s \) for 1,1,1-TCA are 0.20 \( \mu \text{mol/mg TSS/h} \) and 12 \( \mu \text{M} \), respectively.

As discussed above, the variation patterns of both \( k_{\text{max}}^{\text{app}} \) and \( K_{\text{app}}^{\text{opp}} \) (with increasing \( I_1 \)) can be used to identify the inhibition type. Direct linear plots with only the best estimate (median) point \( (k_{\text{max}}^{\text{app}}, K_{\text{app}}^{\text{opp}}) \) plotted at various levels of \( I_1 \) result in shifting directions of the best estimate point \( (k_{\text{max}}^{\text{app}}, K_{\text{app}}^{\text{opp}}) \) (Fig. 2B). Note that axis titles in Figure 2B are different from those in Figure 2A, because only best estimate values for \( k_{\text{max}}^{\text{app}} \) and \( K_{\text{app}}^{\text{opp}} \) are plotted in Figure 2B. By definition, for competitive inhibition, the shift is to the right; for uncompetitive inhibition, it is directly toward the origin; for mixed inhibition, it is intermediate between these extremes; and for noncompetitive inhibition (a special case of mixed inhibition), it shifts down vertically (Cornish-Bowden, 1994).

**Determination of \( k_{\text{max}}, K_s, K_{ic}, \) and \( K_{iu} \)**

For single compound batch kinetic studies, \( k_{\text{max}} \) and \( K_s \) were determined by fitting the kinetic data to Eq. (3) using NLSR in S-PLUS (MathSoft Inc., Cambridge, MA). To estimate the \( k_{\text{max}} \) and \( K_s \) values by the NLSR routine, degradation/transformation rates along with corresponding substrate and inhibitor concentrations were given as an input data, and Eq. (3) was used to obtain a best fit to the data. Initial estimates (guesses) of \( k_{\text{max}} \) and \( K_s \) for NLSR routine were obtained by Monod plots of rate vs. substrate concentration.

In the inhibition studies, \( k_{\text{max}}, K_s, K_{ic}, \) and \( K_{iu} \) were determined by NLSR analysis for the inhibition model determined by the direct linear plot method. Linear regression of the linearized forms presented in Table I was used to obtain the initial guesses of all kinetic parameters for the NLSR fitting. The derivations of the linearized forms in Table I are presented by Kim (2000). Mixed inhibition is a more complex kinetic expression with more parameters compared to the other kinetic equations. The linearized forms provided good initial parameter guesses that are needed for the NLSR method to converge, especially for the case of mixed inhibition.

**Method Used in This Study**

A combined method for determining kinetic parameters, inhibition type, and inhibition coefficients of aerobic CAH transformation is presented in this study. Plots of \( k_{\text{max}}^{\text{app}} \) vs. \( K_{\text{app}}^{\text{opp}} \) (derived from direct linear plots) at various levels of \( I_1 \) (Fig. 2B) were used to visually identify the inhibition type (Cornish-Bowden, 1994). After identifying the inhibition type, the appropriate linearized equations in Table I were used to obtain initial guesses of kinetic parameters for NLSR analysis. The \( k_{\text{max}}^{\text{app}} \) and \( K_{\text{app}}^{\text{opp}} \) values obtained from direct linear plots were used as data inputs for regression of the linearized equations for all inhibition cases. In the case of mixed inhibition, \( 1/k_{\text{max}}^{\text{app}} \) was plotted against \( I_1 \). The slope and y intercept represents \( 1/(k_{\text{max}}K_{\text{iu}}) \) and \( 1/k_{\text{max}} \), respectively. From these values, \( k_{\text{max}} \) and \( K_{\text{iu}} \) were calculated. The values for \( K_s \) and \( K_{ic} \) were obtained from a second plot of \( K_{\text{app}}^{\text{opp}} / k_{\text{app}}^{\text{opp}} \) vs. \( I_1 \). Thus, for the mixed inhibition case all the parameters were obtained from these two linearized plots. In the case of competitive inhibition, \( K_s^{\text{app}} \) was plotted against \( I_1 \). The slope and intercept represent \( K_s / K_{ic} \) and \( K_s \), respectively. From these values, \( K_s \) and \( K_{ic} \) were calculated. The \( k_{\text{max}} \) was calculated by averaging the values of \( k_{\text{max}}^{\text{app}} \) obtained from direct linear plot. The same procedures can be applied to the other inhibition cases to obtain kinetic parameters.

**RESULTS**

**Acetylene Blocking Experiment**

Results of the acetylene inactivation experiment performed to evaluate the kinetic diversity of butane-oxidizers in the mixed culture are presented in Figure 3. A progressive loss of butane uptake rate as a function of time was observed. The natural log of residual butane uptake activity [rate at time \( t \) \((r)\) to rate at time zero \((r_0)\)] is plotted as a function of time. The results fit a first-order kinetics inactivation model well (\( R^2 = 0.96 \)), and were consistent with observations of Keener et al. (1998).

![Figure 3](attachment:image.jpg)

**Figure 3.** Loss of butane degradation rate as a function of time after acetylene exposure. Data were fit to first order decay model. The ratio of the rate at time \( t \) to that at time zero is represented by \( r_t/r_0 \).
with nitrifying bacteria. This fit to a first-order kinetics inactivation model indicates that kinetically similar monoxygenases are present in a butane-grown mixed culture. Thus, applying the inhibition kinetics developed for homogenous enzyme systems was a reasonable assumption.

**k**\textsubscript{max} and K\textsubscript{s} for Butane and 1,1,1-TCA

k\textsubscript{max} and K\textsubscript{s} for butane and 1,1,1-TCA were determined in single compound tests with the butane-grown mixed culture. In butane batch kinetic tests, the amount of cell mass increase resulting from butane degradation was less than 5% of initial cell mass added. Thus, cell growth had little effect on initial rate of butane degradation. The measured degradation rates of butane (A) and transformation rates of 1,1,1-TCA (B) vs. concentration are provided in Figure 4, along with the best-fit curves to Eq. (3) achieved by NLSR. Excellent agreement to the equation was achieved. The estimated k\textsubscript{max} and the 95% confidence intervals for butane and 1,1,1-TCA were 2.6 ± 0.14 and 0.19 ± 0.01 μmol/mg TSS/h, respectively, whereas the K\textsubscript{s} values were 19 ± 3.3 and 12 ± 2.8 μM, respectively (Table II). For 1,1,1-TCA, k\textsubscript{max} and K\textsubscript{s} were in excellent agreement with those determined from the direct linear plot (Fig. 2A). Although the K\textsubscript{s} values for butane and 1,1,1-TCA are in a similar range, the k\textsubscript{max} for butane degradation is over an order of magnitude greater than the k\textsubscript{max} for 1,1,1-TCA transformation.

**Inhibition Types and Inhibition Coefficients**

**1,1,1-TCA Inhibition on Butane Degradation**

The inhibition of butane degradation by 1,1,1-TCA was first investigated by measuring initial butane degradation rates at four butane concentrations (3.2, 12, 23, 40 μM) and five different inhibitor (1,1,1-TCA) concentrations (0.184, 548, 902, 1228 μM). Butane concentrations were chosen to include values ranging from 0.2 to 2.0 the K\textsubscript{s} for butane. The direct linear plot showing inhibition of 1,1,1-TCA on butane degradation is presented in Figure 5A. The points of intersection, shown as smaller symbols, give the estimate of k\textsuperscript{app} and K\textsuperscript{app}. The best estimate of k\textsubscript{max} and K\textsubscript{app}, shown as the larger symbols, are the medians of the individual values at the various I\textsubscript{L} concentrations (1,1,1-TCA). As the inhibitor concentration increases, the K\textsubscript{app} for butane also increases, whereas k\textsubscript{max} for butane remains essentially constant, indicating competitive inhibition of 1,1,1-TCA on butane degradation.

Kinetic parameters (K\textsubscript{IC} and K\textsubscript{s}) were graphically estimated by the plot of K\textsubscript{app} vs. I\textsubscript{L} (Fig. 5B). For the competitive inhibition model the plot of these variables

| Table II. | Comparison of k\textsubscript{max}, K\textsubscript{IC}, K\textsubscript{s} that are separately estimated from single-compound rate studies, and linear plots and NLSR analysis using rate data in the presence of inhibitors. |
|-----------|-------------------------------|-------------------------------|-------------------------------------------------|
| Method    | k\textsubscript{max} (μmol/mg TSS/h) | K\textsubscript{s} (μM) | k\textsubscript{max} (μmol/mg TSS/h) | K\textsubscript{s} (μM) | K\textsubscript{IC} (μM) |
| Single compound test | 2.6 ± 0.14* | 19 ± 3.3 | 0.19 ± 0.01 | 12 ± 2.8 | |
| Linearized equation | 2.4 | 12 | 0.23 | 18 | 0.52 | 0.36 | 350 |
| NLSR | 2.5 ± 0.31 | 13 ± 4.4 | 0.20 ± 0.007 | 19 ± 2.1 | 0.28 ± 0.1 | 0.51 ± 0.1 | 313 ± 88 |

*The errors represent 95% confidence intervals.
The linear regression values of \( k_{\max} \), \( K_a \), and \( K_{ic} \) (Fig. 5B) were used as initial guesses for the NLSR fitting analysis. The data converged with very small residual standard error (RSE) of 0.073 (Fig. 5C), yielding a \( K_{ic} \) value of 313 ± 8 µM for 1,1,1-TCA, which was in the range of the linearized \( K_{ic} \) value of 350 µM used as the initial guess. \( k_{\max} \) and \( K_a \) for butane were 2.5 ± 0.31 µmol/mg TSS/h and 13 ± 4.4 µM, respectively, in good agreement with the values from the single compound tests.

**Butane Inhibition of 1,1,1-TCA Transformation**

Inhibition of butane on 1,1,1-TCA transformation was also evaluated. As shown in Figure 6A, the shift pattern of \( k_{\max} \) and \( K_{ic} \) with increasing inhibitor (butane) concentration can be interpreted as mixed, uncompetitive, or noncompetitive inhibition. Uncompetitive inhibition is not an appropriate model in this case, because an uncompetitive inhibitor only binds to ES (enzyme substrate complex). That is, it only binds and degrades in the presence of substrate. However, butane degrades in the absence of 1,1,1-TCA, so butane is not an uncompetitive inhibitor on 1,1,1-TCA transformation. Mixed inhibition is more inclusive than noncompetitive inhibition; thus, we interpret it as mixed inhibition of butane on 1,1,1-TCA transformation. The kinetic parameters (\( k_{\max} \), \( K_a \), \( K_{iu} \), and \( K_{ic} \)) were estimated by linear regression of the equations in Table I for the mixed inhibition case (Fig. 6B). Excellent fits to the linearized forms were obtained. The \( k_{\max} \) and \( K_a \) values for 1,1,1-TCA were 0.23 µmol/mg TSS/h and 18 µM, respectively, which agreed well with the values obtained in the single compound tests. The \( K_{iu} \) and \( K_{ic} \) for butane were 0.52 µM and 0.36 µM, respectively. NLSR analysis (shown in Fig. 6C) yielded an excellent agreement to the mixed inhibition model. Both inhibition coefficients (\( K_{iu} = 0.51 ± 0.1 \) µM and \( K_{ic} = 0.28 ± 0.1 \) µM) were similar to the values obtained from the linear plot and used as initial guesses for NLSR (Table II).

**Comparison of Kinetic Parameters Determined with Different Methods**

Table II summarizes \( k_{\max} \) and \( K_a \) values determined from single compound tests and from inhibition tests using linearized equation plots and NLSR analysis. The \( K_a \) of butane determined by both the linear plots and NLSR analyses are slightly lower than the values obtained from the single compound tests. All methods yielded very comparable \( K_a \) values for 1,1,1-TCA. The \( k_{\max} \) values determined by all three methods are in excellent agreement. The results suggest that \( k_{\max} \) and \( K_a \) values can be very effectively determined from inhibition test results by either the linear plot method, or NLSR.
analysis using initial guesses obtained from the linear plot.

**Effects of k_max and/or K_s on Inhibition Coefficients in NLSR Analysis**

In the previous NLSR analyses all kinetic parameters in the inhibition equations were permitted to vary in obtaining the model fit to the experimental data. To evaluate how k_max and/or K_s affect the determination of inhibition coefficients (K_{ic} and K_{in}) during NLSR analysis, k_max and/or K_s obtained from the single compound tests were provided as constants for the NLSR analysis. Four different NLSR analyses were performed: (1) varying all kinetic parameters (k_max, K_s, K_{ic}, and/or K_{in}); (2) constant k_max and K_s and varying inhibition coefficients; (3) constant k_max and varying K_s and inhibition coefficients; and (4) constant K_s and varying k_max and inhibition coefficients.

Table III presents the inhibition coefficients (K_{ic} and/or K_{in}), determined with the four different NLSR analyses using initial guesses of the inhibition parameters from the linearized plot. For both inhibition cases, the NLSR analyses yielded estimated inhibition coefficients the 95% confidence intervals of which overlapped each other. The results suggest that prior determination of k_max and/or K_s in single compound tests is not required.
Table III. Comparison of $K_{ic}$ and $K_{ii}$ obtained with different methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Butane</th>
<th>Butane</th>
<th>1,1,1-TCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_{ec}$ (µM)</td>
<td>$K_{ic}$ (µM)</td>
<td>$K_{ii}$ (µM)</td>
</tr>
<tr>
<td>NLSR $^a$</td>
<td>0.28 ± 0.1</td>
<td>0.51 ± 0.1</td>
<td>313 ± 88</td>
</tr>
<tr>
<td>NLSR $k_{max}$</td>
<td>0.25 ± 0.1</td>
<td>0.56 ± 0.1</td>
<td>419 ± 65</td>
</tr>
<tr>
<td>NLSR $k_{max}$</td>
<td>0.24 ± 0.1</td>
<td>0.56 ± 0.1</td>
<td>318 ± 72</td>
</tr>
<tr>
<td>NLSR $K_c$</td>
<td>0.25 ± 0.1</td>
<td>0.53 ± 0.1</td>
<td>356 ± 64</td>
</tr>
</tbody>
</table>

$^a$Kinetic parameter are presented with 95% confidence interval.
NLSR with all kinetic parameters varying ($k_{max}$, $K_c$, $K_i$, and/or $K_{ii}$).
NLSR with constant $k_{max}$ and $K_c$ and with two inhibition coefficients varying.
NLSR with constant $k_{max}$ and with $K_c$ and two inhibition coefficients varying.
NLSR with constant $K_i$, and with $k_{max}$ and two inhibition coefficients varying.

for determining the inhibition coefficients ($K_{ec}$ and $K_{ii}$). The good agreement of $k_{max}$ and $K_c$ values obtained using different methods, shown in Table II, indicate that this would be the likely result of this analysis.

NLSR Analysis with Different Inhibition Models

To evaluate how the data fit to different inhibition models and how the models affect the estimated kinetic parameters, NLSR analysis using three different inhibition model equations was performed with the inhibition data. For this comparison all kinetic parameters were varied and minimized in the NLSR analysis. This analysis evaluated how the data best fit by a mixed inhibition model would be fit using a competitive inhibition model and vice versa, or how data best fit by a mixed inhibition model fit a noncompetitive inhibition model.

The mixed inhibition and the noncompetitive inhibition model NLSR fits to the data showing competitive inhibition of 1,1,1-TCA on butane degradation (Fig. 5A) are tested, and Table IV summarizes the kinetic and inhibition parameters in this fitting exercise. The inhibition data was also well fit by mixed and noncompetitive inhibition models, having the comparable RSE as competitive inhibition fit. For butane, the $k_{max}$ and $K_c$ values were in the range determined using the competitive inhibition model. This result illustrates the importance of using the direct linear plot to identify the inhibition type, since NLSR does not clearly distinguish between inhibition types based on the RSE values. For the case of mixed inhibition model, $K_{ic}$ was in the range determined using the competitive inhibition model. However, the estimated $K_{ii}$ value was a factor of 26 times greater than $K_{ic}$, and $K_{ii}$ value had much greater error than the $K_{ic}$ estimate. Data showing competitive inhibition is represented by a mixed inhibition model having a very high $K_{ii}$ to compensate for the inhibitor effect on $K_{max}$.

To noncompetitive inhibition and competitive inhibition models. Data showing mixed inhibition of butane on 1,1,1-TCA transformation (Fig. 6A) were fit by NLSR. The data could also be well fit to the noncompetitive inhibition model, having the same RSE as mixed inhibition fit. Data when fit to the competitive inhibition model resulted in a higher RSE than those achieved with the mixed and noncompetitive inhibition models. The $k_{max}$, $K_c$, and/or $K_{ii}$ obtained from noncompetitive and competitive inhibition model fits were in the range of the values determined from the fit to the mixed inhibition model (Table II). The reason for the good fit of the noncompetitive inhibition model is that $K_{ic}$ and $K_{ii}$ for butane were very close, thus $K_{opp}$ is equal to $K_c$ resulting in noncompetitive inhibition model as shown in Table I. This result is consistent with the near vertical shift in $k_{max}$ shown Figure 6A, indicating that it is difficult to distinguish between mixed and noncompetitive inhibition for this case.

DISCUSSION

A systematic kinetic and inhibition study of aerobic cometabolism of 1,1,1-TCA, by a butane-grown mixed culture is presented. The method that combined both direct linear plots to identify the inhibition type and NLSR analysis to estimate the kinetic parameters, using

Table IV. Kinetic parameters ($k_{max}$ and $K_c$) for a substrate and inhibition coefficients for an inhibitor obtained from NLSR analysis by fitting different inhibition models to the data.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Inhibition type</th>
<th>$k_{max}$ (µmol/mg TSS/hr)</th>
<th>$K_c$ (µM)</th>
<th>$K_{ic}$ (µM)</th>
<th>$K_{ii}$ (µM)</th>
<th>Residual standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butane</td>
<td>1,1,1-TCA</td>
<td>Com $^a$</td>
<td>2.53 ± 0.31</td>
<td>13.1 ± 4.4</td>
<td>313 ± 88</td>
<td>–</td>
<td>0.073</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mix</td>
<td>2.6 ± 0.38</td>
<td>13.6 ± 5.3</td>
<td>346 ± 189</td>
<td>8915 ± 42184</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NC</td>
<td>3.1 ± 0.53</td>
<td>216 ± 7.7</td>
<td>–</td>
<td>964 ± 215</td>
<td>0.093</td>
</tr>
<tr>
<td>1,1,1-TCA</td>
<td>Butane</td>
<td>Mix $^a$</td>
<td>0.20 ± 0.001</td>
<td>12.7 ± 2.1</td>
<td>0.28 ± 0.1</td>
<td>0.51 ± 0.1</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NC</td>
<td>0.20 ± 0.001</td>
<td>19.1 ± 4.3</td>
<td>–</td>
<td>0.50 ± 0.08</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Com</td>
<td>0.18 ± 0.01</td>
<td>9.4 ± 3.3</td>
<td>0.05 ± 0.02</td>
<td>–</td>
<td>0.011</td>
</tr>
</tbody>
</table>

$^a$Inhibition type determined by the direct linear plot.
graphically estimated kinetic parameters as initial guesses, proved to be very effective. The inhibition of butane degradation by 1,1,1-TCA demonstrates the usefulness of this combined approach. The data are consistent with competitive inhibition and all three methods (single compound test, linear plot, and NLSR analysis) gave similar values of $k_{\text{max}}$ and $K_s$. However, the data also reveal the importance of combining a graphical representation of the data with a NLSR analysis when determining the inhibition type. Note that good fits (based on RSE) were obtained with all three models (Table IV).

NLSR analysis requires initial guesses of the parameters, which, if good, will result in a convergence to best-fit estimates at a global minimum of RSE instead of a local minimum. The linearized equations presented in Table I are useful in providing these guesses. Shown in Table II are the comparison of values obtained from the linearized equations, single compound kinetic tests, and the final values obtained by NLSR. Most of the estimates obtained from the linearized method and single compound kinetic tests for $k_{\text{max}}$ and $K_s$ are in the range achieved by NLSR, indicating convergence to best-fit estimates at a global minimum of RSE. The direct linear plots also provide visual insight into the inhibition type and initial guesses needed for linear and NLSR estimates. For most cases this approach resulted in a distribution of data that was amenable to linear regression of the equations in Table I.

Our kinetic results with a butane-grown mixed culture can be compared to other cometabolic systems. Kinetic parameters for 1,1,1-TCA were determined with *M. trichosporium* OB3b producing soluble methane monooxygenase (sMMO) (Oldenhuis et al., 1991) and with a mutant methanotroph *M. trichosporium* OB3b PP358 constitutively expressing sMMO (Aziz et al., 1999). The $K_s$ for 1,1,1-TCA measured here was a factor of 20 lower than the value Oldenhuis et al. (1991) reported, whereas $k_{\text{max}}$ was an order of magnitude lower. Little transformation of 1,1,1-TCA and very low affinity of sMMO for 1,1,1-TCA was reported with *M. trichosporum* OB3b PP358. Thus, this comparison suggests that the butane-grown culture also has higher affinity for 1,1,1-TCA, but potentially slower maximum transformation rates.

With respect to in situ cometabolic treatment of CAHs, the contaminant concentrations are often much lower than the $K_s$. Thus, the pseudo first-order rate ($k_i = k_{\text{max}}/K_s$) is a more important parameter. The $k_i$ for 1,1,1-TCA obtained with our enrichment is comparable to that with *M. trichosporum* OB3b (Oldenhuis et al., 1991), and it is much higher than that with mutant methanotroph *M. trichosporium* OB3b PP358 constitutively expressing sMMO (Aziz et al., 1999). The $k_i$ values obtained with methane- or propane-grown mixed cultures studied by Strand et al. (1990) and Keenan et al. (1994) are 2 to 4 orders of magnitude lower than observed here. Thus, the butane-grown culture studied here has potential advantages for bioremediation of 1,1,1-TCA.

Inhibition of CAHs on growth substrate utilization or vice versa is an important consideration in the design of effective systems for cometabolizing CAHs, because it is strongly related to microorganism growth and/or viability and enzyme activity (Alvarez-Cohen and McCarty, 1991; Anderson and McCarty, 1996; van Hylckama Vlieg et al., 1997). Most modeling of inhibition of CAH cometabolism has assumed or found competitive inhibition kinetics (Anderson and McCarty, 1996; Chang and Alvarez-Cohen, 1995, 1996; Chang and Cridle, 1997; Semprini, 1995). However, there are reports that other inhibition models may apply. For example, the inhibitory effect of 1,1,1-TCA on methane consumption (Broholm et al., 1992) and propane on 1,1,1-TCA transformation (Keenan et al., 1994) did not fit a competitive inhibition model. Also, in a study with *N. europaea*, the ability of several alternative substrates to inhibit AMO oxidation of NH$_3$ was tested (Keener and Arp, 1993). Some nonhalogenated C1 and C2 compounds, such as ethylene and methane, were competitive inhibitors of NH$_3$ oxidation. Larger nonhalogenated compounds such as propane, butane, and monohalogenated compounds such as chloromethane and chloroethane noncompetitively (in this study, defined as mixed inhibition) inhibited NH$_3$ oxidation. In results presented here, competitive inhibition of 1,1,1-TCA on butane degradation was observed. However, butane showed mixed inhibition on 1,1,1-TCA transformation. Thus, competitive inhibition and mixed inhibition are both important in the cometabolism of CAHs. The types of inhibition mechanisms observed may differ with different microorganisms, growth substrates, and CAHs.

The butane-degradation dependent inactivation of cells by acetylene was well fit by a first-order-decay model. These results suggest that a kinetically similar population of butane-oxidizers was responsible for the degradation of butane and 1,1,1-TCA. Thus, mixed inhibition of butane on CAHs transformation does not likely result from the kinetic diversity of mixed culture. Other mechanisms cannot be ruled out, because the measurements were done with whole cells and not with purified enzymes. Thus, substrate transport to the enzyme and other cell dynamic processes may have an influence on the inhibition observed. Detailed studies with pure cultures and with purified enzymes are needed to further demonstrate the utility of this method.

This article has not been reviewed by the U.S. Environmental Protection Agency, and no official endorsement should be inferred. We acknowledge the help of Dr. James M. Tiedje for discussions on the interpretation of these data. We also thank Adisorn Tovanaboot, Dr. Mark Dolan, George Pon, and Incheol Pang for helping to measure overall mass transfer coefficients.
NOMENCLATURE

\[ H_s \] dimensionless Henry's constant of substrate (–)
\[ H_i \] dimensionless Henry's constant of inhibitor (–)
\[ I_i \] inhibitor concentrations in liquid phase (µM)
\[ k_{app\ max} \] apparent maximum degradation/transformation rates (µmol/mg TSS/h)
\[ k_{app} \] apparent half-saturation coefficient (µM)
\[ K_i \] competitive inhibition coefficient (µM)
\[ K_u \] uncompetitive inhibition coefficient (µM)
\[ k_{max} \] maximum degradation/transformation rates (µmol substrate/mg TSS/h)
\[ K_s \] half-saturation coefficient (µM)
\[ M_i \] total inhibitor mass (µmol)
\[ M_s \] total substrate mass (µmol)
\[ S_i \] substrate concentrations in liquid phase (µM)
\[ t \] time (minutes or hour)
\[ v \] specific substrate degradation rate (µmol/mg TSS/h)
\[ V_i \] volume of liquid phase (L)
\[ V_g \] volume of gas phase (L)

References


