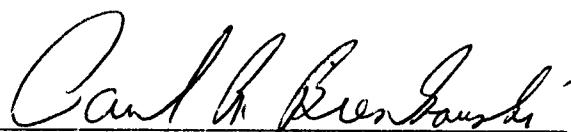
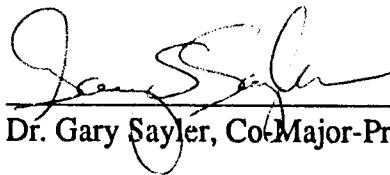


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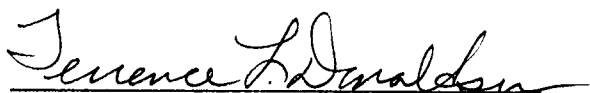


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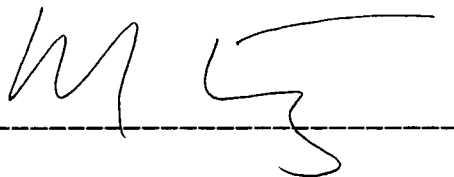
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**Development of a Fully-Suspended, Multi-Stage Bioreactor
System for Trichloroethylene Degradation Based on
Soluble Monooxygenase Cometabolism**

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Michael Ford Tschantz

May 1994

Acknowledgments

I would like to thank everyone directly and indirectly involved in helping me obtain this Master's Degree. I would like to thank Dr. Sayler for the support, patience, and insight that he has provided me. I would like to thank Dr. Bienkowski for the academic and technical advice he has given me. I would like to thank Dr. Donaldson for being on my committee and aiding me in modeling, presentations, etc. The biological work associated with this research by Dr. John Bowman and all of the help given to me by Doug Fielden and Larry Smith is most appreciated. I am thankful for the gracious support provided to me by my girl friend, Heather Rader. I am especially thankful and grateful for all of the advice and support given to me by my parents, Bruce and Penny Tschantz, while obtaining my B.S. and M.S. degrees.

Abstract

A dual-stage suspended-growth bioreactor, utilizing methanotrophs for TCE co-oxidation, was designed, constructed, and demonstrated. The bioreactor was designed to incorporate previous batch and bioreactor knowledge of the physiological and kinetic requirements to maximize sMMO expression and TCE degradation by the utilized methanotroph, *Methylosinus trichosporium* OB3b. The system was designed to minimize abiotic TCE losses through adsorption and stripping and to maximize the growth of the methanotroph and the production of sMMO. The system provides adequate monitoring capabilities for eventual modeling and optimization. Abiotic experiments have demonstrated a closed material balance on TCE and biological data has been acquired for continuously fed TCE concentrations ranging between 0.2 mg/L and 3 mg/L with removal efficiencies ranging between 100% and 82%, respectively.

Table of Contents

Section	Page
I. INTRODUCTION	1
II. BACKGROUND	15
Sparged Biofilm Reactor	15
Biofilm Reactor for Treatment of TCE in Gas Streams	18
Fixed-Film, Packed-Bed Bioreactor	21
Methanotrophic Attached-Film Expanded-Bed Bioreactor ...	22
Dual-Stage Dispersed-Growth Bioreactor	25
Literature Interpretation and Study Objectives.....	27
III. EXPERIMENTAL	31
Bioreactor Design Considerations	31
Bioreactor Inoculation and Routine Operation	36
Sample Acquisition	38
Analytical Procedures	38
Abiotic Experiments	39
TCE Degradation Experiments	40
IV. RESULTS AND DISCUSSION	42
Background Bioreactor Conditions and Performance	42
Abiotic Mass Balance	45
Continuous TCE Introduction	49
0.2 mg/L Nominal TCE Feed Concentration	49
1 mg/L Nominal TCE Feed Concentration	49
3 mg/L Nominal TCE Feed Concentration	58
V. CONCLUSIONS	64
BIBLIOGRAPHY	66
VITA	70

List of Figures

Figure	Page
1. Detailed Schematic of the Dual-Stage Recirculating Reactor System for Methanotrophic TCE Biooxidation.....	32
2. Detailed Schematic of the CSTR.....	34
3. Abiotic Control Data.....	46
4. TCE Concentrations Throughout Bioreactor for TCE Feed Concentrations of 0.2 mg/L.....	50
5. sMMO Activity Throughout Bioreactor for TCE Feed Concentrations of 0.2 mg/L.....	51
6. Determination of Maximum Rate of TCE Degradation for TCE Feed Concentrations of 0.2, 1, and 3 mg/L.....	52
7. TCE Concentrations Throughout Bioreactor for TCE Feed Concentration of 1 mg/L.....	53
8. Determination of Q_{feed} and Q_{eff} for a Nominal TCE Feed Concentration of 1 mg/L	56
9. Determination of Q_{int} for a Nominal TCE Feed Concentration of 1 mg/L and 3 mg/L	57
10. sMMO Activity Throughout Bioreactor for TCE Feed Concentration of 1 mg/L.....	59
11. TCE Concentrations Throughout Bioreactor for TCE Feed Concentration of 3 mg/L.....	61
12. Determination of Q_{feed} and Q_{eff} for a Nominal TCE Feed Concentration of 3 mg/L	62
13. sMMO Activity Throughout Bioreactor for TCE Feed Concentration of 3 mg/L.....	63

List of Nomenclature

English Symbols

C	concentration of contaminant
E	sMMO concentration (mg/L); enzyme
F_T	volumetric flow rate (L/h)
G	Gas flow rate (L/d)
H	Henry's constant
I	inhibitor
k	maximum rate of contaminant transformation (d ⁻¹)
k₁	rate constant; first order rate constant
k₋₁	rate constant
k₂	rate constant
k_m	maximum rate of methane consumption (d ⁻¹)
K_I	overall inhibitory rate constant
K_m	half-velocity constant for methane (mg/L)
K'_m	Michaelis-Menten rate constant
K'_{m,app}	apparent Michaelis-Menten rate constant
K_s	half-velocity constant for TCE (mg/L)
L	Liquid flow rate (L/d)
P	product
Q_{CSTR}	total mass of TCE in the CSTR (mg)
Q_{deg}	total mass of TCE degraded (mg)
Q_{eff}	total mass of TCE in the effluent (mg)
Q_{int}	total mass of TCE remaining in reactor (mg)
Q_{PFR}	total mass of TCE in the plug-flow columns (mg)
r_m	rate of methane consumption (mg/L/d)
r_s	rate of TCE transformation (mg/L/d)
S	TCE concentration (mg/L)
S₀	initial TCE concentration after dilution (mg/L)
S_e	TCE concentration in effluent (mg/L)
S_f	TCE concentration in feed stream (mg/L)
S_m	steady-state methane solution concentration (mg/L)
S_{sub}	substrate or cosubstrate
t	time
v	rate of enzymatic conversion
V	volume (L)
V_{max}	Michaelis-Menten maximum rate of conversion

x	liquid phase concentration of contaminant (mg/L)
X	active biomass concentration (mg/L)
y	gas phase concentration of contaminant (mg/L)

Greek Symbols

θ	lag-time in plug-flow columns (min)
τ	plug-flow column residence time (min)
v	volumetric flow rate (mL/min)
v_{rec}	volumetric flow rate of recycle stream from CSTR (L/min)
v_f	volumetric flow rate of feed stream (L/min)

I. Introduction

The contamination of the nation's groundwater by trichloroethylene (TCE) and other chlorinated solvents has necessitated the development of cost-effective and efficient means of remediation. This is due to their widespread distribution: over 25 million people are exposed to TCE concentrations of greater than $0.5 \mu\text{g/L}$ in their drinking water and 1.8 million people are exposed to TCE concentrations $>5 \mu\text{g/L}$ (Cothorn et al., 1986). TCE is a primary organic pollutant according to the Environmental Protection Agency and a possible procarcinogen (Baek et al., 1989). TCE has been shown to be toxic to yeast at concentrations of $10 \mu\text{L/mL}$. While TCE itself may not be carcinogenic, the products of its degradation by cytochrome P-450 in mammals have potential mutagenic effects (Ensley, 1991). TCE epoxide formed by cytochrome P-450 in tissues can covalently bind to cellular proteins, DNA, and RNA. Due to the toxicity of TCE and/or its by-products towards humans and other mammals, a variety of remediation schemes and techniques have been proposed, tested, and implemented.

Several sources of TCE and other chlorinated organic solvents in the groundwater supply have been identified (Love et al., 1982). These include industrial discharges onto the land and in dumps, landfill leachates, septic

tank degreasers, sewer leaks, accidental spills, and treated waste used for groundwater recharge. TCE has become a common groundwater contaminant owing to its wide spectrum of uses (Love et al., 1982; Ensley, 1991). TCE has been used as a spot remover, rug cleaner, air freshener, dry-cleaning fluid, industrial metal degreaser and polisher, electronics degreaser, refrigerant, insect and rodent fumigant, cleaning and rinsing agent for tanks and machinery, and anesthetic.

Volatile organic compounds (VOCs), including TCE, are not removed by the coagulation, sedimentation, and filtration processes of a conventional water treatment plant; therefore, other physical/chemical methods have been utilized for the removal of VOCs. The United States EPA has identified counter-current air-stripping and granulated-activated carbon (GAC) adsorption as the most economical and effective means of treating groundwater contaminated by synthetic organic chemicals (EPA [1984] Publication 600/9/84/004; Nirmalakhandan et al., 1992; Love et al., 1982).

Due to the high volatility of chlorinated solvents, on-line aeration has been utilized as a method of VOC removal from drinking water supplies (Willey et al., 1986; Norwood et al., 1988). Love et al. (1982) have shown that a water stream containing 1,1-dichloroethylene, 1,1,1-trichloroethane, tetrachloroethylene, trichloroethylene, cis-1,2-dichloroethylene, 1,1-

dichloroethane, and 1,2-dichloroethane at a total VOC concentration of 460 µg/L had a VOC removal efficiency of 92% after treatment in an aeration column, and the ease of stripping a VOC increases with increasing Henry's constant (McCarty et al., 1979). The equilibrium between the gas phase, y , and the liquid phase, x , may be expressed by:

$$y = H x \quad (1)$$

where H is the Henry's Law constant. Thus, in order to drive x in the effluent to zero, then y must approach zero. This demonstrates why counter-current operation of the air stripping column is favorable over co-current operation. The column must be long enough (providing an adequate mass transfer time) to allow x to approach zero. The cost of the process is proportional to the amount of air used for stripping; therefore the minimum amount of air that may be used is the most economical. The minimum gas-to-liquid ratio $(G/L)_{\min}$ is the reciprocal of the Henry's law constant (Fang et al., 1989):

$$(G/L)_{\min} = 1/H \quad (2)$$

However, as the volume of air used in the stripping process decreases, the concentration of VOCs in the gas-phase increases. Often in industrial processes, this gas-phase concentration is too large to emit to the atmosphere, and secondary treatment of the gas phase is necessary. A balance must be made between the stripping cost and the secondary treatment/disposal cost.

Adsorption of VOCs onto activated carbon has been shown to be a proven method of the remediation of VOC contaminated groundwater; however, this method has been limited to granulated activated carbon (GAC) and not powdered activated carbon, such as that used in taste and odor control (Love et al., 1982). GAC adsorption is also used as a secondary treatment system for air and steam stripping processes, where the lack of an organic condensate of stripping off-gas may be isolated and the off-gas can not be emitted to the atmosphere (Crittendon et al., 1988). Problems associated with the use of GAC adsorption include the effect of water quality on adsorption performance (Love et al., 1982). The adsorption capacity of the GAC may be reduced with an increase in the pH by lime softening of the water feed stream. Furthermore, competition may occur between contaminants and solubilized organic and inorganic matter for adsorption sites on the GAC. Another concern with the use of GAC is that the contaminant may concentrate onto the GAC, then detach due to

changing conditions. This may cause contaminant effluent concentrations to exceed contaminant feed concentrations. This detachment may also occur when the contaminant feed concentrations decline, resulting in an increase in contaminant effluent concentrations due to adsorption equilibrium theory.

Using GAC is generally more expensive than using stripping alone. The cost of using stripping and then GAC as a secondary treatment would be additive. Nirmalakhandan et al. (1992) performed cost analyses on GAC and stripping technologies for four common groundwater contaminants: xylene, 1,2-dichloropropane, ethylene dibromide, and 1,2-dibromo-3-chloropropane. Henry's Law constants were utilized as a measure of strippability, and isotherm parameters were used for adsorbability. Due to a high volatility and a low adsorbability, conventional stripping was less expensive than utilizing GAC for the first three of the above four contaminants. But for the case of 1,2-dibromo-3-chloropropane, which had a low volatility and a high adsorbability, GAC was shown to be less expensive. Therefore, the cost is contaminant specific. Love et al. (1982) performed a similar cost study on TCE and found that for a TCE feed concentration of 1 mg/L and an effluent concentration of 1 μ g/L, aeration was five times less expensive than utilizing GAC.

The above cost studies do not take into consideration the regeneration of the GAC or the disposal of the TCE after treatment. Therefore, the cost of GAC adsorption is dramatically higher than that shown by Love et al. (1982) and Nirmalakhandan et al. (1992). Furthermore, neither of these technologies chemically reduces the contaminants to less harmful compounds, but merely re compartmentalizes the contaminants between media. By offering a remediation system with low cost feedstocks and energy requirements, and by eliminating the need to dispose of the concentrated contaminant(s) by producing harmless end products, a low cost treatment system for the remediation of contaminated groundwater would find widespread use in public water works and in the clean-up of contaminated sites. Bioremediation has been shown to be a cost-effective alternative to traditional remediation processes.

The biological processes of TCE degradation may be described as reductive dehalogenation and co-oxidation (or cometabolism) processes (Ensley, 1991). Many aquifers are anaerobic or oxygen limited, and the biological process of reductive dechlorination by anaerobes involves the transfer of electrons to chlorinated ethylenes. This redox potential is provided by biochemical processes in which the reduction of the chlorinated hydrocarbon is coupled to the oxidation of electron transfer

agents involved in methane biosynthesis. The biochemical pathway of PCE and TCE dehalogenation follows the pathway:



Because vinyl chloride is more carcinogenic than PCE or TCE, a greater health hazard is posed during anaerobic dechlorination processes.

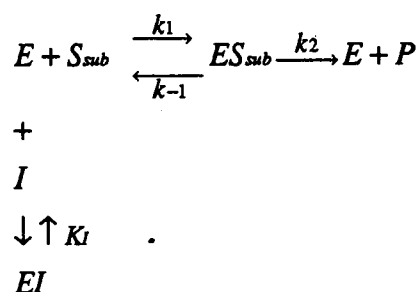
Therefore, research has been aimed at aerobic cometabolic processes, where vinyl chloride is not produced as an intermediate, and the contaminants are mineralized to CO_2 , a harmless end-product.

Nitrosomonas europaea has been shown capable of degrading TCE, cis- and trans-DCE, and vinyl chloride, through the enzyme ammonia monooxygenase. The substrate of ammonia monooxygenase is ammonia; however, carbon monoxide, methane, methanol, ethylene, propylene, and bromoethane are co-oxidized by ammonia monooxygenase. This spectrum of co-metabolites relates to the broad specificity of this enzyme system, which also enables it to oxidize TCE, with a whole cell rate of TCE degradation of 1 nmol/min/mg protein (Arciero et al., 1989).

Toluene oxygenases, in strains such as *Pseudomonas putida* (containing toluene dioxygenase) and *Pseudomonas mendocina* (containing toluene para-monooxygenase), have broad substrate specificity and can

fortuitously oxidize TCE. Phenol or toluene are necessary inducers of the enzyme and stimulate TCE oxidation. Therefore, competitive inhibition for the toluene oxygenase exists, limiting the rate of TCE degradation by these enzymes (Ensley, 1991).

Competitive inhibition is caused by substrate analogs that compete with the substrate for the active site of the enzyme. Competitive inhibition may be described (Shuler et al., 1992):



where E is the enzyme, S_{sub} is the substrate, P is the product, and I is the substrate analog (competitive inhibitor). k_1 , k_{-1} , k_2 , and K_I are rate constants. Assuming rapid equilibrium:

$$K'_m = \frac{k_{-1}}{k_1} = \frac{[E][S_{sub}]}{[ES_{sub}]} \quad (3)$$

$$K_I = \frac{[E][I]}{[EI]} \quad (4)$$

where K'_m is the Michaelis-Menten rate constant. Assuming that the enzyme is conserved:

$$[E_0] = [E] + [ES_{sub}] + [EI] \quad (5)$$

where $[E_0]$ is the overall enzyme concentration. Assuming the rate of enzymatic conversion of substrate, v , is limited by:

$$v = k_2[ES_{sub}] \quad (6)$$

Then the rate of enzymatic conversion with competitive inhibition may be expressed as:

$$v = \frac{V_{max}[S_{sub}]}{K'_{m,app} + [S_{sub}]} \quad (7)$$

$$K'_{m,app} = K'_m \left[1 + \frac{[I]}{K_I} \right] \quad (8)$$

where V_{max} is the Michaelis-Menten maximum forward velocity and $K'_{m,app}$ is the apparent Michaelis-Menten constant. From equations 7 and 8, it is found that as the concentration of the competitive inhibitor is increased, $K'_{m,app}$ increases, which causes a subsequent decrease in v . The effect of competitive inhibition is diluted by increasing S . In order to reduce the effects of competitive inhibition to increase TCE degradation, then the inhibitory component, in this case toluene or phenol, I , should be minimized.

The K_s and V_{max} of *ortho*-monooxygenase for phenol oxidation are 8.5 μM and 466 nmol/min/mg protein, respectively, and the K_s and V_{max} of this same enzyme for TCE oxidation are 3 μM and 8 nmol/min/mg protein.

TCE oxidation by toluene oxygenases produces no energy (thus no NADH) and reductant supplies or substrate must be added to maintain high rates of degradation. The toluene system is inducible and must be continued being fed toluene to maintain enzyme activity.

Methylosinus trichosporium OB3b is a methanotroph capable of cometabolic TCE degradation mediated by the enzyme soluble methane monooxygenase (sMMO), which is produced under copper-limiting conditions (Stanley et al., 1983) and is thereby suitable for the remediation of TCE contaminated groundwater at low copper concentrations. Wilson and Wilson (1985) were the first to study the use of methanotrophs for the cometabolism of chlorinated solvents in soil columns. In the presence of copper, sMMO synthesis is suppressed, while the synthesis of a membrane associated methane monooxygenase, referred to as particulate methane monooxygenase (pMMO), is promoted. It has been shown previously that the sMMO of *Methylosinus trichosporium* OB3b degrades TCE at a much higher rate ($V_{\max} = 220 \text{ nmol TCE/min/mg cells}$) (Brusseu et al., 1990) than pMMO ($V_{\max} < 0.5 \text{ nmol TCE/min/mg cells}$) (DiSpirito et al., 1992). During TCE oxidation, sMMO oxidizes TCE to an epoxide, which spontaneously breaks down to chloroacetate, glyoxylate, formate, and carbon monoxide. The conversion products of TCE degradation (epoxide

or acylchlorides) can react with the sMMO hydroxylase component of the enzyme, rendering it inactive for further degradation (Ensley, 1991).

If it is assumed that the kinetics of TCE degradation in the bioreactor follow a second-order Monod equation (Alvarez-Cohen et al., 1991b):

$$r_s = \frac{kXS}{K_s + S} \quad (9)$$

where r_s is the rate of degradation of TCE, k is the maximum rate of contaminant transformation, X is the active biomass concentration, S is the TCE concentration, and K_s is the half-velocity constant for TCE, then in order to maximize the rate of degradation, the active biomass concentration should be maximized. Active biomass is defined as biomass containing active pools of sMMO. But since the sMMO activity varies due to the detrimental effects of the TCE by-products on the enzyme, X in equation 9 should be substituted with an sMMO concentration, E , resulting in:

$$r_s = \frac{kES}{K_s + S} \quad (10)$$

According to equation 10, as sMMO pools are depleted, the rate of TCE transformation decreases (as it also decreases as the TCE concentration itself decreases). In order to maintain high rates of TCE degradation and to drive the TCE concentration to low levels, the maintenance of sMMO pools is necessary. This may be performed by either decreasing the toxic effects of TCE degradation, or by maintaining sMMO production by the bacteria.

The normal substrate of sMMO is methane, and the biological process for methane metabolism follows the pathway:



The last two steps reduce NAD^+ to NADH. Formate has been shown to increase the specific activity (or rate of substrate metabolism as shown by the naphthalene oxidation assay [Brusseau et al., 1990]). It has been theorized that this increased activity is due to an increase in available NADH to the cell. Formate dehydrogenase oxidizes formate to carbon dioxide forming NADH. This increased activity lessens the impact of deactivation of a proportion of the sMMO pools by TCE. Furthermore, the increased reducing supplies produced by formate metabolism allows for an increased rate in protein turnover, including sMMO. NADH is required for

both the degradation and production of the proteins. Damaged proteins may be replaced more rapidly, lessening the impact of the deactivation of sMMO by the breakdown products of TCE oxidation (acylchlorides or epoxide). This will reduce the detrimental kinetic effects of TCE toxicity on TCE degradation.

By increasing the biomass concentration (assuming sMMO activity is constant throughout the biomass), the sMMO concentration will increase. The rate of substrate consumption, in this case methane, is described by (Alvarez-Cohen et al., 1991b):

$$r_m = \frac{k_m X S_m}{K_m + S_m} \quad (11)$$

where r_m is the rate of methane consumption, k_m is the maximum rate of methane consumption, S_m is the steady-state methane solution concentration, and K_m is the half-velocity constant for methane. Assuming that methane consumption results in biomass production (during cellular growth phase, where the growth yield is 0.65 mg VS/mg CH_4 (Speitel et al., 1993)), then as the methane concentration is increased, the biomass concentration will increase. The increase in the biomass concentration will have a diluting effect on the loss of sMMO activity by the TCE by-products

by allowing a lower proportion of the biomass to be lost to TCE toxicity, resulting in a less dramatic effect on the rate of TCE degradation by equations 9 and 10.

Several bioreactors utilizing methanotrophs have been proposed, but many have limitations. Many of the limitations may be reduced or eliminated by designing the bioreactor around the biological necessities and characteristics of the methanotrophs. The goal of this research was to design and construct a bioreactor that will maximize the utility of methanotrophs in the co-oxidation of TCE to CO_2 and to demonstrate that a mass balance on TCE may be closed in the system. Another goal was to provide ample monitoring capabilities to allow eventual modeling and optimization of the complete TCE degrading system. Further goals are to demonstrate the functionality of the bioreactor to reduce TCE feed concentrations to suitable effluent concentrations.

II. Background

Several bioreactor designs for the degradation of TCE have been studied, including attached-film, expanded-bed, and fluidized systems (Strandberg et al., 1989; Phelps et al., 1991; Strand et al., 1991; Fennell et al., 1993); and two-stage CSTR plug-flow systems (Alvarez-Cohen et al., 1991a; McFarland et al., 1992). The work of several investigators is examined. By determining the limitations and problems associated with the past and recent work and by identifying the key biological requirements of methanotrophs to maximize TCE degradation, both physiologically and kinetically, a bioreactor was developed that will allow efficient TCE degradation with the necessary monitoring capabilities to ensure that the system may be modeled and optimized.

Sparged Biofilm Reactor

Strand et al. (1991) tested a submerged, upflow, packed-bed biofilm reactor, sparged with air and methane, for the treatment of TCE and 1,1,1-trichloroethane (TCA), utilizing methanotrophs. The proposed reactor was constructed to overcome problems associated with the high volatility of these compounds and the low solubility of methane, and to maximize the

biomass available for degradation. In an attempt to minimize competitive inhibition between methane and the chlorinated solvents for available sMMO, the reactor used a continuous supply of methane at 2% (v/v air) in the gas phase. Previous research had shown that competitive inhibition was negligible at gas phase methane concentrations of less than 5% (v/v air) (Strand et al, 1990).

The reactor consisted of a 1 L (empty volume) packed bed reactor, filled with 0.6 mm diameter glass beads, on which the biomass was grown. Liquid (containing a nutrient feed, TCE, and TCA) and gas (methane and oxygen) were sparged at the bottom of the reactor, and the liquid and gas was recycled (20:1) from the top of the reactor. Effluent liquid also escaped at the top of the reactor.

TCE feed concentrations varied between 10 and 5100 $\mu\text{g/L}$, and TCE degradation rates were determined after steady-state effluent TCE concentrations were achieved. The hydraulic retention time in the packed bed reactor was 1.5 h. The gas-phase loop in the system was a closed loop; therefore, the degradation rate was determined by subtracting the effluent TCE concentration, S_e , from the feed concentration, S_f , and dividing by the hydraulic retention time, τ .

$$r_s = \frac{S_f - S_e}{\tau} \quad (12)$$

The biomass concentrations varied throughout the reactor, ranging between 8300 mg VSS/mL at the bottom of the reactor to 2100 mg VSS/mL at the top of the reactor, based on empty bed reactor volume. The TCE concentration in the effluent ranged between 5 and 3000 µg/L, and the removal efficiencies averaged 60±5%. First order kinetics was used to estimate the rate constant at TCE concentration less than 1000 µg/L, yielding a rate constant of 0.40 h⁻¹; and for TCE concentrations greater than 1000 µg/L, an average maximum rate of degradation of 400 µg/L h was determined.

At these TCE concentrations, Strand et al. found that no competitive inhibition occurred, that there was an adequate supply of methane to support the biomass, and that reasonable rates of degradation occurred. Based on the rates obtained experimentally, the retention time necessary to reduce a TCE feed concentration of 500 µg/L to 5 µg/L was 11.5 h.

Biofilm Reactor for Treatment of TCE in Gas Streams

The research (Speitel et al., 1993) utilized *Methylosinus trichosporium* OB3b in a 0.9 m by 5 cm reactor packed with Celite Biocatalyse Carrier R-635 (Manville, Denver, CO), on which the biofilm grew. The liquid and gas streams flowed cocurrently from top to bottom. The gas stream was comprised of air, natural gas, and a chlorinated solvent (TCE and DCA). The liquid stream provided microbial growth nutritional requirements and wetted the packing material.

The initial DCA introduction caused a dramatic drop in methane consumption (roughly a tenth of the original consumption), suggesting that competitive inhibition was occurring or that sMMO pools had diminished due to toxicity of the DCA or by-products of degradation. When DCA was again added, the methane consumption doubled, suggesting an adaptive response by the bacteria towards the toxic solvent. In between the two experiments, and again afterward, the methane consumption increased, suggesting that competitive inhibition was also occurring. In all of the experiments with TCE added, there was also a dramatic decline in methane consumption relative to consumption with no TCE added, suggesting that competitive inhibition and toxicity was occurring.

The DCA feed concentrations for the two experiments were 786 $\mu\text{g/L}$ and 973 $\mu\text{g/L}$; but with a Henry's constant of 0.04744 (Convery et al. 1980), the liquid phase concentrations were 16.7 mg/L and 20.5 mg/L, respectively. The gas-phase residence times in the reactor were 5.7 min and 12.2 min, respectively, and the removal efficiency (from the gas phase) was 20.4% and 81%, respectively. The increase in the removal efficiency was due to a more active biomass and a larger residence time.

TCE was fed at three concentrations: 327 $\mu\text{g/L}$, 395 $\mu\text{g/L}$, and 720 $\mu\text{g/L}$. The dimensionless Henry's constant for TCE is 0.3726 (Gossett et al., 1986), resulting in lower liquid phase concentrations, compared to DCA, of 878 $\mu\text{g/L}$, 1060 $\mu\text{g/L}$, and 1932 $\mu\text{g/L}$, respectively. The gas-phase residence times for the three experiments were 9.6 min, 9.9 min, and 9.0 min, respectively. The maximum removal efficiencies were 27.6%, 62.3%, and 20.7%, respectively. During the first two TCE fed experiments, the removal performance of the reactor declined over time. Speitel used Alvarez-Cohen's et al. (1991) concept of a finite transformation capacity constant, which is the maximum mass of TCE that may be degraded by a mass of TCE (Alvarez-Cohen et al. computed this to be 0.036 and 0.042 mg TCE/mg cells for separate experiments), to explain the decline. The assumption that volatile solids are 90% of the total solids yields transformation capacities of 0.040 and 0.047 mg TCE/mg VS,

respectively. Using the second TCE experiment as an example, an average of 230 mg VS/day would have been inactivated. Using a growth-yield coefficient of 0.65 mg VS/mg CH₄, the determined methane removal rate would have produced 200 mg VS/d. The rate of volatile solids production is 87% the rate of inactivation explaining the decline in performance. The final TCE experiment showed a constant TCE removal efficiency and resulted in a transformation capacity of 0.047 mg TCE/mg VS, which is similar to that obtained by Alvarez-Cohen et al.

The kinetics of the system were modeled using Monod kinetics and were adapted from submerged-biofilm models (Rittmann et al., 1980; Rittmann et al., 1981). During the final TCE experiment, methane was shut off to determine whether competitive inhibition was occurring. The pseudo-first-order rate constant increased from 0.00075 to 0.0015 L/mg d, indicating that competitive inhibition occurred when methane was present with TCE. The pseudo-first-order rate constant then decreased to 0.0003 L/mg d, due to the methane starvation of the bacteria and due to toxicity of the TCE epoxide on sMMO.

Fixed-Film, Packed-Bed Bioreactor

Strandberg et al. (1989) utilized a 5 cm inner diameter by 110 cm long column packed with 0.6 cm berl saddles, inoculated with a methanotrophic consortium obtained from C.D. Little (1988) for the degradation of TCE and DCE. The feed concentration of each contaminant was ~1 mg/L each and the combined flow rate of the contaminant and the feed stock entering the top of the column ranged between 0.005 and 0.05 L/min. A concurrent gas stream, typically consisting of 4% methane (v/v) and air entered the top of the reactor at 20 mL/min.

Roughly half of the fed TCE was removed during a single pass through the reactor, and greater amounts of DCE were degraded. TCE degradation was first order with respect to TCE over the range of TCE concentrations examined (0.15 to 5 mg/L) and was assumed to follow the first order Monod kinetic equation for degradation:

$$\frac{dC}{dt} = -k_1 C \quad (13)$$

where C is the concentration of the contaminant, t is time, and k_1 is the first-order rate constant. The first-order rate constant was determined for

the examined flow rates and ranged between 0.016 and 0.024 min⁻¹, except during the case with a flow rate of 0.050 L/min, where the first-order rate constant was 0.046 L/min. As the flow rate increased, the total degradation increased, due to an increase in the rate of degradation as TCE and DCE concentrations increased throughout the reactor. By recycling the effluent through the reactor, TCE concentrations of 50-100 µg/L could be achieved after 1.5 h of operation, but could not be lowered any more.

The effects of competitive inhibition were tested by both shutting the methane introduction completely off and by reducing the methane concentration to 4% methane to air (v/v). No significant increases in rates of degradation or removal efficiencies were found, reinforcing the fact that at methane concentrations <5% (v/v), competitive inhibition may be avoided. It should be noted, though, that when the methane was turned off, the rate of degradation remained constant for ~4 h, then declined to <20% its original value after 18-48 h. Furthermore, when the methane concentration was reduced to 2% (v/v), the rate declined after 4 to 5 days.

Methanotrophic Attached-film Expanded-Bed Bioreactor

The employed bioreactor featured a 3 L expanded bed, containing diatomaceous earth and adsorbing granular activated carbon (Fennell et al.,

1993). Separate oxygen and methane countercurrent diffuser columns, one providing oxygen and the other methane, were utilized and pumps carried the liquid with dissolved gases to the bottom of the expanded bed column. By separating the oxygen introduction from the methane introduction, the explosion hazard of the system was lowered. One third of the expanded bed column contained bed material, and the rest of the column served as a clarifier zone for the recycling of the liquid effluent.

Batch runs were performed on the system with TCE concentrations ranging from 0.56 to 13 mg/L. Continuously fed TCE concentrations ranging between 0.1 and 40 mg/L were also examined.

The concentration of the mixed methanotrophic biomass ranged from 8 to 75 g VS/L with a typical concentration of 40 g VS/L. 10-30% of the total biomass was present in the diffusers and as a free suspension in the liquid. The maximum observed growth rate was 0.15 d^{-1} , and a maximum growth yield of 0.48 g VS/g CH_4 was found.

During the batch experiments, the maximum TCE degradation rate was determined to be $10.6 \pm 1.9 \text{ mg TCE/g VS d}$ and a half-velocity coefficient of $2.8 \pm 1.8 \text{ mg TCE/L}$. During continuous operation, TCE removal efficiencies ranged from 25 to 61% with hydraulic retention times ranging between 1.2 and 11 h. The removal efficiencies were 48%, 40%, and 38% with hydraulic retention times of 1.9 h, 2.5 h, and 1.2 h,

respectively, for TCE feed concentrations of 0.46 mg/L, 1.07 mg/L, and 2.78 mg/L, respectively. TCE degradation rates varied between 0.26 and 2.7 mg TCE/g VS d or from 6.7 to 91 mg TCE/L d. The ratio of methane consumed to TCE degraded ranged between 23 and 1200 g CH₄/g TCE. The addition of yeast extract at 20 mg/L caused copper to accumulate at 0.012 mg/L, causing pMMO to be produced. This production of pMMO over sMMO caused a reduced maximum TCE degradation rate of 0.66 ± 0.3 mg TCE/g VS d as compared to 2.9 ± 0.007 mg TCE/g VS d when a lower yeast extract concentration of 7.5 mg/L was employed. The loss in the maximum rate of TCE degradation shows the high sensitivity of the methanotrophs to low copper concentrations.

Methane inhibition was also characterized for the system. When the methane concentration was near zero, at 0.01 mg/L, the rate of TCE degradation was 2.5 mg TCE/g VS d; but when the methane concentration was increased to 5.4 mg/L, the rate of TCE degradation dropped considerably to 0.3 mg TCE/g VS d. This work also demonstrates that if high rates of degradation are to be achieved, low methane concentrations must exist when the methanotrophs are in the presence of TCE.

Dual-Stage Dispersed-Growth Bioreactor

A two-stage dispersed-growth bioreactor and model has been proposed by Alvarez-Cohen and McCarty (1991), which separates growth from TCE oxidation, eliminating competitive inhibition between methane and TCE for available sMMO, and discards the TCE exposed cells with the effluent stream. After the cells are cultivated, they contact methane in a continuously stirred tank reactor (CSTR) and are fed to a plug-flow column, where a TCE stream is also introduced. The effluent of the plug-flow column is then discarded.

A two-stage bioreactor system utilizing methanotrophs for TCE cometabolism was constructed by McFarland et al. (1992). They examined the effects on TCE removal rates caused by eliminating competitive inhibition for available pools of sMMO through the separation of growth with methane introduction from TCE degradation. They also studied the effects of formate addition. Tsien et al. (1989) reported that addition of formate, a reducing equivalent, may increase the rate of TCE degradation in pure cultures of methanotrophs. Formate acts to stimulate the activity of available pools of sMMO but does not compete with the active sites of the sMMO.

The bioreactor was composed of two units, a 1.4 L methanotrophic growth unit with a hydraulic retention time of 24 h, and a 400 mL plug-flow reactor with a hydraulic retention time of 40 min. The flow rate from the growth reactor into the plug-flow column was 10 mL/min. The effluent from the plug-flow column was stripped to remove residual TCE and was recycled back into the growth unit. Pure TCE was pumped into the system prior to the plug-flow column to bring the TCE concentration to 15 mg/L.

Initially the bacteria successfully removed TCE to a concentration of ~5 mg/L, but the concentrations then increased. This was partly due to the settling of the biomass in the plug-flow column, where, at the bottom of the column, the biomass concentration was 1.2 - 1.6 g VS/L, while at the top of the column, the biomass concentration was 0.01 g/L. Eventually the TCE concentration of the effluent reached 30 mg/L, indicating that sorption of TCE to the biomass was occurring.

Formate was then added to the cell/TCE stream prior to the plug-flow column to bring the formate concentration to 20 mM. TCE effluent concentrations dramatically decreased to 1.4 mg/L within 24 h. This led to a maximum rate of TCE degradation of 21.1 mg TCE/g VS d. After 2 d of formate addition, the formate addition was ceased, which led to another increase in the TCE effluent concentration.

In order to determine the effects of competitive inhibition by methane on the rate of TCE degradation, methane introduction was ceased and formate was again added. The rate of TCE degradation increased to 25.9 mg TCE/g VS d.

McFarland et al. also reinforced the fact that without periodic inputs of methane, the enzymes would be degraded. They suggested stripping the methane out of solution prior to TCE degradation and/or inputting oxygen periodically into the plug-flow column, which would increase the rate of methane metabolism by the bacteria.

Literature Interpretation and Study Objectives

The work of Strand and Strandberg shows that at methane concentrations less than 5% (v/v air), competitive inhibition between methane and TCE for the available sMMO may be avoided. However, Strandberg's and McFarland's work went on to demonstrate that there is a finite period during which the methanotrophs may be absent from methane before starvation and enzyme activity loss occurs. If the reactor is set up as proposed by Alvarez-Cohen and McFarland in a dual-stage manner, then this finite period is the maximum residence time that the cells may be exposed to the TCE away from methane in the plug-flow columns.

Speitel's and McFarland's work further reinforced the fact that at higher than 5% methane in air (v/v) competitive inhibition occurs, lowering the rate of TCE degradation. Their work also indicated that the toxicity of the TCE epoxide towards sMMO plays an important part in the rate of TCE degradation; that is, unless the rate of biomass or sMMO generation is as great as or greater than the rate of sMMO depletion, then the rate of degradation will decline.

It may be derived that treatment may be made easier by operating solely in the liquid phase, rather than the gas phase. This is deduced based on partition coefficients, where methane tends towards the liquid phase with a partition coefficient of 23.3 liquid:air, and TCE tends toward the gas phase with a partition coefficient of 0.17 liquid:air (Gossett et al., 1987; Broholm et al., 1992). By operating completely in the liquid phase without headspace, the methane concentration may be easily controlled, without large changes in liquid phase concentration after small changes in gas-phase concentrations, and without mass transfer limitations imposed between the partitioning of TCE between the gas and liquid phases. If it is necessary to continually wet a biofilm, as implied by Speitel, for nutritional requirements of the biomass, then one must deal with the partitioning of the TCE between the two phases, and then deal with treatment of the off-gas, where Speitel found that 10% of the fed TCE escaped with the off-gas.

If the TCE was completely dissolved in the liquid phase, then the mass transfer limitations of TCE between the two phases could be eliminated, and the only mass transfer would be between the liquid and the cell. Furthermore, the mass balance and secondary treatment of the gas-phase would be simplified. By operating the system under pressure, an adequate supply of oxygen may be maintained in the plug-flow column.

The objective of this research was to design a bioreactor around the biology of sMMO production by *Methylosinus trichosporium* to enhance the efficiency and stability of TCE degradation. In order to maximize the utility of methanotrophs for TCE degradation, TCE contacting and degradation should be decoupled from cell growth and sMMO production, reducing competitive inhibition. Additionally, the rate of mass transfer of methane and oxygen to the methanotrophs is increased by utilizing the cells in an aqueous suspension rather than a fixed-film. Methane-limiting conditions for methanotrophic growth may be avoided by operating the reactor system under pressure to increase gaseous oxygen and methane mass transfer, due to a greater liquid-phase gas solubility according to Henry's Law.

In order to ensure a closed mass balance, the reactor should minimize TCE adsorption and have no head-space (minimizing partitioning between the liquid-phase and the gas-phase). Abiotic experiments were

performed to demonstrate the minimization of non-degradative TCE losses (i.e. adsorption and stripping to the gas-phase).

Utilizing batch culture data for growth and sMMO production, the methane and oxygen concentrations were maintained in the bioreactor to ensure maximal expression, and various feed TCE concentrations, ranging from 0.2 mg/L to 3 mg/L were examined.

The objectives of the research may be summarized as follows:

- (1) Design and construct a two-staged dispersed-growth bioreactor with recycle and effluent filtration.
- (2) Perform abiotic experiments, demonstrating a TCE material balance may be closed, and quality biological data and TCE losses may be accurately measured.
- (3) Operate the bioreactor for TCE feed concentrations ranging from 0.2 mg/L to 3 mg/L and measure key biological data (i.e. biomass concentrations, sMMO activities, methane and oxygen concentrations) and TCE concentrations.

III. Experimental

Bioreactor Design Considerations

In order to reduce competitive inhibition between methane and TCE, a bioreactor was developed to separate cell growth and sMMO synthesis from TCE contacting and degradation. Additionally, the system was designed to have no gaseous head-space, eliminating problems associated with stripping of TCE to the gas phase, and thus allowing accurate mass balances to be determined. The complete bioreactor system is depicted in Figure 1. All internal surfaces of the system potentially in contact with TCE were composed of 316 stainless steel, glass, teflon, or viton rubber, minimizing TCE adsorption and cell adhesion. The reactor was pressure tested to 15 atm without leakage, but normal operating pressure is in the range of 1 to 3 atm.

The first compartment included a pressurized 2.4 L continuously stirred tank reactor (CSTR) in which cells were continuously cultivated with methane and oxygen. The CSTR was a liquid-filled unit constructed of 316 stainless steel containing two six-bladed downward thrust agitators and a six-bladed radial agitator connected to an exogeneous motor

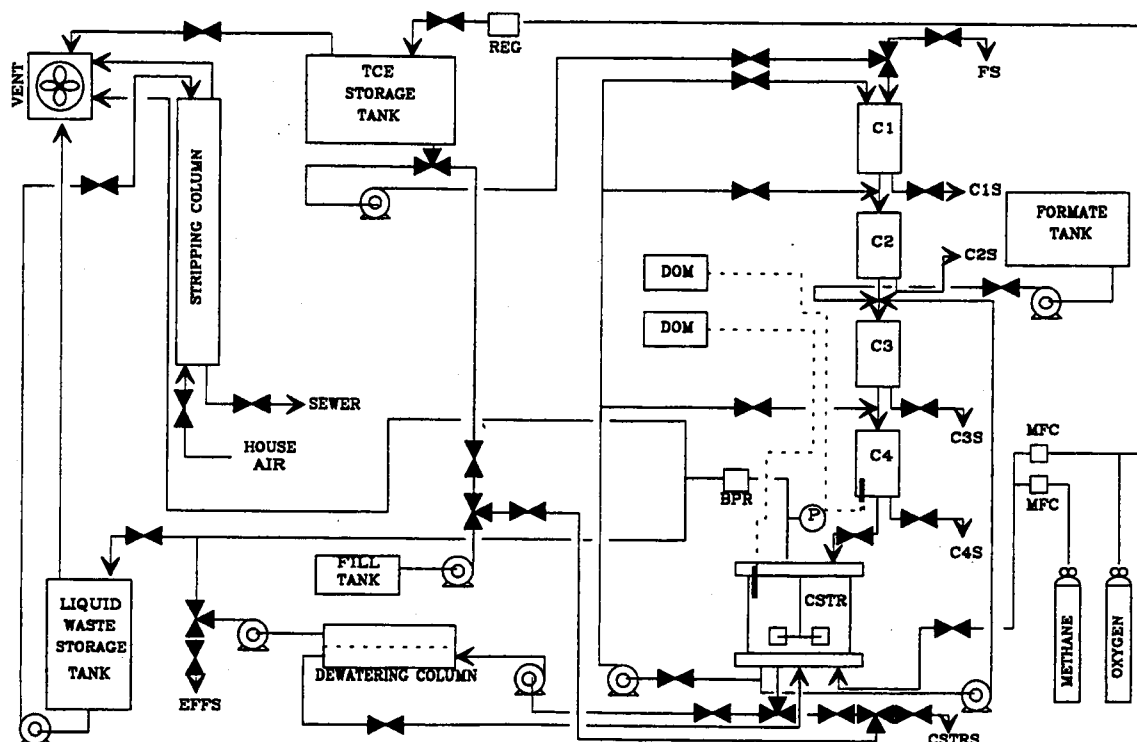


Figure 1. Detailed Schematic of the Dual-Stage Recirculating Reactor System for Methanotrophic TCE Biooxidation. (Liquid is pumped from the CSTR to any variety of the plug-flow columns, C1 - C4. Liquid samples are obtained at the bottom of each plug-flow column (C1S - C4S), from the CSTR (CSTRS), feed stream (FS), and effluent stream (EFFS). The system is fed methane and oxygen, which is regulated by mass flow controllers (MFC) and reactor pressure is controlled by a back-pressure relief valve (BPR). Dissolved oxygen meters (DOM) and probes monitor oxygen concentrations in the CSTR and column 4.

(Figure 2). A mechanical seal, consisting of a ceramic seat and a graphite head, was used to maintain a pressure-tight seal around the agitator shaft as it exits the CSTR. A thrust bearing pressed into the mechanical seal shroud allowed a minimal friction surface for the agitator shaft to press against, while providing a contacting force between the head and the seat of the seal. A radial bearing in the mechanical seal shroud and a teflon bushing in the base of the CSTR allowed pivot-free rotation of the shaft. Furthermore, a universal joint in series between the agitator motor and shaft allowed the shaft to remain vertical during small alignment variations between the agitator motor and the CSTR. Pressure control for the entire system occurred in the CSTR, via a variable tension backpressure relief valve (BPR), allowing undissolved gases to escape from the system to a vent, and allowing excess liquid to escape to a waste tank in a self-regulating manner. Dissolved oxygen concentration in the CSTR was measured by a stainless steel oxygen probe (Ingold) connected to a dissolved oxygen meter (DOM) (Cole-Parmer, Chicago, Ill.). A glass window in the CSTR allowed liquid level in the CSTR to be monitored. Methane and oxygen introduction into the CSTR occurred through two model 8272 mass flow controllers (MFC) (Matheson Gas Products, Montgomeryville, Penn.) coupled to a Matheson model 8284 dynablender. The gases were mixed in a manifold and entered the CSTR through a

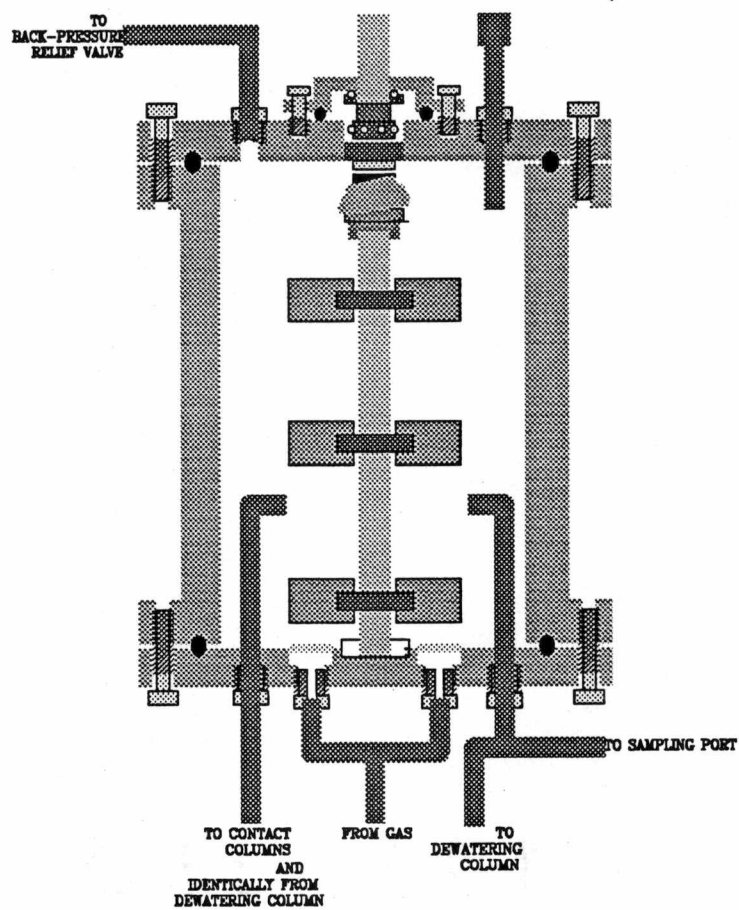


Figure 2. Detailed Schematic of the CSTR

porous metal diffuser, located at the bottom of the CSTR. Check valves between the mass-flow controllers and the manifold prevented liquid from backing into the mass-flow controllers.

In order to keep cells from escaping with the effluent stream, a tangential flow dewatering device with feed-side recycle was used. Liquid was recycled from and to the CSTR through a 6.35 cm I.D. stainless steel pipe 30.5 cm in length by a FMI model QD metering lab pump. A FMI model QV metering lab pump delivered liquid (as the reactor effluent stream) from a 3.81 cm O.D. porous metal filter, 15.2 cm in length, located inside the pipe, to a waste storage tank.

Fluid, composed of media and cells, was pumped through 0.32 cm O.D. stainless steel tubing from the CSTR to the second section of the bioreactor, which consisted of a series of four plug-flow contacting columns (C1-C4) (volumes 0.56 L, 0.80 L, 0.88 L, and 1.04 L, respectively), in which methane-limiting conditions presumably existed and TCE degradation occurred. These TCE contact columns were placed in series to allow versatility for future experiments, additions and modifications, and to allow easy manipulation of residence times. The columns were connected to one another in series by 0.635 cm stainless steel tubing. Valves allowed control of flow from the CSTR to any configuration of the four columns. In addition, the placement of discrete

sampling points allowed monitoring throughout the length of the TCE contacting columns for accurate analysis of the degradation kinetics of the system. Another Ingold dissolved oxygen probe was positioned in the lowest column for measuring the oxygen concentration after contact of the biomass with TCE. The TCE contacting columns emptied into the CSTR.

Synthetic TCE contaminated water was pumped by a Cole-Parmer centrifugal pump to a stainless steel storage tank under a 1.7 atm oxygen blanket for subsequent addition to the bioreactor. The contaminated water was delivered to the top of the uppermost TCE contacting column by a Gilson model 302 high performance pump or a FMI model QV metering lab pump through a series of 0.3175 cm and 0.1588 cm stainless steel tubing.

Bioreactor Inoculation and Routine Operation

Methylosinus trichosporium OB3b mutant PP358 was provided by the University of Texas, Austin, Tex. The strain was maintained and cultivated during these studies courtesy of Dr. John Bowman, Center for Environmental Biotechnology, The University of Tennessee. This strain was cultivated either in liquid or on agar-solidified NMS media (Bowman et al., 1993), supplemented with 0.025% (w/v) yeast extract and vitamins (Balch et al., 1976), under a 1:4 methane:air headspace at 28°C for 3-5 d.

The CSTR was inoculated with 1 L of PP358 culture ($A_{600} \sim 0.5$), and NMS media was then added to fill the CSTR and the remaining sections of the bioreactor completely. The CSTR agitator was rotated at 138 rpm. Cells were recycled through the contact columns at a rate of 10 mL/min. Methane and oxygen were initially added at 10 mL/min each, and the flow rates were adjusted to final flow rates of 60 mL/min and 5 mL/min, respectively, as the biomass concentration in the reactor increased. The oxygen concentration was maintained at 5-15% saturation at 3 atm (1.4 - 4.2 mg/L). PP358 was recirculated throughout the bioreactor in the absence of TCE addition until it had reached stationary growth phase. The simulated TCE contaminated feed was prepared with NMS media and TCE saturated water solution (8.31 mM at 25°C) to a specified TCE concentration. The TCE feed was then pumped into the uppermost TCE contacting column at a rate of 2.1 ± 0.1 mL/min. Biomass was partially retained, and effluent was removed through the use of a dewatering device. Liquid from the CSTR was recycled through a 6.3 cm by 30.5 cm stainless steel pipe. Filtered effluent was pumped through a 2.54 cm O.D., 15.2 cm long, porous metal filter covered with a 0.2 μ m teflon membrane, located inside the pipe (at 1.0-2.0 mL/min), to a waste storage tank. To maintain a constant volume of liquid in the bioreactor, excess liquid escaped through the back-pressure relief valve to a waste storage tank.

Sample Acquisition

Valved liquid-phase sampling ports were located at the bottom of the CSTR, at the bottom of each of the TCE contacting columns, along the simulated TCE wastewater feed line, and in the dewatering line prior to deposition into the waste tank. Liquid bioreactor samples (4-6 mL) were obtained via 16-gauge needles inserted into screw cap septum vials sealed with Teflon-lined silicon seals (32 mL; Pierce, Rockford, IL).

Analytical Procedures

PP358 culture optical density was determined spectrophotometrically by measuring absorbance at 600 nm and comparing with a standard curve of optical density vs. dry weight (mg/L). The sMMO activity was determined by the naphthalene oxidation assay (Koh et al., 1993), whereby naphthalene is oxidized by sMMO to form naphthol (naphthalene is not oxidized by pMMO). The sMMO activity was normalized to cell dry weight.

TCE levels were calculated by gas chromatographic analysis of vial headspace samples. Analysis was performed on a Shimadzu GC 9AM gas chromatograph (Shimadzu Analytical Instruments Co., Kyoto, Japan) equipped with a split injector port operated at 250°C. A 60 m x 0.53 mm

I.D. RTX volatiles capillary column (Restek Corp., Bellefonte, PA) was used isothermally at 120°C, with electron capture detection at 300°C. Nitrogen was used as the carrier gas. The peak areas were integrated with a Shimadzu C-R6A Chromatopac. The concentration of methane in samples was determined by analyzing head space samples by gas chromatography using a Shimadzu GC 9 AM chromatograph equipped with a flame ionization detector and a 15m x 0.53 mm I.D. AT-1 capillary column (Alltech, Deerfield, IL) maintained at 60°C using nitrogen as the carrier and makeup gas (1 mL/min). The bioreactor liquid TCE and methane concentration were then determined by Henry's Law (Gossett, 1987; Atkins, 1986) with normalization of chromatographic peaks to a uniform ratio of gas-phase and liquid-phase volume. Oxygen concentrations were determined by percentage of saturation of oxygen from the oxygen probe readout. Saturation concentrations of oxygen were then calculated by an empirical correlation based on Henry's constant (Franson, 1985).

Abiotic Experiments

A series of abiotic TCE mass balance experiments was performed on the system, by first filling the reactor with water and recycling the water through the columns and back into the reactor at 10 mL/min, and then

adding saturated TCE in water to bring the reactor TCE concentration to 3 mg/L or 0.150 mg/L. The system was run for two days at 3.04 atm for each experiment. Three replicate liquid samples were taken at 0 and 48 h for each experiment. Samples were analyzed for TCE content. A second experiment examined the time required for the TCE concentration to obtain a level equal to the inlet concentration throughout the entire reactor. A TCE inlet concentration of 1 mg/L at a flow rate of 2.1 ± 0.1 mL/min was utilized with a 10 mL/min recycle rate, monitored by a Key Instrument FR4000 Series Flowmeter (Key Instruments, Trevose, PA). The effluent was self-regulating through the back-pressure relief valve at 2 mL/min. TCE levels were monitored for the inlet stream, all four contacting columns, and the CSTR.

TCE Degradation Experiments

Several experiments were performed with different feed concentrations of TCE, ranging from 0.2 to 3 mg/L. The runtime of these experiments varied, with the runtime for each of the above TCE inlet concentrations being at least 90 h and ranging up to 432 h. Liquid samples were obtained from the inlet stream, all four contacting columns, and the CSTR on a regular basis. The following parameters were routinely

determined: biomass levels; TCE, methane, and oxygen concentrations; and sMMO activity level.

IV. Results and Discussion

Background Bioreactor Conditions and Performance

Recently, mutants of *Methylosinus trichosporium* OB3b, capable of producing sMMO but not pMMO, have been developed through the use of chemical mutagenesis (Phelps et al., 1992). Characterization of these mutants showed they were unable to take up or metabolize copper, thus preventing pMMO synthesis (Fitch et al., 1993). By using one of these mutants, PP358, problems associated with copper suppression of sMMO could be avoided. Unlike the wild type, the nutritional requirements of PP358 are significantly more fastidious, probably induced through the chemical mutagenesis. To obtain growth rates comparable to the wild-type strain, the NMS media had to be supplemented with yeast extract and a vitamin solution. With a dilution rate of 0.02 h^{-1} , and CSTR liquid methane and oxygen concentrations kept at 3-5 mg/L and 1-4 mg/L respectively, biomass levels of 200-400 mg/L were maintained in the reactor. Methane made up 80-90% of the off-gas molar composition. Cell growth rates were found to range from 0.074 -0.125 mg/L/min, and the resultant cell yield ranged from 0.26-0.32 mg cells/mg methane. Though the system was maintained non-aseptically in all experiments, the background growth of

contaminants never exceeded 10% of the total population as determined by plate counts on various media, including nutrient agar (Difco) and R2A medium (Difco, Detroit, MI). No contaminants able to grow on methanol or methane were ever observed. The contaminants were growing mostly on the yeast extract being introduced in the feedstock and were almost entirely non-methylotrophic Gram-positive bacteria of airborne origin, i.e.

Curtobacterium, *Aureobacterium*, *Arthrobacter*, *Pimelobacter*, etc. These contaminants did not seem to have a significant effect on the bioreactor operation and TCE degradation.

The various TCE feedstock experiments were performed utilizing the dewatering/filtration column. However, rapid clogging of the 0.2 μm Teflon filter membrane by a biofilm resulted in the majority of the effluent leaving the system to escape through the back-pressure relief valve, where no filtration occurred. In some experiments, where little filtration occurred, the biomass levels in the system declined due to these dilution effects. Throughout most of the experiments, a higher biomass was sustained in column 1 than in the rest of the reactor. This was probably due to residual methane in solution, causing a biofilm of PP358 to form in the column.

In order to obtain maximal levels of sMMO activity, a variety of criteria had to be implemented. These criteria have been defined in batch

culture optimization studies (Bowman et al., 1993). The most critical factor implemented was maintaining a high concentration of methane in the CSTR of the bioreactor. This allowed maximal growth of PP358 and synthesis of sMMO, prevented exhaustion of reductant pools (i.e., NADH) needed for methane oxidation and TCE degradation, and would help recovery of sMMO activity if TCE induced any toxic effects on the cells or on sMMO. Most of the methane was consumed by the second contacting column, and thus competitive inhibition of TCE degradation was never observed. Brusseau et al. (1990) found that methane at 5-10% of solution saturation (1.6-3.2 mg/L methane) was optimal for TCE degradation by sMMO, whereas Broholm et al. (1992) found that methane at levels less than 1.5 mg/L did not significantly affect TCE degradation. In the contacting columns of the bioreactor, in which most TCE degradation takes place, methane levels ranged from 0-0.9 mg/L.

A second factor implemented was maintenance of oxygen levels between 2 and 4 mg/L in the CSTR. This range was optimal for PP358 growth and sMMO activity (Bowman et al., 1993). When oxygen exceeds 5 mg/L, lower growth rates and a reduction in sMMO activity occur. All oxygen was consumed by column 4 ($\tau_{\text{PFR}} = 275$ min). The medium input maintained nitrate (2 mM), phosphate (2 mM), iron (50 M), and magnesium (150 μM) levels well above limitation. Limitation of these

nutrients results in a decline in growth and sMMO activity. During the various bioreactor experiments, these nutrients were always present in the bioreactor at levels adequate for high sMMO activity as determined from batch culture studies (Bowman et al., 1993).

Abiotic Mass Balance

The abiotic experiments demonstrated that the reactor held pressure for at least 48 h and that TCE loss, due to stripping or adsorption, was negligible. The first experiment used an initial TCE concentration of 3.13 ± 0.07 mg/L. After 48 h, 3.05 ± 0.11 mg/L of the TCE was recovered. The second abiotic experiment used a lower initial TCE concentration of 0.123 ± 0.010 mg/L. After 48 h, analysis revealed that 0.126 ± 0.007 mg/L was still present in the reactor.

Figure 3 shows the TCE concentrations converging towards the TCE feed concentration of 1 mg/L throughout the reactor. A time period of approximately 92 h was needed for TCE concentrations throughout the bioreactor to reach 95% of the feed TCE concentration. The hydraulic dilution characteristics of the system were modeled as a 2.4 L CSTR in series with a plug-flow column of volume 3.28 L, with recycle; and also as a series of 5 CSTRs of volumes 0.56 L, 0.8 L, 0.88 L, 1.04 L, and 2.4 L,

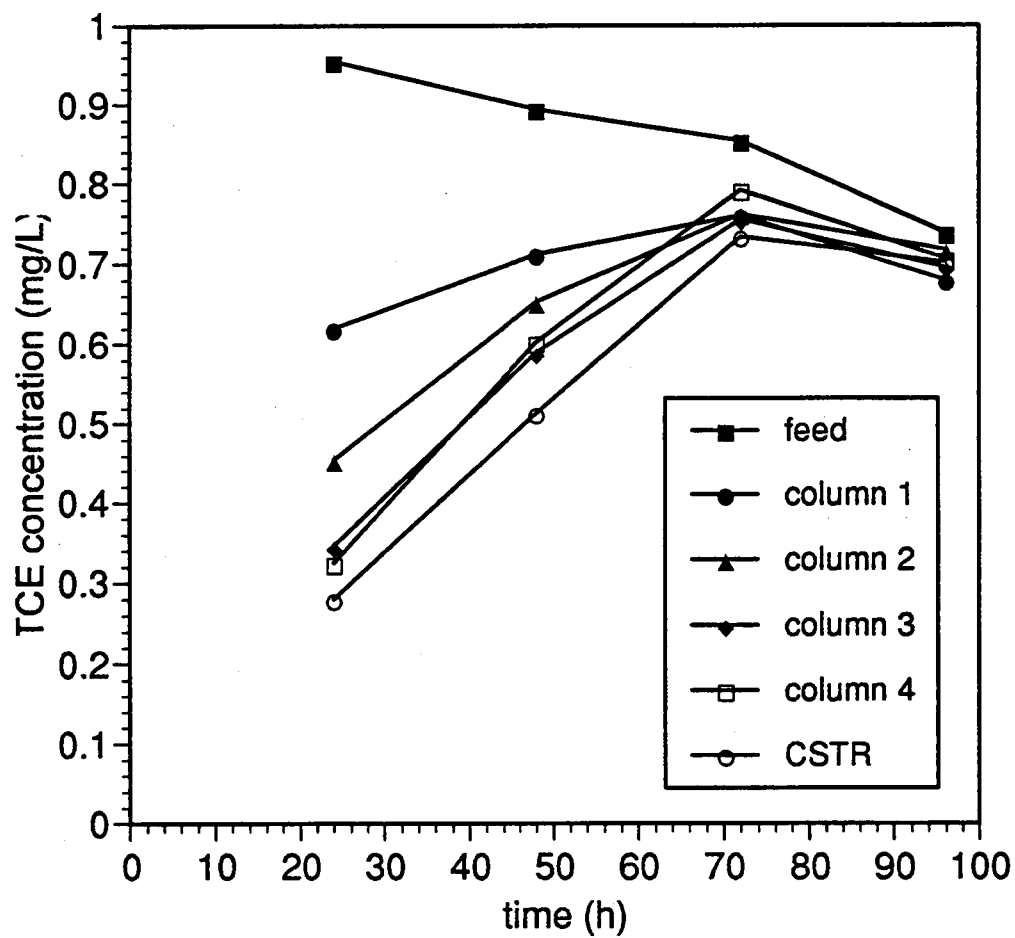


Figure 3. Abiotic Control Data

with recycle. The concentration of TCE in the CSTR, S , is related to the concentration of TCE fed into the CSTR, S_0 , by:

$$\frac{dS}{dt} = v \frac{S_0 - S}{V} \quad (14)$$

where v is the volumetric flow rate, V is the CSTR volume, and there is no generation of TCE ($r_s=0$). This equation was utilized for both models. The equation may then be integrated over the range of S , and from time $t=0$ to $t=t$:

$$S(t) = S_0(t) - \exp\left\{\ln[S_0(t)] - \frac{vt}{V}\right\} \quad (15)$$

where S_0 may be calculated from a dilution equation:

$$S_0(t) = \frac{v_{rec}}{v} S(t - \theta) + \frac{v_f}{v} S_f \quad (16)$$

where v_{rec} is the volumetric flow rate of the cell feed stream to the plug-

flow columns, v_f is the volumetric flow rate of the feed stream, θ is the lag-time, due to the plug-flow columns, and S_f is the TCE concentration of the feed stream.

The model predicted that if the system behaved ideally as configured (CSTR in series with plug-flow columns), 81.4 h of continuous abiotic operation would be required for the system to reach steady-state (minimum of 95% of fed TCE concentration throughout the reactor). The model predicted that 130 h of continuous abiotic operation would be required if the reactor behaved as five CSTRs in series with recycle. The experiment showed that 92 h was required to obtain a steady state (within 95%). The fact that the hydraulic characteristics of the bioreactor fell in-between the two ideal models is normal. It further reinforces the fact that there is little adsorption of the TCE to reactor surfaces or stripping of TCE to the off-gas. If this were the case, then the time necessary for the TCE concentrations to converge to the feed concentration would be much greater. Off-gas analysis from the back pressure relief valve line showed that only a negligible level of TCE was being lost through stripping, due to the addition of methane and oxygen in the CSTR.

Continuous TCE Introduction

0.2 mg/L nominal TCE feed concentration. 100% of the fed TCE was degraded throughout the 432 h of continuous operation by the bottom of the third column, as shown in Figure 4. TCE feed concentrations varied between 0.14 ± 0.02 mg/L and 0.23 ± 0.02 mg/L, where variations were due to partitioning of the TCE between the liquid-phase and the gas-phase in the TCE feed tank as the liquid level declined. Increases in feed concentrations were due to filling of the TCE feed tank. The initial biomass concentration, throughout the reactor, ranged from 500-600 mg/L but declined to an average biomass concentration of 240 mg/L. Shown in Figure 5, the sMMO activity remained stable between 54 ± 6 to 67 ± 4 nmol naphthol formed/h/mg cells throughout the experiment. The maximal rate of degradation was calculated from Figure 6 to be 0.08 mg TCE/L/d, obtained by fitting TCE mass flux [$F_T \cdot S$] along the plug-flow columns vs. residence time, τ , to a second-order polynomial (DeltaGraph Professional, DeltaPoint, Inc., Monterey, CA), taking the derivative, and obtaining the rate at $\tau = 0$ min, where the slope is maximum.

1 mg/L nominal TCE feed concentration. Figure 7 shows the drop in TCE concentrations throughout the reactor during the 428 h in which

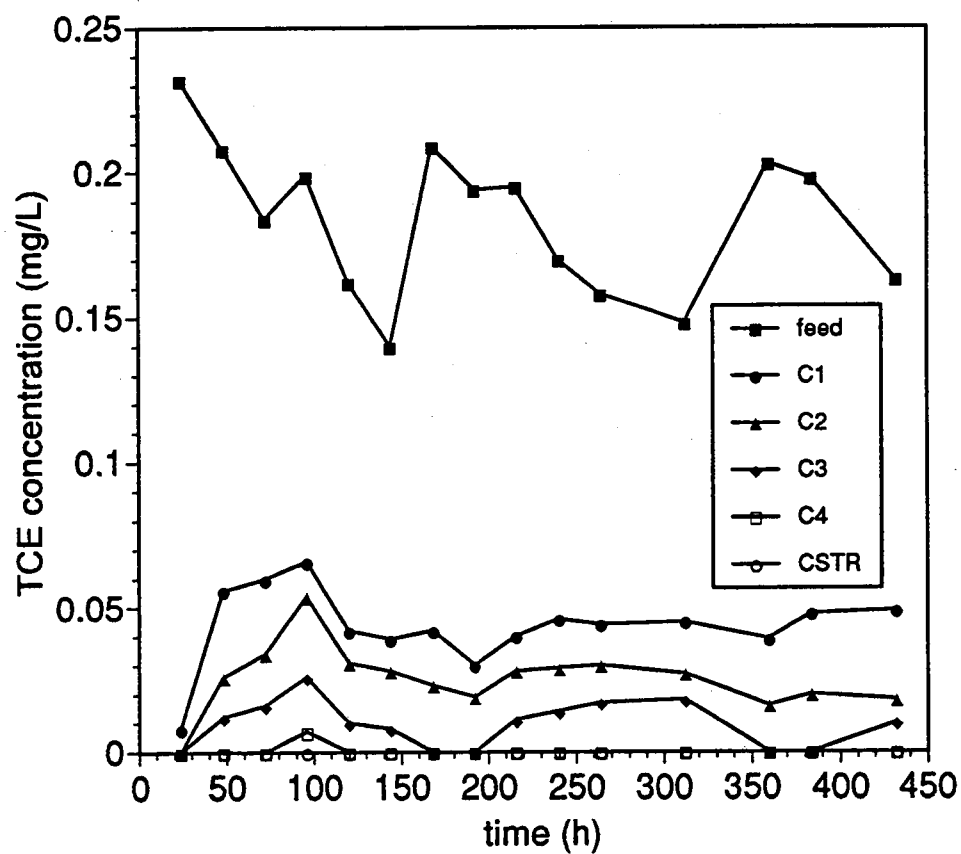


Figure 4. TCE Concentrations Throughout Bioreactor for TCE Feed Concentration of 0.2 mg/L

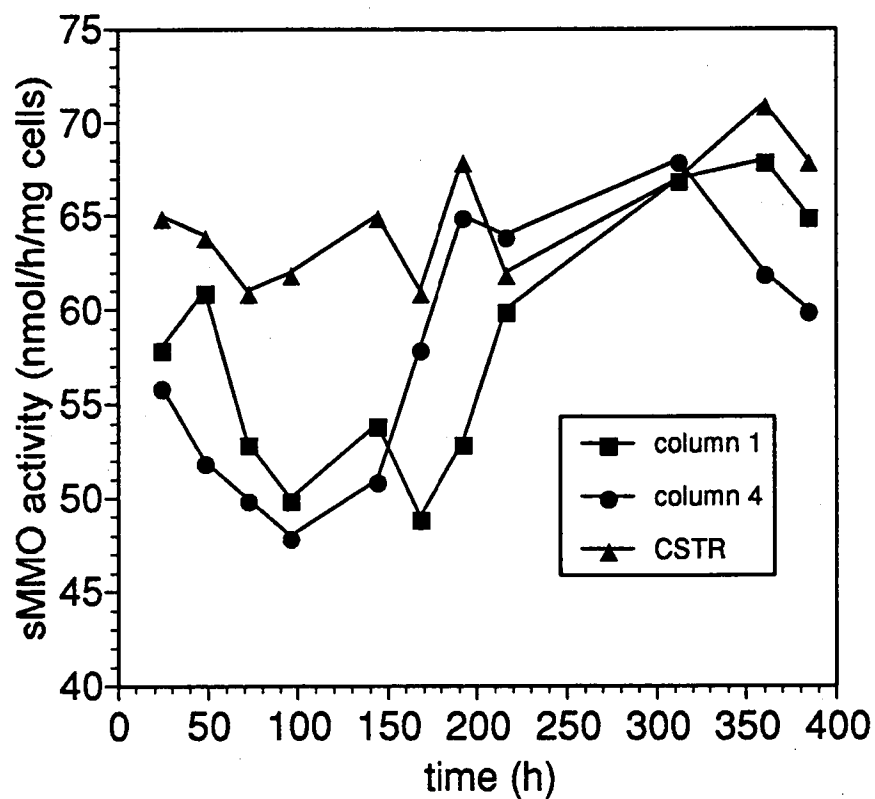


**Figure 5. sMMO Activity Throughout Bioreactor
for TCE Feed Concentration of 0.2 mg/L**

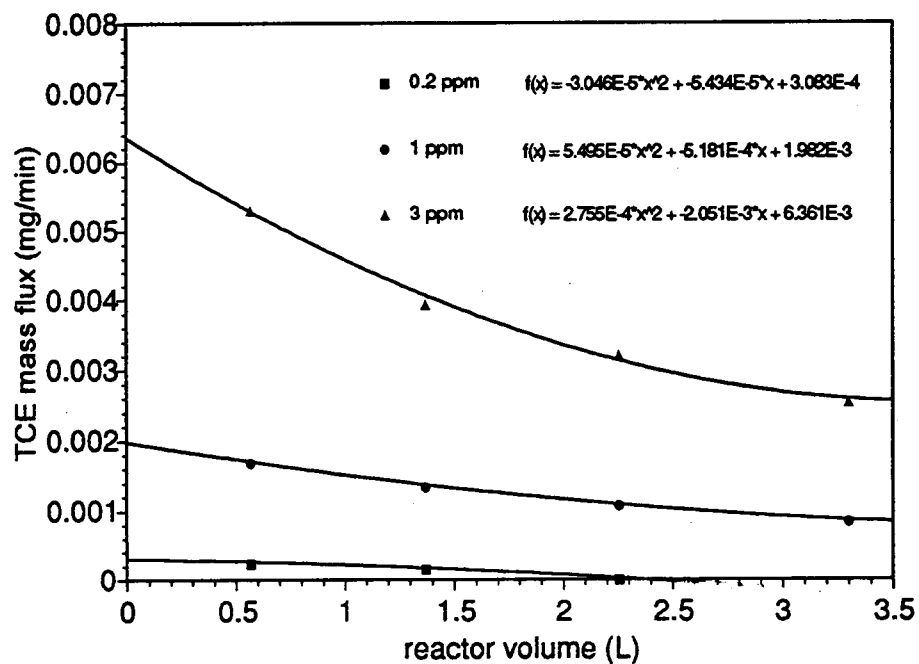


Figure 6. Determination of Maximum Rate of TCE Degradation for TCE Feed Concentrations of 0.2 mg/L, 1 mg/L, and 3 mg/L

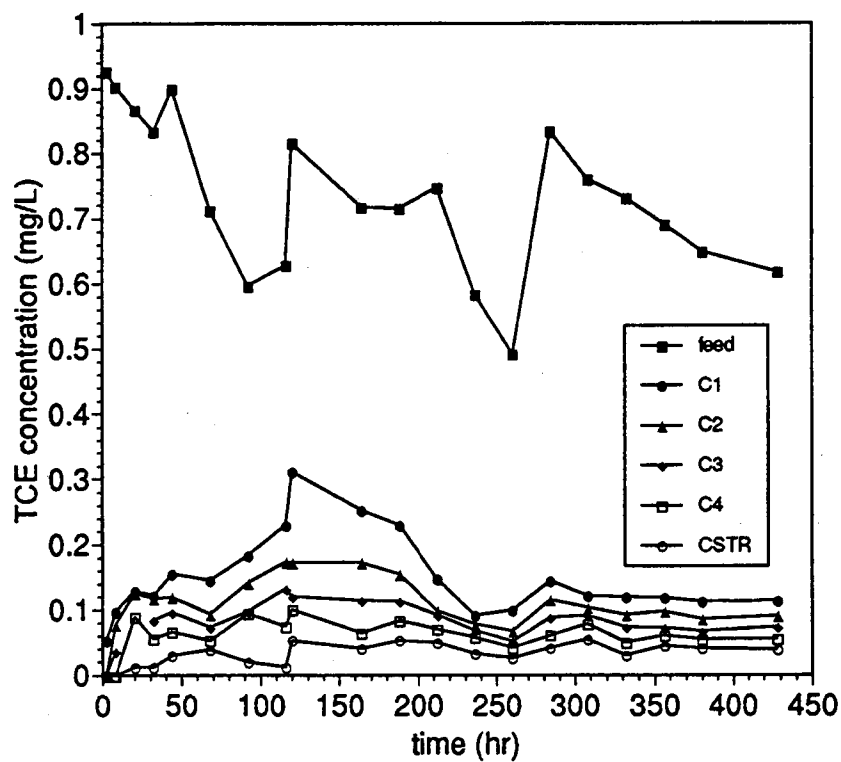


**Figure 7. TCE Concentrations Throughout Bioreactor
for TCE Feed Concentration of 1 mg/L**

TCE was fed at a nominal concentration of 1 mg/L. In order to determine the total amount of TCE degraded by the methanotrophs, a mass balance on TCE over the entire system was performed:

$$Q_{\text{feed}} = Q_{\text{int}} + Q_{\text{eff}} + Q_{\text{deg}} \quad (17)$$

where Q_{feed} is the total mass of TCE fed to the system, Q_{int} is the total mass of TCE remaining in the reactor, Q_{eff} is the total mass of TCE that was present in the effluent, and Q_{deg} is the total mass of TCE that was degraded. This mass balance assumes that no TCE adsorption occurs to reactor surfaces or is adsorbed to biomass without degradation. Both of these assumptions are valid. The abiotic experiments demonstrated there were negligible abiotic losses. Koh et al. (1993) found that over 80% of initial TCE concentrations was extractable in heat killed strains of OB3b, where some of the loss was attributable to leaks in the system. Equation 17 may be rearranged to obtain an equation for the total mass of TCE degraded:

$$Q_{\text{deg}} = Q_{\text{feed}} - Q_{\text{int}} - Q_{\text{eff}} \quad (18)$$

Q_{feed} was obtained in Figure 8 by fitting the TCE feed concentrations to a sixth order polynomial (DeltaGraph Professional, DeltaPoint, Inc., Monterey, CA), integrating the polynomial, and multiplying the result by the volumetric flow rate of the feed ($Q_{\text{feed}} = 36.56$ mg TCE). Q_{eff} was determined similarly, by fitting a sixth order polynomial to the TCE effluent concentrations, integrating the polynomial, and multiplying the result by the volumetric flow rate of the effluent stream ($Q_{\text{eff}} = 1.86$ mg TCE). The mass of TCE retained in the reactor is comprised of a mass of TCE in the CSTR, Q_{CSTR} , and a mass of TCE in the plug-flow columns, Q_{PFR} :

$$Q_{\text{int}} = Q_{\text{CSTR}} + Q_{\text{PFR}} \quad (19)$$

Q_{CSTR} was computed by determining the average TCE concentration in the CSTR during steady-state (0.042 mg/L) and multiplying by the volume of the CSTR (2.4 L). Q_{PFR} was obtained in Figure 9 by averaging the concentrations of TCE in the plug-flow columns during steady-state operation and plotting these concentrations vs. reactor volume, and a best-fit second order polynomial was obtained. Integration resulted in a total retained TCE mass in the plug-flow columns of 0.30 mg, and from equation 19, the total retained mass of TCE, Q_{int} , was calculated to be 0.40 mg.

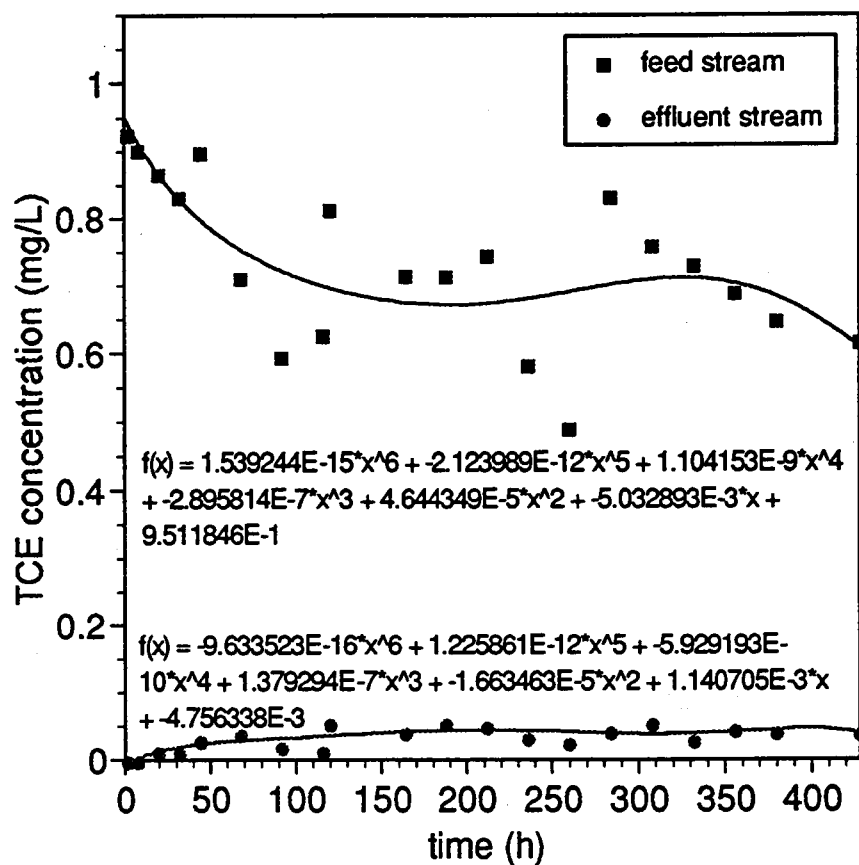


Figure 8. Determination of Q_{feed} and Q_{eff} for a Nominal TCE Feed Concentration of 1 mg/L

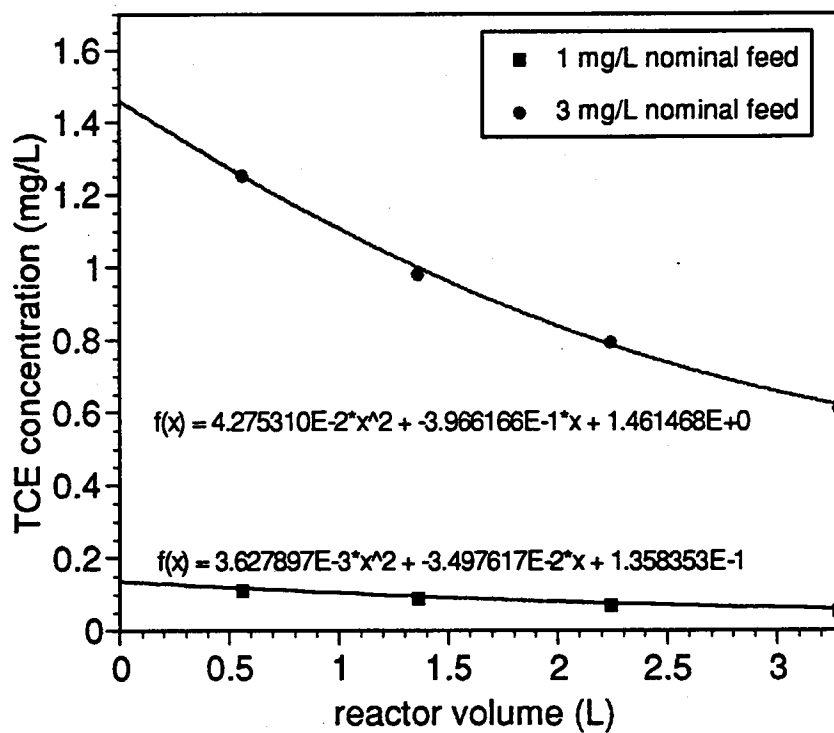


**Figure 9. Determination of Q_{int} for
TCE Feed Concentrations of 1 mg/L and 3 mg/L**

From equation 18, the total mass of TCE that was degraded was 34.30 mg, resulting in a total removal efficiency of 93.8%. The steady-state removal efficiency may be expressed:

$$\text{removal efficiency} = \frac{S_f - S_e}{S_f} * 100\% \quad (20)$$

The steady-state removal efficiency was computed to be 93.9%.

A pseudo-steady-state was reached after 12 d, where the effluent concentration (that of the CSTR) stabilized around 0.04 mg/L. Feed concentrations ranged between 0.49 ± 0.01 mg/L and 0.93 ± 0.02 mg/L. Biomass concentrations again declined rapidly during the first three days of operation, from an initial concentration of 600 mg/L to a stable concentration 360 ± 30 mg/L, after 4 d. As shown in Figure 10, the sMMO activity remained relatively stable between 52 ± 5 and 64 ± 4 nmol naphthol formed/h/mg cells. The average sMMO activity decreased by ~10% over the course of the experiment, but the variation in sampling (error bars not shown) was ~10%. There was no statistical decrease in sMMO activity. The maximum rate of degradation was calculated to be 0.75 mg TCE/L/d by fitting the data to a second-order polynomial in Figure 6.

3 mg/L nominal TCE feed concentration. The reactor was continuously operated for 90 h, and the concentrations throughout the

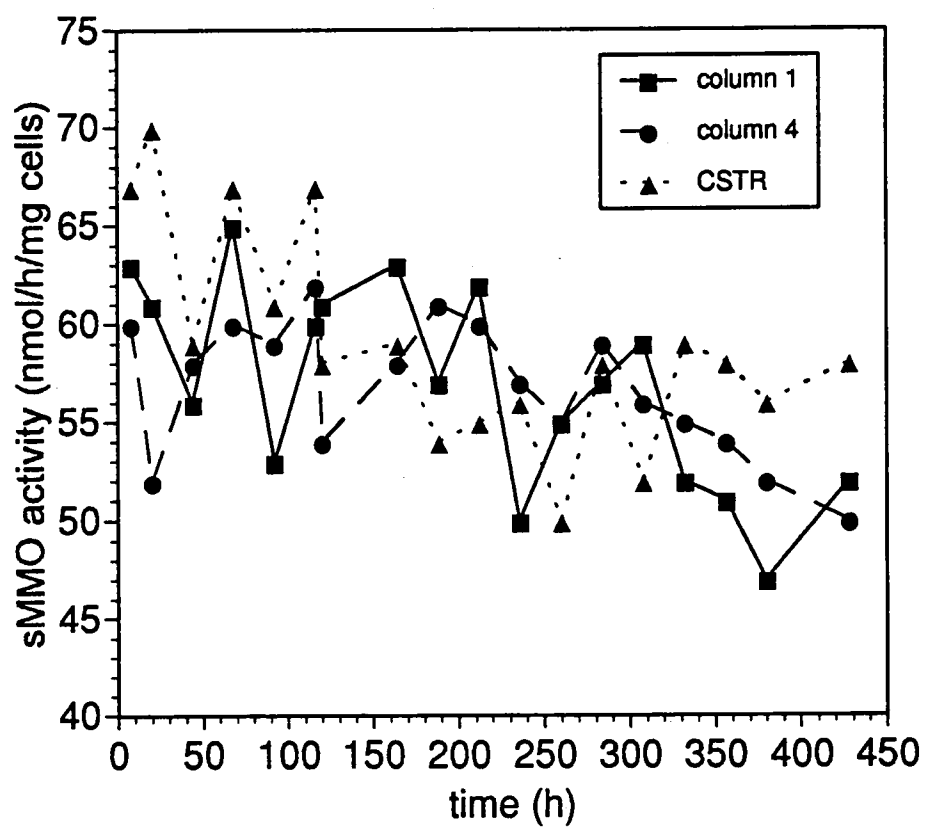


**Figure 10. sMMO Activity Throughout Bioreactor
for TCE Feed Concentration of 1 mg/L**

reactor are shown in Figure 11. Figure 12 shows the best-fit sixth-order polynomials integrated to show that a total of 26.61 mg of TCE was fed into the reactor (Q_{feed}), with TCE feed concentrations ranging between 2.34 ± 0.17 mg/L and 2.73 ± 0.54 mg/L. Q_{eff} was calculated to be 2.44 mg TCE, and Q_{int} was calculated to be 19.96 mg TCE. A total of 19.96 mg of TCE was degraded, Q_{deg} , throughout the 90 h, resulting in 75.0% of the total fed TCE being lost through biodegradation. However, the removal efficiency increases with the length in time of the experiment, because the ratio of $Q_{\text{int}}:Q_{\text{feed}}$ decreases, assuming that Q_{int} reaches a steady-state value. The steady-state removal efficiency is 81.7%. Effluent concentrations rose from 0 mg/L to 0.46 ± 0.07 mg/L as the biomass concentrations stabilized to 320 ± 35 mg/L, with relatively stable sMMO activities (as shown in Figure 13), ranging between 63 ± 4 to 71 ± 3 nmol naphthol formed/h/mg cells. The maximal rate of degradation was calculated to be 2.95 mg TCE/L/d by a second-order polynomial as shown in Figure 6.

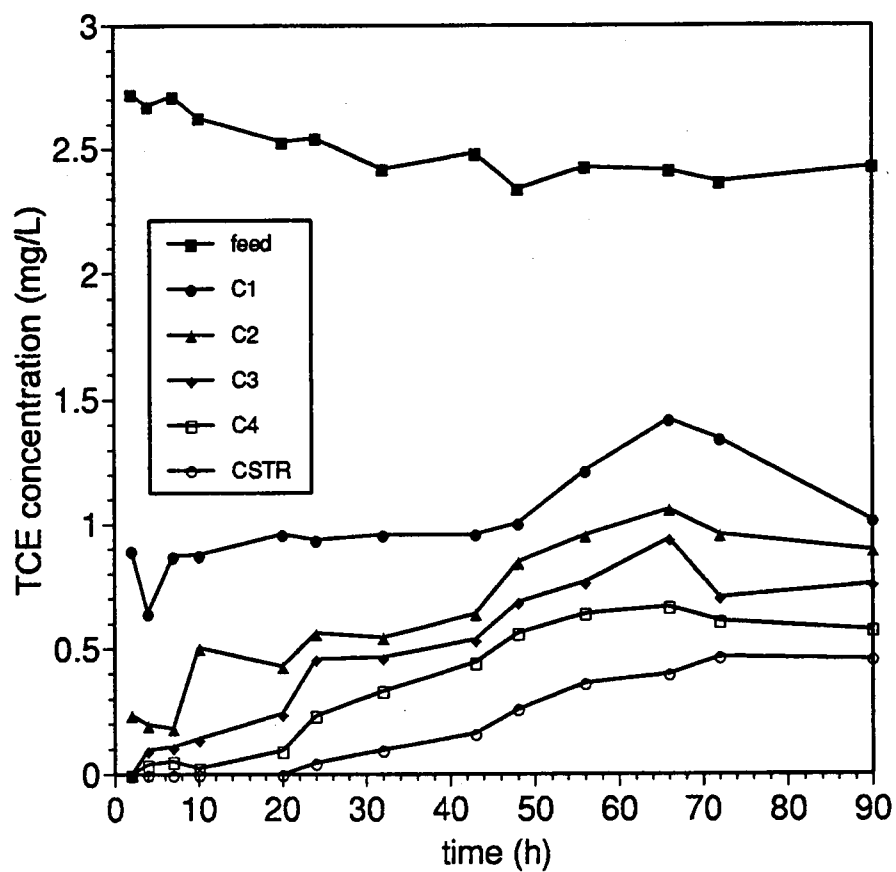


**Figure 11. TCE Concentrations Throughout Bioreactor
for TCE Feed Concentration of 3 mg/L**

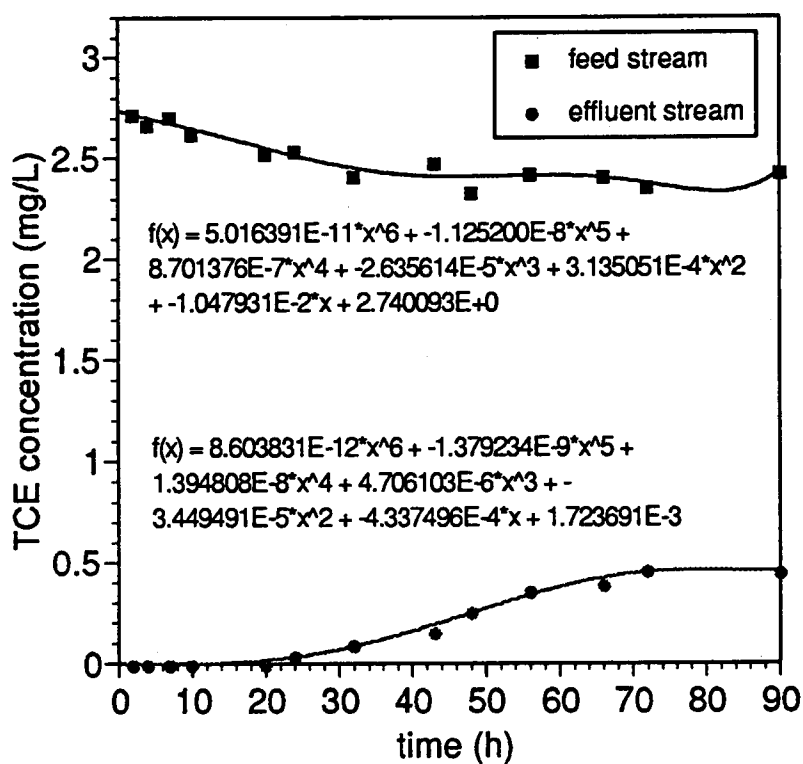


Figure 12. Determination of Q_{feed} and Q_{eff} for a Nominal TCE Feed Concentration of 3 mg/L

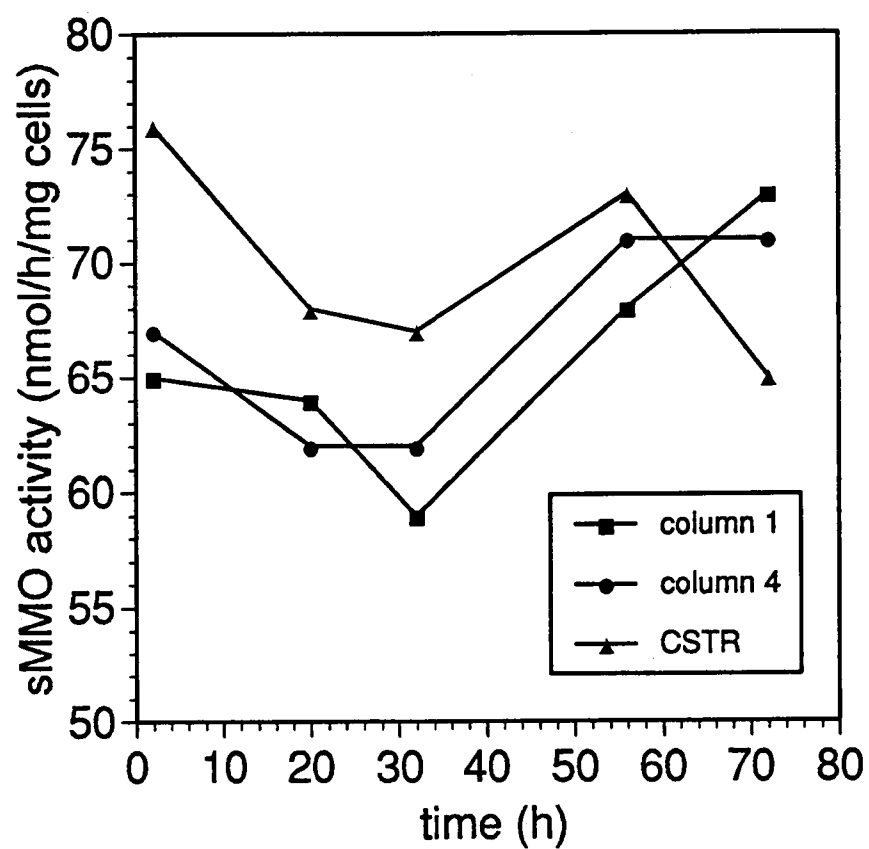


**Figure 13. sMMO Activity Throughout Bioreactor
for TCE Feed Concentration of 3 mg/L**

V. Conclusions

A pressurized dual-stage suspended-growth bioreactor system with cell recycle has been developed at the bench scale and successfully operated for methanotrophic co-metabolic oxidation of TCE at TCE feed concentrations ranging from 0.2 mg/L to 3 mg/L. It is noteworthy that 100% to 82% steady-state removal efficiencies were achieved for TCE feed concentrations ranging from 0.2 mg/L to 3 mg/L in only 4 h. Lower effluent concentrations can thus be achieved by lengthening the total residence times in the plug-flow columns and/or increasing the reactor biomass concentration.

Both batch and continually operated abiotic experiments have shown that the bioreactor was effective at maintaining a complete mass balance for TCE degradation. During continuous biological operation, the system maintained biomass concentrations of approximately 350 mg/L. Experiments with TCE continuously introduced showed a high level of degradation, with the relative amount of degradation declining with increasing TCE feed concentrations. However, the maximum rate of TCE degradation increased from 0.08 mg TCE/L/d to 2.95 mg TCE/L/d, as expected from the second-order Monod equations. Complete removal of TCE fed at 0.2 mg/L was achieved with the bioreactor.

It was interesting to observe that sMMO activity was relatively constant (50-70 nmol naphthol formed/hr/mg cell) throughout the entire bioreactor experiments using TCE feed levels of 3 mg/L and less (Figures 5, 10, and 13). The level of sMMO activity attained was approximately 30-35% of the maximal PP358 sMMO specific activity, as measured by the naphthalene oxidation assay, using whole cells in the presence of 10 mM sodium formate ($V_{\max} = 200$ nmol naphthol formed/hr/mg cells) (Bowman et al., 1993). Batch culture studies have shown that sMMO specific activity tends to peak in early stationary growth phase, then declines to a stable level. This trend is equivalent to what is observed in the bioreactor studied here. The stabilized sMMO activity can be maintained for several weeks in batch culture with periodical inputs of methane and nutrients (Bowman et al., 1993). Further optimization of TCE degradation in this bioreactor would be best accomplished through the increase of biomass levels during operation achieved by retaining a high proportion of the biomass through the use of filtration. A level of cell wastage would still be needed to maintain an actively growing segment of the bioreactor population.

The reactor has been proven to show high levels of degradation and a closure of the mass balance on TCE. The monitoring capabilities of the reactor are sufficient to completely describe the kinetics and characteristics of the system for future modeling and optimization.

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Vita

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