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Biodegradation of Trichloroethylene by *Alcaligenes eutrophus* JMP134 in a Laboratory Scale Bioreactor

DANIEL J. McKAY,[1] JOHN S. MORSE,[1] and TERRY C. HAZEN[2]

[1]*Department of Mechanical Engineering*
University of South Carolina
Columbia, SC
[2]*Savannah River Technology Center*
Westinghouse Savannah River Company
Aiken, SC

ABSTRACT

A single stage recirculating bioreactor with a pure culture of *Alcaligenes eutrophus* JMP134 and a packed gravel bed was operated for a two week period during which a maximum biodegradation of 88.4% of the influent trichloroethylene was observed with average performance of 71.8% at 8.4 hour hydraulic retention time. The reactor was then operated for a seven day period with the gravel bed removed, demonstrating a maximum degradation of 97.4% and an average of 95.6%. Average influent and effluent concentrations for the second case were 5.97 mg/l and 145 μg/l with a mean retention time of 14.1 hours. Phenol, supplied as the sole source of carbon and energy, was degraded below levels of detection (< 1.6 μM) in the effluent.

INTRODUCTION

Trichloroethylene (TCE) is a volatile chlorinated hydrocarbon which has been widely used by industry as a solvent in degreasing operations (1). Waste disposal methods have resulted in the widespread contamination of groundwater with TCE. The recalcitrance and mobility in soils of this suspected carcinogen have prompted the study and development of numerous remediation techniques including biological transformation. Several strains of bacteria have been shown to be capable of fortuitous degradation of TCE via aromatic induction of a catabolic pathway (2). Among these is the JMP134 strain of *Alcaligenes eutrophus* which can degrade TCE by a phenol-dependent pathway (3). Another such bacterium is *Pseudomonas cepacia* G4 which utilizes a toluene-degradative pathway (4). Shields, *et al* (5) recently reported that a revertant of a Tn5-induced mutant of G4 was capable of oxidizing TCE without aromatic induction, making it a potentially suitable candidate for in-situ remediation strategies. However, for ex-situ treatment in bioreactors, phenol is readily degraded by bacterium such as G4 or JMP134 and can be monitored to ensure complete oxidation.

This study had two basic objectives. One was to determine if *A. eutrophas* JMP134 could degrade TCE in a flow-through bioreactor and to map out one set of operating conditions. The second was to evaluate the effect of two substratum types. The first substratum was a pea gravel bed, while the second was a liquid culture, with no bed. These two types were compared under conditions of identical total reactor volumes. On a full-scale bioreactor, one of the key factors is the total reactor volume, not pore volume, since total reactor volume is a major determining factor in reactor cost.

MATERIALS AND METHODS

Bacterial strain and culture conditions

A. eutrophus JMP134 was provided by A. R. Harker of Oklahoma State University and maintained on TGE solid medium (6). The bioreactor medium consisted of a mineral salts solution developed by Repaske and Mayer (7,8). Reagent grade phenol and TCE (Mallinckrodt, Paris, KY) were added to concentrations between 720-1540 mg/l and 3.2-7.4 mg/l respectively. Neither the medium nor the bioreactor was sterilized prior to inoculation with JMP134. The bioreactor was filled with fresh medium and then approximately 100 ml of JMP134 in a liquid culture was added from a 24 hr old transfer. Within 48 hours the bioreactor was fully colonized and the TCE flow was initiated.

Experimental equipment and procedures

The bioreactor was designed for continuous fluid circulation at a flowrate up to 500 times the inlet rate. Construction materials consisted entirely of stainless steel. Originally, bacteria were cultivated on pea gravel having nominal diameters ranging from 3 to 9 mm bedded inside a vertically mounted 1.5 m length of 5.1 cm diameter pipe (Fig. 1). A second column, identical to the first but without the pea gravel, was placed parallel to the reactor and served to furnish a gaseous space for oxygen recharge of the liquid. The two columns were connected at the top by 9.5 mm diameter tubing. Fluid was circulated by a 0.37 kW centrifugal pump having stainless steel wetted parts while a recirculation loop controlled the flow rate to the reactor. An identical poisoned control was used to measure abiotic losses.

Two feeds were used: (a) concentrated mineral salts and (b) distilled water containing appropriate amounts of phenol and TCE. The two flows were kept separate to ensure a low cell population in the influent. The latter flow was delivered via gravity and controlled by a fine metering valve, whereas the mineral salts were fed by a variable speed peristaltic pump. The discharge line, open to atmospheric pressure, was raised to the desired height of the water in the recharge column to maintain constant volume in the bioreactor. Siphoning was prevented by a vacuum break at the highest point in the line. The inlet TCE concentration was measured at a point before the two separate feeds were mixed by making triplicate analyses of a single daily sample. The TCE concentration actually entering the bioreactor was calculated by multiplying the measured concentration by the ratio of feed (b) to the total inlet flowrate. This elaborate procedure was necessary to ensure the measurement was made before any significant degradation of the TCE had occurred. Since the bioreactor contents were well-mixed, the effluent concentration was obtained from triplicate analyses of a sample withdrawn from the base of the reactor column.

Oxygen was sparged inside the bottom of the reactor column through a 2 micron sintered filter element. A two-stage regulator controlled upstream line pressure as oxygen flowed through 6.1 m of 0.76 mm stainless steel tubing. The pressure drop was measured with a manometer and related to flowrate via the isothermal pipe flow equation. A fine metering valve was used to control the oxygen flow. The recharge column was vented through a 1.6 mm diameter orifice at the top to allow the escape of carbon dioxide and excess oxygen. Flowrates measured with a soap bubble flowmeter at the vent on the control bioreactor were within 2.4% of the calculated oxygen flowrate.

Analytical methods

Analyses of samples for TCE concentration were performed on a Hewlett Packard 5890 series II gas chromatograph with electron capture detector (ECD) in conjunction with a 19395A headspace sampler, 3396A integrator and a 60 m x 0.75 mm x 1.5 µm film VOCOL capillary column (Supelco, Bellefonte, PA). Carrier was ultra high purity (UHP) nitrogen at 20 ml/min through the column while makeup was UHP nitrogen supplied at 60 ml/min. Oven temperature was held at 35°C for 5 min, then ramped to 60°C at 4°C/min. Samples were placed in 20 ml headspace vials with Teflon lined septa and crimp top caps (Wheaton, Millville, NJ). Dilutions to 10 ml final volume were performed as necessary to ensure a TCE concentration below 145 ppb (upper limit for the ECD). Samples were spiked with 0.2% sodium azide, then equilibrated for a minimum of four hours at 75°C before being analyzed.

Detection for phenol was performed by direct liquid injection of 6.6 µl on a Hewlett Packard 5890 series II gas chromatograph with flame ionization detector and 2.44 m x 3.18 mm stainless steel

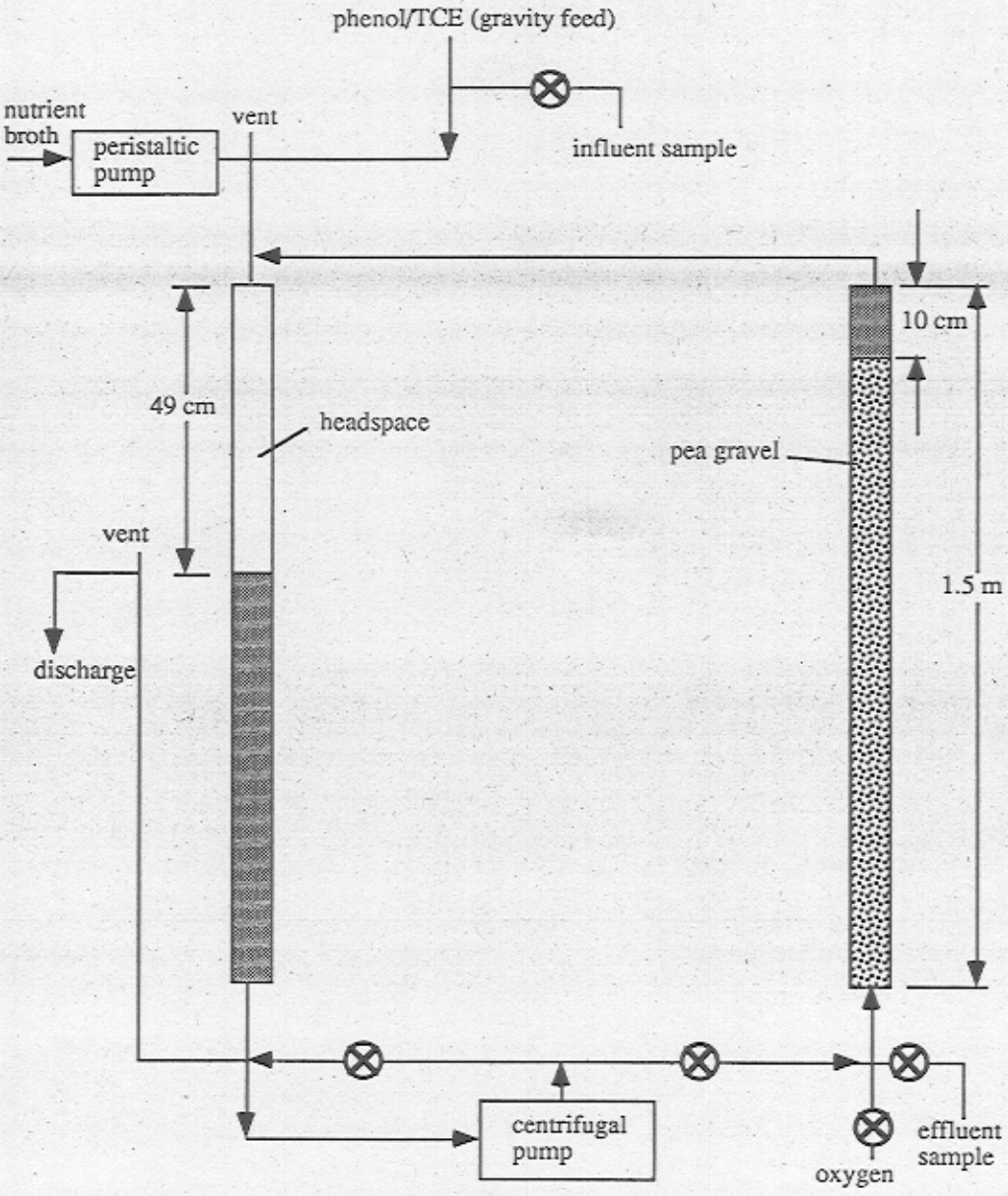

Figure 1
Flow schematic of bioreactor. Liquid volume was 4.2 liters with gravel bed, 6.5 liters without bed. Headspace volume was 1.1 liters. Influent concentration was calculated by multiplying the sample concentration by the ratio of substrate feed to total feed rate. Discharge concentration was measured at base of reactor column.

column with 5% OV-73 on 100/120 Supelcoport (Supelco, Bellefonte, PA). Carrier was zero grade helium at 30 ml/min while oven temperature was isothermal at 120°C. Under those conditions, the minimum detection level was 1.6 μM. Calibrations for phenol and TCE were performed approximately every four days by multiple analyses of standards.

The gas chromatographic method used for TCE analysis will also quantify all other C_1 and C_2 chlorinated hydrocarbons. None of these were ever detected in any measurements, so only TCE results were reported.

Attempts to measure chloride concentration in the bioreactor effluent were made. Such measurements could be used to determine the extent of mineralization of the TCE. However, the very high

background levels of chloride in the media overwhelmed the chloride resulting from TCE mineralization. At 6 mg/liter of TCE loading, complete mineralization results in 1.4E-4 chloride ions per liter, while the media contributes over 2.2E-2 chloride ions per liter. At this ratio of nearly 160:1, the background chloride renders the chloride measurements useless for determining the extent of mineralization.

Transformation calculations

Determination of biodegradation required quantification of TCE in both liquid and headspace regions of the bioreactor and distinction between biotic and abiotic losses. The former was estimated by Henry's law while the latter was estimated by comparison to a poisoned control. Henry's Law constants were obtained by linear interpolation between two published values: 9.9E-3 atm m^3/mol at 20°C (9) and 1.96E-2 atm m^3/mol at 37°C (10).

The biodegradation rate under non-steady conditions was determined by modeling the bioreactor as a well stirred tank with one inlet and one outlet. The period between two sampling times was denoted as Δt (hours). Additional variables were defined to develop a mass balance equation:

$Q_L(t)$ - aqueous quantity of TCE in bioreactor during $0 < t \leq \Delta t$, mg

$Q_V(t)$ - gaseous quantity of TCE in bioreactor during $0 < t \leq \Delta t$, mg

Q_0 - aqueous quantity of TCE in bioreactor at $t = 0$, mg

Q - aqueous quantity of TCE in bioreactor at $t = \Delta t$, mg

Q_T - total of TCE losses due to stripping and biodegradation, mg

C_0 - influent TCE concentration at $t = 0$, mg/l

$C_{\Delta t}$ - influent TCE concentration at $t = \Delta t$, mg/l

I - hydraulic flowrate of influent (and effluent) l/hr.

The total rate of TCE loss, dQ_T/dt, was assumed to be constant during the sampling interval. Influent concentration was assumed to vary linearly with time such that TCE entered at the rate of $I[C_0 + (C_{\Delta t} - C_0)t/\Delta t]$. The rate of flow in was equal to the rate of flow out. Therefore the volume of the liquid in the bioreactor stayed constant at V_L and TCE left the bioreactor at the rate of IQ_L/V_L. The mass ratio of TCE in the headspace to that in the liquid, $Q_V(t)/Q_L(t)$, was assumed constant for a given temperature in accordance with Henry's law and assigned the value m_r. The rate of change of total TCE mass inside the bioreactor was then $(1+m_r)dQ_L/dt$. The resulting mass balance expression was

$$(1+m_r)dQ_L/dt = I[C_0 + (C_{\Delta t}-C_0)t/\Delta t] - IQ_L/V_L - dQ_T/dt \qquad (0 \leq t \leq \Delta t). \qquad [1]$$

Equation (1) is a first order, linear ordinary differential equation for which the solution is

$$Q_L(t) = C_0V_L + [(1+m_r)^{-1}It/V_L - 1](C_{\Delta t}-C_0)(1+m_r)V_L^2/(I\Delta t) +$$
$$[(C_{\Delta t}-C_0)(1+m_r)V_L^2/(I\Delta t) - C_0V_L + Q_0 + V_L/I\, dQ_T/dt]\exp[-It/V_L(1+m_r)^{-1}]$$
$$- V_L/I\, dQ_T/dt \qquad (0 \leq t \leq \Delta t). \qquad [2]$$

Eqn. [2] was rearranged to solve for dQ_T/dt and then integrated to obtain $Q_T(t)$ after setting $Q_L(t) = Q$ at $t = \Delta t$. As mentioned previously, abiotic losses were assessed by use of a control bioreactor poisoned by addition of 0.2% sodium azide. Designating the cultured reactor as 1 and the control as 2, the quantity lost due to abiotic factors over the interval Δt was estimated as

$$Q_{A1} = Q_{T2}[\int Q_{V1}dt / \int Q_{V2}dt] \qquad [3]$$

by recognizing that TCE was vented at the headspace concentration. The expression in brackets is the ratio of average headspace concentrations in the bioreactor and control. Since both systems had equal liquid and gas phase volumes, equation (3) was rewritten in terms of liquid phase concentrations:

$$Q_{A1} = Q_{T2}[\int Q_{L1}dt / \int Q_{L2}dt]. \qquad [4]$$

Equations [3 & 4] assume equal vent gas flowrates. Because of biological conversion of O_2 to the more soluble CO_2, actual stripping in the cultured bioreactor was probably overestimated. The biodegraded quantity of TCE was calculated as

$$Q_{B1} = Q_{T1} - Q_{A1}. \qquad [5]$$

The percent of influent TCE transformation during the sampling interval Δt was

$$Q_{B\%} = 100 \times Q_{B1}/[I(C_{\Delta t}+C_0)/2] \qquad [6]$$

where the denominator was the TCE influx.

A check on these calculations was made by comparing Q_{T2} (= Q_{A2}) with the amount which was stripped from the control reactor based on Henry's law and the known oxygen influx (equal to vent gas flowrate in the control). The average difference between these two calculations was 8.6%.

The check was not appropriate for Q_{A1} because of the previously mentioned difference between entering and exiting gas flowrates in the cultured bioreactor.

RESULTS AND DISCUSSION

A closed system hydraulic test preceded the degradation study to assess the degree of abiotic losses other than ventilation stripping. A summary of the data indicated 0.51% loss per day in the bioreactor and 0.74% loss per day in the control. The bioreactor and control were then operated for 6 weeks in the gravel bed configuration while temperature and pH were monitored, but not controlled. Average values for temperature and pH were 33°C and 6.18 (measured once per day). Difficulties with mineral salts feedrate control hampered data collection during the first four weeks, producing highly variable data due to resulting dissolved oxygen and pH imbalances. The bioreactor was subsequently operated within the desired range of conditions for two weeks during which average TCE degradation relative to influent concentration was 71.8% (Figures 2 and 3). Phenol was detected in undetermined amounts in the effluent on 5 of those days.

The gravel bed was then removed from the reactor column. There was no visible evidence of an aerobic biofilm on the gravel. Conventional wisdom for bioreactor design suggests that an attached biofilm is normally preferable. However, in this case the bacteria apparently did not attach. What was observed was that unsuspended biomass had settled near the bottom of the reactor column. During the operation of the gravel bed phase, the bioreactor circulation was ever decreasing and the control valves around the pump (Fig. 1) had to be constantly adjusted to maintain the circulation flow. The unsuspended biomass was reasoned to have been responsible for this phenomenon. During this phase of bioreactor operation, an immediate improvement in degradation was also observed, with 95.6% average degradation of influent TCE during one week of operation after bed removal (Figures 4 and 5). The average influent phenol concentration was 871 mg/l. This was oxidized below the level of detection (< 150 µg/l) before discharge.

Table 1 provides the average and standard deviation of the bioreactor parameters for both bed types. It can be readily seen that the only significant differences are in phenol concentration (and variability) and hydraulic residence time, in addition to the aforementioned circulation problems. A thorough regression analysis of phenol concentration versus TCE degradation failed to show any correlation. Therefore the difference in performance is most likely just due to the difference in bed types.

Removing the gravel bed raises the residence time dramatically, since the study was performed under constant total reactor volume, as mentioned previously. An increase in residence time would tend to increase the TCE degradation. The bacteria apparently did not receive any benefit from the presence of the gravel bed, since they did not attach to it. The effect of the unsuspended biomass and

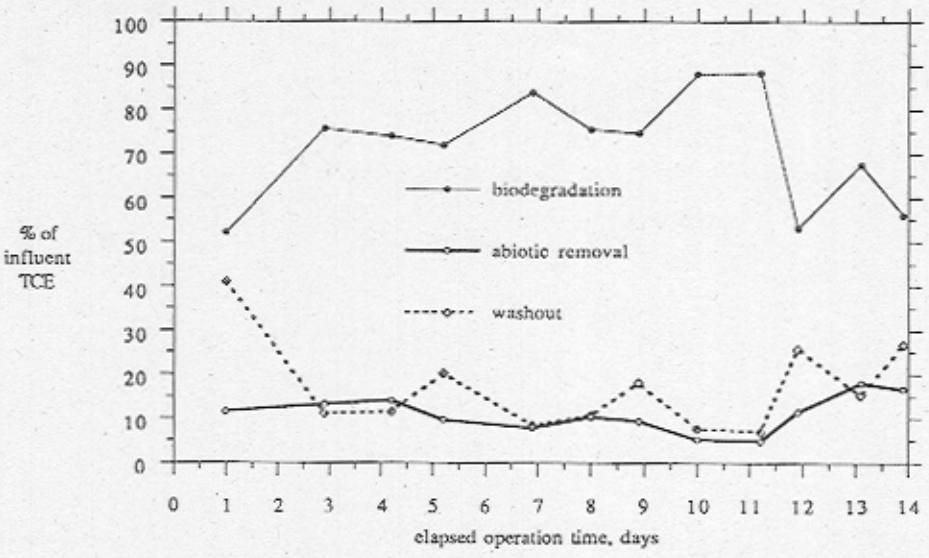

Figure 2
Percent removal of TCE from bioreactor with gravel bed. Each datum represents average of three analyses. Coefficient of variation for washout is ± 2% and is ± 5% for stripping measurement.

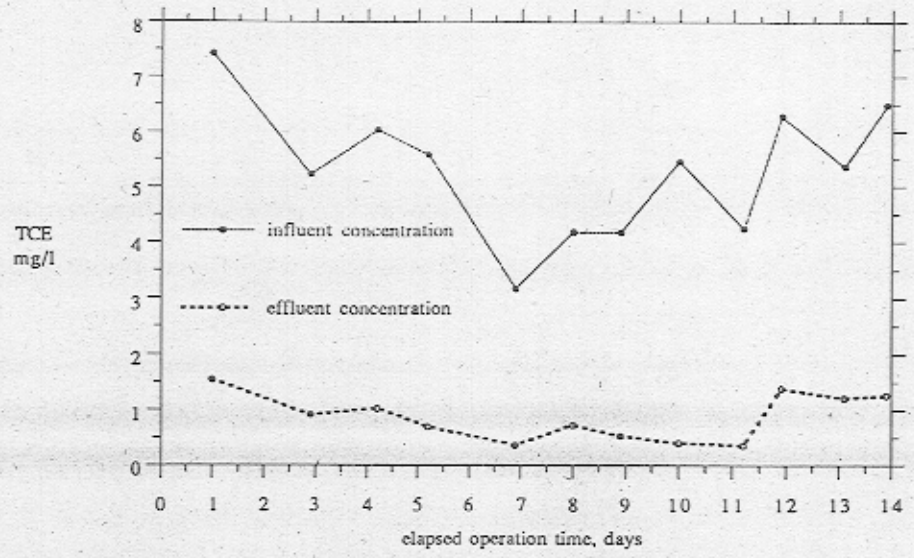

Figure 3
TCE concentration in influent and effluent of bioreactor with gravel. The measured influent concentration was adjusted to account for the mixing of two inlet flows. Each datum is average of three analyses. Coefficient of variation for TCE concentration is ± 3 %.

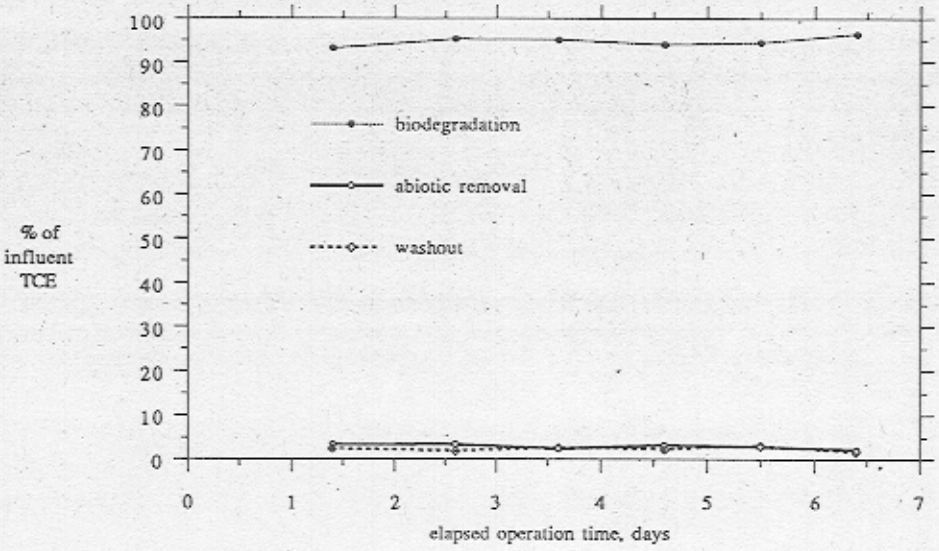

Figure 4
Percent removal from bioreactor without solid support. Each datum is average of three analyses. Coefficient of variation for washout is ± 2% and is ± 5% for stripping measurement.

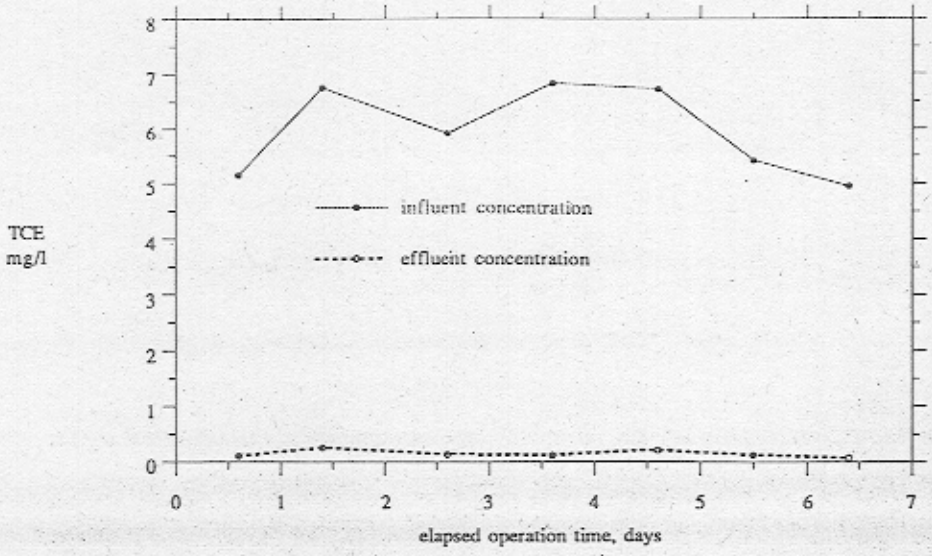

Figure 5
TCE concentration in influent and effluent of bioreactor after removal of gravel bed. The measured influent concentration in the TCE feed was adjusted for the ratio of the two inlet flow rates. Each datum is average of three analyses. Coefficient of variation for TCE concentration is ± 3 %.

TABLE I

Bioreactor Parameters	Gravel Bed Operation	Liquid Culture Operation
pH	6.24 ± 0.076	6.10 ± 0.06
temperature (°C)	32.4 ± 2.05	35.0 ± 2.27
inlet phenol concentration (mg/l)	988 ± 224	871 ± 17.4
inlet TCE concentration (mg/l)	5.23 ± 0.87	5.53 ± 0.83
hydraulic residence time (hr)	7.95 ± 1.27	13.4 ± 0.688
inlet TCE degraded (%)	71.8 ± 12.5	95.6 ± 1.66

resulting circulation problems on performance is unknown. These could have been the cause of the wide swings in TCE degradation observed with the gravel bed.

These preliminary results for TCE degradation compare favorably with the reports of laboratory- and pilot-scale bioreactors by other researchers. Miller, *et al* (11) observed an average degradation of 81.7% of 10.9 mg/l of influent TCE in 20.5 hours. A two-stage anaerobic-aerobic biofilm reactor was reported by Fathepure, *et al* to degrade 96% of TCE in 39.7 hours (12). Phelps, *et al* have demonstrated 95% removal of 20 mg/l after 5 days by a methane and propane oxidizing consortium (13,14). Separating the growth and treatment phases of a bioreactor into two stages may, in some circumstances, minimize competitive inhibition of the degradative enzymes' catalytic sites. Folsom and Chapman (15) described significant phenol-induced degradation of TCE in a two-stage bioreactor in which the typical HRT for the total system was roughly 24 hours. McFarland, *et al* (16) reported degradation from 29.2 to 1.4 mg/l in a two-stage methanotrophic reactor with a HRT of approximately 24 hours. However, notable loss occurred only after sodium formate was added to the treatment unit in order to stimulate non-competitive degradation activity.

CONCLUSIONS

This work demonstrated the first successful degradation of TCE by *Alcaligenes eutrophas* JMP134 in a flow-through bioreactor. Previous studies had demonstrated TCE degradation in a "test-tube" environment. While the study was performed under controlled conditions, i.e. artificial groundwater, it still showed potential for the process to be scaled-up for bioremediation. This work also represents the first TCE flow-through bioreactor that we are aware of that utilizes a non-proprietary strain of non-methanotrophic bacteria.

This study compared two bioreactor bed configurations, a gravel bed and a liquid culture, under conditions of constant overall reactor volume. For purposes of scale-up, overall reactor volume is more important than pore volume in determining costs. A comparison of the two bed types revealed that the liquid culture performed far better than the gravel bed. For the liquid culture, there was no phenol breakthrough, and the TCE degradation was substantially more complete and more consistent. Therefore on the basis of total reactor volume, the liquid culture is the clear winner. The gravel bed apparently provided no benefit to the bacteria, and the increase in liquid volume upon its removal provided a boost in residence time, leading to better performance.

A set of feasible operating conditions was established for the bioreactor. At approximately 30°C, a pH of 6.1, inlet TCE concentration of 5.5 mg/l, inlet phenol concentration of 870 mg/l, and HRT of 13 hours, one can expect approximately 96% TCE degradation. This represents a very high degradation level for a reasonable TCE feed concentration and short hydraulic residence time. This performance could likely be improved through optimization. This system has definite potential for bioremediation scale-up.

FUTURE WORK

It is recognized that one of the principal weaknesses of this study is the method of measuring the abiotic or stripping losses. It would be better to measure them directly by measuring the mass loss of TCE via the vent. This can be accomplished by measuring the vent gas flow rate with a flowmeter and assessing the TCE level with a gas chromatographic analysis of a gas sample from the vent. In order to do this one must prevent diffusion of air into the space where the gas sample is taken. The easiest way to accomplish this is to extend the vent tube upward 3 or 4 m, with the gas sample taken at its base. This method will also eliminate the need for a control bioreactor.

A surface response study is planned to determine the optimal operating conditions for the bioreactor. The variables chosen will be hydraulic residence time, TCE level in the feed, and phenol level in the feed. Work is also planned to determine if the mineral salts solution can be simplified, and what the minimum concentrations are for efficient TCE degradation. In particular, the chloride levels in the media need to be reduced dramatically. Measurements are planned to determine the concentration of bacteria in the bioreactor and to determine if there is any contamination with other microorganisms.

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Reprint Requests:
John Morse
University of South Carolina
Department of Mechanical Engineering
300 South Main Street
Columbia, SC 29208