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The Use of Methanotrophic Bacteria for the Treatment of Groundwater Contaminated with Trichloroethene at the U.S. Department of Energy Kansas City Plant

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Environmental Sciences Division
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ORNL/TM-11084

ENVIRONMENTAL SCIENCES DIVISION

THE USE OF METHANOTROPHIC BACTERIA FOR THE TREATMENT OF GROUNDWATER CONTAMINATED WITH TRICHLOROETHENE AT THE U.S. DEPARTMENT OF ENERGY KANSAS CITY PLANT

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EXECUTIVE SUMMARY

S. B. GARLAND II, A. V. PALUMBO, G. W. STRANDBERG, T. L. DONALDSON, L. L. BOLLA, W. ENG, and C. D. LITTLE. 1989. The use of methanotrophic bacteria for the treatment of groundwater contaminated with trichloroethene at the U.S. Department of Energy Kansas City Plant. ORNL/TM-11084. Oak Ridge National Laboratory, Oak Ridge, Tennessee. 79 pp.

Contamination of groundwater by organic compounds is recognized as a major problem in many areas of the country. Conventional treatment of the contaminated groundwater by air stripping or carbon adsorption removes the organics from the water by transferring them to another medium but does not destroy them. A variety of microorganisms are capable of converting organic chemicals to carbon dioxide, water, and simple inorganic acids, bases, and salts. Frequently, the organics in the groundwater are in insufficient concentration to support microbial growth or are not themselves used for growth and energy, and a primary carbon source is needed, as well as oxygen. A potential application of a supplementary carbon source is the use of methanotrophic microorganisms to degrade halogenated organics such as trichloroethene (TCE) in the presence of methane and oxygen.

This study was conducted to demonstrate the technical feasibility of a trickle-filter methanotrophic bioreactor for the remediation of TCE contamination in groundwater. A bench-scale continuous-flow bioreactor was constructed and operated for several months to treat synthetic contaminated groundwater and to identify the rate of TCE degradation and the parameters that control bioreactor performance. With influent concentrations of TCE and trans-1,2-dichloroethene (DCE) of 1 mg/L each and a residence time of 50 min, approximately 50% of the TCE and 90% of the DCE were degraded in a single pass through the bioreactor. Further degradation of TCE was obtained with liquid recycle.

Simultaneously, batch shake-flask tests were conducted to characterize the substrate and nutritional requirements of the microbial culture in order to guide process development and to estimate potential performance of the bioreactor. Experiments were conducted with mineral salts media and contaminated site groundwater. Selection of a mixed

microbial consortium resistant to the toxic effects of chlorinated hydrocarbons and the identification of appropriate micronutrient conditions, specifically manganese concentration, are two factors identified as being important in optimizing the utility of the microbial consortia. Based on the results of these studies, the presence of high levels of other chlorinated alkenes may reduce the rate of TCE degradation, but the importance of this effect depends upon the specific composition of the contaminated water. These other chlorinated alkenes (e.g., DCE) may actually degrade faster than the TCE.

The performance of the bench-scale bioreactor indicates that bioremediation of TCE-contaminated groundwater is technically feasible. A 3-month pilot plant project to further develop the process is estimated to cost approximately $180,000. A full-scale plant ranging in size from 50 to 700 gal/min is estimated to cost from $180,000 to $1 million to construct and from $4 to $1 per 1000 gal to operate.

INTRODUCTION

The objective of this study is to demonstrate the technical feasibility of a trickle-filter methanotrophic bioreactor for the remediation of groundwater contaminated with trichloroethene (TCE) and trans-1,2-dichloroethene (DCE) at the U.S. Department of Energy (DOE) Kansas City Plant. In order to accomplish this objective, a prototype bench-scale continuous bioreactor was constructed and operated for several months to treat synthetic contaminated groundwater. Following startup of the bioreactor and the development of an adequate microbial culture, the parameters that control bioreactor performance were identified, the rate of TCE destruction was measured, mass transfer limitations were identified, and approaches to reducing mass transfer limitations were evaluated. Concurrently, batch shake-flask tests were performed to characterize the substrate and nutritional requirements of the microbial culture in order to guide process development and to estimate the potential optimal performance of a bioreactor system. The data were used to determine the merit for further process scale-up and to estimate the costs for doing so.

This study was divided into four tasks as described below.

TASK 1: CHARACTERIZATION OF PERFORMANCE OF BENCH-SCALE BIOREACTOR

A bench-scale trickle-filter bioreactor was constructed and inoculated with a TCE-degrading culture isolated from a TCE-contaminated groundwater well in the Oak Ridge area. The best existing information on the requirements for the culture was used to start up the bioreactor. As further understanding was obtained (Task 2), the operating conditions of the bioreactor were modified as appropriate.

Degradation of TCE and DCE in synthetic contaminated groundwater was evaluated, and the effects of various parameters on performance--liquid and gas flow rates and gas phase oxygen and methane content--were investigated. Complete material balances on TCE were attempted. The ultimate objectives were to determine which parameters are performance-controlling and to estimate the nature of the correlation in order to guide further process definition, evaluation, and scale-up.

TASK 2: CHARACTERIZATION OF MICROBIAL CULTURE

The culture used in the prototype fixed-film bioreactor was characterized in batch shake-flask tests to determine the conditions for maximum TCE and DCE degradation. Although work to date at ORNL and other labs has demonstrated that TCE can be degraded, it is not known how to selectively promote this behavior, nor is it known what the potential maximum degradation rate might be. Parameters that are potentially influential include concentrations of methane, oxygen, TCE, and other chlorinated compounds; nutrients such as nitrogen, phosphorus, and trace metals; pH; and temperature. Actual contaminated groundwater from the site was used for much of this work. These tests were performed concurrently with the bioreactor development work (Task 1). As further understanding was gained on the behavior of the culture, it was transferred to the operation of the bioreactor. Furthermore, these tests help to estimate the ultimate potential performance of a full-scale groundwater remediation process.

TASK 3: REVIEW AND DECISION MEETING

Two ORNL staff members met with the Kansas City staff to review the experimental progress over the first 6 months of the project. Based on these results, a decision was made to initiate a preliminary engineering evaluation for a pilot plant and a full-scale plant (Task 4).

TASK 4: PRELIMINARY ENGINEERING EVALUATION

The objective of this task was to determine if this treatment technology appears feasible from a technical and economic perspective. Information and process understanding gained in Tasks 1 and 2 were combined to develop a conceptual process design for treatment of TCE-contaminated groundwater, and a preliminary cost estimate was developed.

A first quarter progress report was submitted in May 1988, and a review of the project (Task 3) was conducted in Kansas City in August 1988. At the review in August, a decision was made to expand Task 4 to include a pilot plant test campaign schedule, a full-scale plant

conceptual design, a cost estimate for a 1 gal/min pilot plant, and an economic evaluation of a conceptual 50 gal/min full-scale plant.

This final report contains background material on TCE degradation, the results of Tasks 1, 2, and 4, and conclusions. Task 3 was the oral presentation and review that took place in Kansas City in August 1988, and no additional information is provided in this report.

BACKGROUND

Contamination of groundwater aquifers by organic chemicals is recognized as a major national problem. TCE and DCE are common pollutants at U.S. Environmental Protection Agency (EPA) Superfund sites and at DOE sites (EPA 1985). These compounds are suspected carcinogens, are resistant to aerobic degradation, and thus threaten water supplies (Infante and Tsongas 1982). Although TCE is an EPA priority pollutant, American industry consumed 178 million pounds in 1985 (Storck 1987).

When contaminated groundwater is pumped to the surface, air stripping and carbon adsorption are effective methods to remove the organics from the water. However, these technologies simply move the contaminant from one phase to another; the organics have not been broken down, and a clean-up and disposal problem still remains. Photolytic and chemical destruction methods are under development but are not-yet-proven technologies. Therefore, a low-cost biological treatment method offers a significant step forward in the remediation of groundwater contaminated with organics, particularly when the organics are present in low concentrations.

A variety of microorganisms have the capacity to use organic chemicals via metabolic pathways forming carbon dioxide, water, and simple inorganic acids, bases, and salts. Since the groundwater environment frequently offers too low a concentration of organics to support microbial growth, a primary carbon source is needed. In addition, oxygen is needed for aerobic biodegradation. An attractive potential treatment scheme is the use of methanotrophic microorganisms to degrade halogenated organics such as TCE in the presence of methane and oxygen. While this can occur in an aboveground bioreactor or in situ, the technology is further advanced for the former. Degradation of TCE has been demonstrated in the laboratory by several investigators (Barrio-Lage et al. 1986, Bouwer and McCarthy 1983, Colby et al. 1977, Eng et al. 1988, Fathepure et al. 1987, Barrio-Lage et al. 1987, Kleopfer et al. 1985, Little et al. 1988, Nelson et al. 1986), including a group at Oak Ridge National Laboratory (ORNL) (Little et al. 1988). Several types of bioreactor systems appear to be feasible for process

applications; however, the technical feasibility has yet to be demonstrated experimentally.

There are reports of biodegradation of TCE by a variety of mechanisms. Some investigators have reported anaerobic degradation of TCE (Fathepure et al. 1987, Kleopfer et al. 1985). DCE and vinyl chloride, a known carcinogen, are apparently produced from anaerobic degradation of TCE (Vogel and McCarty 1985, Maltoni and Lefemine 1974). Degradation of TCE to CO_2 by aerobic mixed cultures has also been reported, but the degradation mechanisms have not been clearly identified (Fogel et al. 1986). TCE biodegradation by aerobic pure cultures of methanotrophs (Little et al. 1988) and pseudomonades (Wacket and Gibson 1988) has also been reported. The toluene dioxygenase enzyme of Pseudomonas has been shown capable of TCE degradation (Nelson et al. 1988, Wacket and Gibson 1988). However, either toluene or phenol was required to induce degradation (Nelson et al. 1986). Pure strains of methanotrophic cultures have also exhibited TCE degradation, apparently by the methane monooxygenase enzyme (Colby et al. 1977, Little et al. 1988, Whittenbury et al. 1970).

TCE degradation by methanotrophs is apparently initiated by the methane monooxygenase with a cometabolic process. Normally, methane is oxidized to methanol by the methane monooxygenase enzyme (Colby et al. 1977), but TCE is also oxidized by the monooxygenase. TCE breakdown may begin with epoxidation of the double bond, eventually resulting in the formation of carbon dioxide, glyoxylic acid, and dichloroacetic acid (Little et al. 1988). A small fraction of the carbon from the TCE is incorporated into the cells (Little et al. 1988).

The DOE Kansas City Plant, which has groundwater contaminated with TCE, is being studied in an effort to model the movement of chlorinated compounds and devise remediation plans. The groundwater at this site contains TCE, DCE, and vinyl chloride in various proportions with increasing concentrations of DCE and vinyl chloride with increasing distance from the spill site; thus the ratio of DCE to TCE in the groundwater can vary over a wide range. The objectives of the research presented here are to evaluate TCE and DCE degradation of several methane-utilizing consortia for use in a proposed aboveground bioreactor

(Strandberg, personal communication) for treatment of the contaminated groundwater at the site and to determine operating parameters (e.g., effect of amounts of TCE, methane, oxygen, DCE, and manganese) for the bioreactor.

TASK 1

CHARACTERIZATION OF PERFORMANCE OF BENCH-SCALE REACTOR

INTRODUCTION

A trickle-type packed-bed bioreactor was constructed and operated to evaluate the technical feasibility of bioremediation of TCE-contaminated groundwater by methanotrophic microorganisms. The performance of this bench-scale bioreactor system is described in this section, along with observations on the kinetics of the degradation of TCE and DCE.

METHODS AND MATERIALS

Bioreactor Design and Operation

The bioreactor (Fig. 1) consisted of a 5-cm ID x 110-cm-long glass column packed with 0.6-cm ceramic berl saddles (Scientific Products, McGaw Park, Illinois) as a support matrix for the biofilm. Viton tubing was used for all streams containing TCE.

A concentrated feed solution containing mineral salts (Little et al. 1988) and TCE, DCE, or both was continuously bled into a stream of process water by a peristaltic pump. The mixture was distributed (generally at 10 mL/min) over the top of the packing. Flow rates were measured daily by collecting liquid in a graduate for a short period of time. The influent concentrations of TCE and DCE were controlled by adjusting their concentrations in the feed concentrate and by varying the dilution with process water. A gas stream (usually 25 mL/min) containing methane (4% v/v unless noted otherwise) and air was also introduced at the top of the bioreactor. This type of bioreactor and mode of operation were chosen to promote transfer of oxygen and methane to the biofilm.

The bioreactor system was operated continuously at nominal steady state under conditions noted above for most of this feasibility study. However, deviations were made on occasion to examine the effects of changing various parameters. Oxygen and methane concentrations from 10 to 50% methane in air were tested. The bioreactor was operated for several days on several occasions with methane and oxygen predissolved

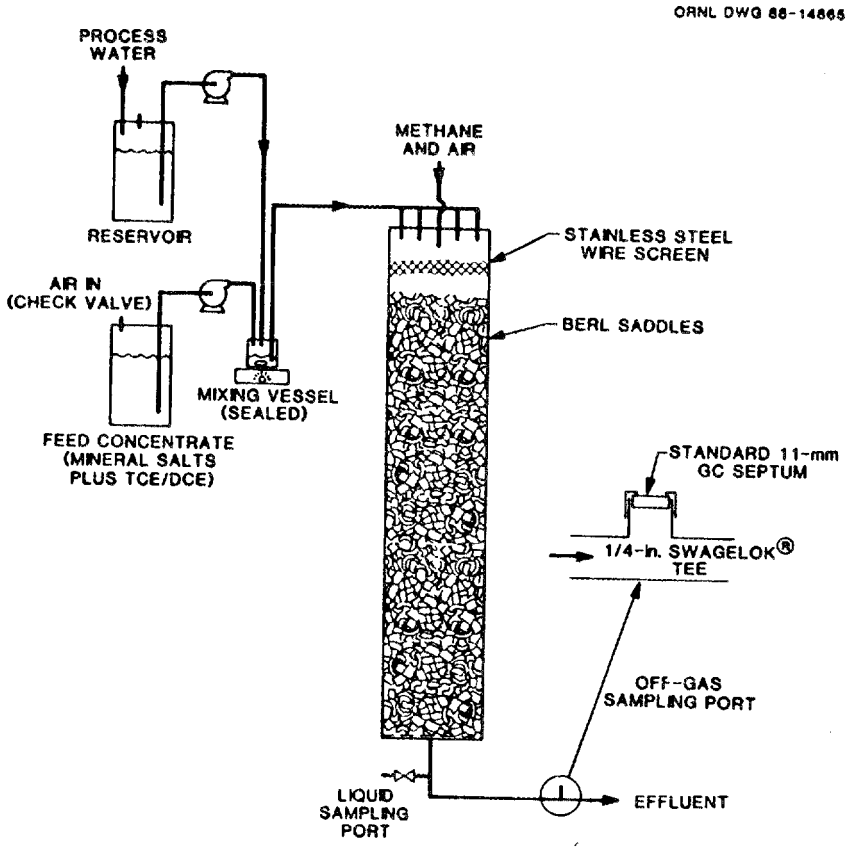

Fig. 1. Schematic diagram of the trickle-type methanotrophic bioreactor system.

in the feed stream and no flowing gas phase. Total liquid recycle was used on several occasions to extend the liquid residence time and simulate a batch experiment.

Controls were done to test the TCE material balance in the absence of biological activity. An empty column with no packing was used, and the packed column containing biofilm was tested in the absence of methane and air to eliminate TCE degrading activity.

The average liquid residence time and holdup were estimated at several flow rates by using a salt tracer method (Levenspiel 1972). This technique involves monitoring the electrical conductivity of the effluent liquid following an input pulse of NaCl.

Bioreactor Startup

The microbial consortium originated from a TCE-contaminated groundwater monitoring well on the Oak Ridge Reservation. The culture was maintained in a mineral salts medium (Little et al. 1988) under an atmosphere of 20% methane and 80% air. The microbial population in the bioreactor was established by adding approximately 50 mL of an actively growing culture and 150 mL fresh medium and then operating the system at ~99% liquid recycle at 10 mL/min. Fresh medium containing 1 mg/L of TCE was introduced at 0.1 mL/min. The gas stream (10 mL/min) contained 20% methane and 80% air. After 3 to 4 weeks, a substantial growth of salmon-colored biomass was visible throughout the column. The recycle was then switched off, and the system was operated routinely in a single-pass mode except for several periods when liquid recycle was employed.

Analytical Procedures

TCE and DCE were analyzed by gas chromatography with a Varian 3700 gas chromatograph (Palo Alto, California) equipped with an electron capture detector. Separation was achieved with a DB+1 megabore column (J&W Scientific, Folsom, California) operated isothermally at 40°C with nitrogen as the carrier gas (3 to 4 mL/min). Liquid samples (20 mL) were collected daily and placed in 65-mL amber bottles sealed with a Teflon-lined septum closure. The bottles were placed on a rotator (20

rpm, Cole-Palmer, Chicago, Illinois), and the contents were allowed to equilibrate for 1 h. The headspace gas was then assayed. Samples (5 μL) of the headspace gas or the bioreactor off-gas were injected directly onto the column. Quantification was based on integrated detector responses to headspace gas when known quantities of TCE and DCE were diluted in 20 mL of the mineral salts--process water feed. The detection limit was $\approx$10 μg/L, and the detector response was linear over the concentration range from 0 to 10 mg/L of TCE or DCE (initial liquid phase concentrations).

Mercuric chloride was used to inhibit biological activity in the samples during the 1-h equilibration. However, the results were comparable to no use of mercuric chloride, and thus it was not used routinely.

RESULTS AND DISCUSSION

Single-Pass Mode

Removal of TCE is illustrated by the data shown in Table 1 and Fig. 2. Typically about half of the TCE was lost from the liquid phase when the influent TCE concentration was about 1 mg/L, whereas DCE was removed more rapidly and to a greater extent. The TCE removal rate appeared to be first order with respect to TCE concentration over the range of 0.15 to 5 mg/L (Fig. 2).

Bioreactor performance in terms of TCE and DCE degradation was measured at liquid flow rates of 5, 10, 20, 35, and 50 mL/min (Tables 1 and 2). The mean residence time at each flow rate was estimated from residence-time distribution studies (see below). Removal of TCE was consistent with first-order kinetics; that is, a first-order rate constant of 0.016 to 0.024 min^{-1} was found throughout this range of flow rates, except for a single high value of 0.046 min^{-1} at 50 mL/min. The DCE removal rate may also be first order in DCE concentration; however, the limited data to date are inconclusive. Although the TCE and DCE concentrations in the effluent rose with increasing flow rate as expected, the total removal increased, presumably due to the higher average concentrations of TCE and DCE in the bioreactor.

Table 1. Degradation of TCE in a trickle bioreactor

Flow rate (L/min)	t[a] (min)	Influent (mg/L)	Effluent (mg/L)	Off-gas[b] (mg/L)	Degradation rate[c] (mg/d)	k[d,e] (min^{-1})
0.005	75	0.9	0.2	0.02	5	0.020
		1.3	0.3	0.02	6	0.020
0.010	50	1.0	0.3	0.06	7	0.024
		1.1	0.5	0.05	6	0.016
0.020	30	1.0	0.5	0.06	12	0.023
		1.3	0.7	0.06	15	0.021
0.035	16	1.1	0.8	0.14	14	0.020
		1.3	1.0	0.10	10	0.016
0.050	11	1.0	0.6	0.04	29	0.046
		1.0	0.6	0.03	29	0.046

[a]Mean residence time (see text).

[b]Gas flow rate was 20 mL/min, 4% methane in air.

[c]Corrected for small loses of TCE in the off-gas.

[d]$C_{effluent}/C_{influent} = \exp(-kt)$, where k is the overall apparent rate constant.

[e]Also see Fig. 2; the kt band from 0.5 to 1.2 corresponds to k values from 0.01 to 0.024 min^{-1}.

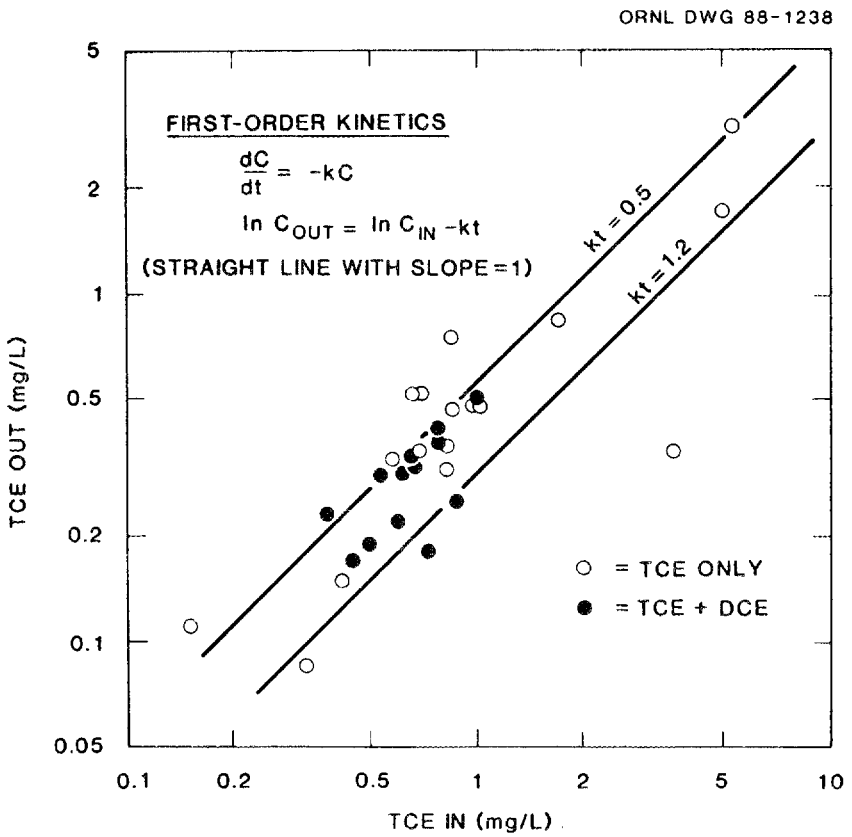

Fig. 2. Performance of the trickle bioreactor at steady state (t is the mean liquid residence time). Liquid flow rate = 10 mL/min; gas flow rate = 25 mL/min, 4 to 20% methane in air.

Table 2. Degradation of DCE in a trickle bioreactor

Flow rate (L/min)	t[a] (min)	Influent (mg/L)	Effluent (mg/L)	Off-gas[b] (mg/L)	Degradation rate[c] (mg/d)	k[d] (min^{-1})
0.005	75	1.1	<0.01	0.003	≥ 8	≥0.063
		1.7	0.05	0.002	10	0.047
0.010	50	1.2	<0.01	0.005	≥17	≥0.095
		1.0	0.01	0.01	13	0.092
0.020	30	1.2	0.03	0.02	33	0.12
		1.6	0.08	0.02	21	0.10
0.035	16	0.8	0.03	0.01	40	0.21
		3.2	0.04	0.01	159	0.27
0.050	11	1.0	0.02	0.01	69	0.35
		1.0	0.02	0.01	69	0.35

[a]Mean residence time (see text).
[b]Gas flow rate was 20 mL/min, 4% methane in air.
[c]Corrected for small loses of DCE in the off-gas.
[d]$C_{effluent}/C_{influent} = \exp(-kt)$, where k is the overall apparent rate constant.

Mean residence times were estimated by monitoring the effluent conductivity following pulses of NaCl (Levenspiel 1972). At flow rates of 11, 32, and 66 mL/min, the mean residence times were found to be 47, 17, and 10 min, respectively. The liquid holdup was thus about 500 to 650 mL, depending upon the flow rate. The conductivity response indicated a large degree of backmixing (meaning relatively inert or inactive regions) in the bioreactor. This suggests that the reactor may be equally active with considerably less biomass in the column.

Recycle Mode

The data in Table 1 and Fig. 2 show that ~50% of the TCE was removed during a single pass through the bioreactor. To determine if the TCE concentration could be reduced further, recycle of the liquid effluent was used to extend the residence time. The normal feed flow was stopped and a fresh 1 mg/L solution of TCE in salt medium was recycled through the bioreactor. The gas flow was maintained at 20 mL/min. After 1.5 h the TCE concentration had decreased to and remained at 50 to 100 μg/L (Fig. 3).

Approximately 10% of the TCE and DCE was volatilized by the methane-air gas stream (Tables 1 and 2). In practice, this could necessitate either off-gas treatment to remove these pollutants or perhaps recycle of the off-gas. To avoid a gas phase in the bioreactor, an attempt was made to operate the system by sparging the process water diluent stream with methane and oxygen before TCE and DCE were added. At all methane levels tested (from 10 to 50% of the sparge gas; remainder air), the TCE-DCE degradation rate remained constant for approximately 4 h but fell significantly in 18 to 48 h (Table 3). Apparently there was insufficient dissolved methane or O_2 or both to maintain enzyme production (see below). The degradation rate rose to the original levels within 2 to 3 h after restoring the flow of the methane-air gas stream.

As noted earlier, it is generally believed that methane monooxygenase is responsible for the initial oxidation of TCE and DCE. Thus, it could be hypothesized that there is competition between methane and TCE and DCE for the enzyme. This hypothesis suggests that it might

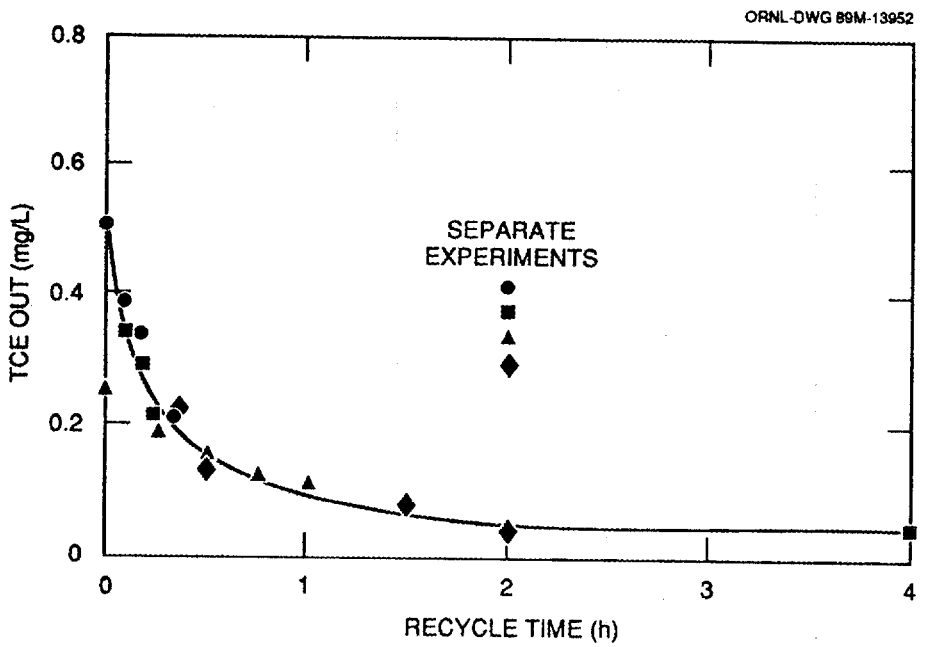

Fig. 3. Degradation of TCE in the trickle bioreactor at total recycle. The initial TCE concentration was ~1 mg/L for each experiment. Liquid flow rate = 10 mL/min; gas flow rate = 25 mL/min, 4% methane in air.

Table 3. Performance of bioreactor with methane and air predissolved in feedwater[a]

Time (h)	TCE Influent (mg/L)	TCE Effluent (mg/L)	TCE Degradation Rate[b] (mg/d)	DCE Influent (mg/L)	DCE Effluent (mg/L)	DCE Degradation Rate[b] (mg/d)
10% methane in air						
Initial[c]	0.8	0.3	4	0.8	0	≤11
2	0.6	0.3	4	0.8	0.03	11
3	0.8	0.4	6	1.1	0.05	15
4	0.7	0.5	3	1.1	0.07	14
22	0.8	0.8	0	1.1	0.9	4
20% methane in air						
Initial[c]	1.8	1.0	12	3.6	0.05	51
2	1.2	0.9	4	2.2	0.5	24
3	1.0	0.8	3	1.7	0.4	19
5	1.2	1.0	3	2.1	0.4	24
22	1.0	1.2	0	1.9	2.2	0
50% methane in air						
Initial[c]	0.9	0.6	[d]	0.9	0.08	11
2	1.2	0.8	6	1.2	0.4	12
3	1.1	0.7	6	1.0	0.2	12
4	1.1	0.8	4	1.0	0.2	12
5	1.2	0.8	6	1.1	0.1	14
22	1.4	1.1	4	1.3	1.1	3

[a]Liquid flow rate = 10 mL/min.
[b]Corrected for loss of TCE in off-gas initially (no off-gas at subsequent times).
[c]Immediately before changeover to sparged feedwater, 4% methane in air at 20 mL/min.
[d]Uncertain due to abnormally high off-gas concentration.

be advantageous to periodically restrict the supply of methane to achieve a greater TCE degradation rate. With TCE as the sole chlorinated alkene (no DCE), the methane (4% v/v) supply was shut off. Unexpectedly the TCE degradation rate did not rise but remained constant for about 4 h and decreased by about one-third in 16 to 18 h (Table 4).

This behavior is similar to what was observed during attempts to operate the system with methane- and O_2-presaturated process water. Perhaps at normal operating conditions the liquid-phase methane concentration was just sufficient to maintain growth and enzyme activity, and the TCE was in relatively great excess from a kinetic standpoint. In this case, any short-term effect of methane removal on the TCE degradation rate would be too small to detect. A likely explanation for the loss of activity is the cessation of enzyme production due to substrate (methane) limitation and the natural decay of the existing enzyme (i.e., protein turnover).

At times there were indications that the rate of TCE degradation was depressed by DCE. Experiments to verify this inhibition in the bioreactor were inconclusive. However, inhibition of TCE degradation by DCE was observed in shake-flask experiments that use, among others, a culture initiated from the bioreactor (see Task 2 discussion).

Although TCE removal was obtained at gas phase methane concentrations of up to 20% (v/v), 4% methane was sufficient to maintain the microbial population and bioreactor activity. When the methane concentration was decreased to 2%, TCE degradation was maintained for 4 to 5 d but then appeared to decrease somewhat (the data are inconclusive). At the same time, a bright yellow microbial growth began to appear. The organism, a Gram-negative bacterium, was isolated by streaking cultures on agar; it neither utilized methane nor degraded TCE. In this regard, six to eight different organisms were isolated from the consortium by plating on a variety of media. Only one methane-utilizing organism (salmon pink) appeared to be present based on cell and colony morphology. This was also the predominant organism visually in the consortium. The biofilm did not adhere tightly to the packing; rather, the culture appeared to be growing as dense masses in the void spaces between the packing elements.

Table 4. Effect of no methane and air to the bioreactor[a]

Time (d)	TCE Influent (mg/L)	TCE Effluent (mg/L)	TCE Off-gas (mg/L)	TCE Degradation Rate[b] (mg/d)
-1	0.7	0.3	0.04	5
0 (before gas off)	0.5	0.2	0.06	3
+2	0.5	0.4	-	1
2 + 2 h after gas back on	0.8	0.3	0.04	6
-1	0.8	0.4	0.04	5
0	1.3	1.0	-	4
+3 (a.m.)	0.6	0.4	-	3
+3 (p.m. after gas back on)	1.1	0.5	0.07	7

[a]Liquid flow rate = 10 mL/min, 4% methane in air, 20 mL/min, before the gas was shut off (time < 0).
[b]Corrected for loss of TCE in the off-gas.

Liquid effluent from the bioreactor was assayed twice for priority pollutants [purge and trap followed by gas chromatography/mass spectroscopy (GC/MS)]. No priority pollutants other than TCE and DCE were detected. Although vinyl chloride has been shown to be a product of chlorinated-alkene degradation by anaerobic organisms, it is not produced by aerobic, methanotrophic organisms (Little et al. 1988).

One additional chromatographic peak was often observed during the course of bioreactor operation with DCE and batch-type DCE degradation experiments. Its retention time was 1.6 min, intermediate between DCE (1.1 min) and TCE (2.0 min). It decreased with time during recycle and batch experiments. Mass spectrometric analysis revealed that the compound had a mass of 112 or greater and likely contained two carbon atoms, two chlorine atoms and possibly an oxygen atom. Although the compound was not identified, its characteristics are consistent with the DCE epoxide recently found when methanotrophs were exposed to DCE (Jansen et al. 1987). On occasion, particularly during recycle, other chromatographic peaks whose elution times were between those of TCE and DCE were observed. These peaks also appeared to arise as a result of DCE metabolism. They were not noted when only TCE was fed to the system. During recycle, these peaks gradually disappeared.

Finally, three independent observations indicate that TCE disappearance was in fact due to microbial action. Greater than 90% of the influent TCE was accounted for in the effluent liquid and off-gas by using both a blank column (without packing) and when the biological activity was virtually eliminated by shutting off the gas flow. Shake-flask experiments with the mixed culture from the bioreactor that used ^{14}C-labeled TCE showed that in excess of 60% of the TCE was mineralized to CO_2 (see Task 2 discussion). The rest of the label appeared in the cell (~25%) and in water-soluble products (5-10%).

CONCLUSIONS

The performance of the bench-scale bioreactor indicates that bioremediation of TCE-contaminated groundwater is technically feasible, but further development and demonstration of this new technology is needed at the pilot scale. Alternate packing materials for support of

the biofilm in the bioreactor should be tested. Laboratory studies are needed to address the apparent lower limit of TCE in the effluent and to further characterize the optimal operating conditions.

TASK 2
CHARACTERIZATION OF MICROBIAL CULTURE

INTRODUCTION

Batch studies were conducted to determine what cultures and conditions would result in the greatest degree of TCE and DCE degradation. A comparison the TCE and DCE degradation capacities among mixed and pure cultures in mineral salts media was made. Growth was also examined in groundwater obtained from the site. Since previous experiments had shown that manganese, a monooxygenase cofactor, has an effect on TCE degradation by pure cultures (Palumbo, personal communication), the effect of manganese on a mixed culture was determined. In an effort to increase the extent and rate of TCE degradation by methane-utilizing cultures, the effects of methane, oxygen, phosphate, ammonia and methanol on TCE degradation were also tested.

MATERIALS AND METHODS

Microbial Cultures

Three pure cultures and four consortia were used in the experiments. Pure methanotroph strains 46-1 and 68-1 were previously isolated from well water contaminated with chlorinated compounds from an industrial waste disposal site in Oak Ridge, Tennessee (Little et al. 1988). Both strain 46-1 and 68-1 are type I methanotrophs as indicated by their internal stacked membranes and the ribulose monophosphate pathway of formaldehyde fixation (Little et al. 1988). Methylosinus trichosporium (strain OB3B) was provided by the California Institute of Technology. The JS mixed culture was also isolated from the waste disposal site in Oak Ridge (Little et al. 1988) and is currently being used in bioreactor studies of TCE degradation (Strandberg, personal communication). The DT1 and DT2 mixed cultures are bacteria-amoeba consortia isolated from an Oak Ridge site. The S1 consortia was obtained during this study from contaminated groundwater at the DOE Kansas City Plant.

In all experiments unless otherwise noted, 100 mL of sterile mineral salts media, NATE, (Whittenbury et al. 1970) containing 50 μg/L

$CUSO_4 \cdot 5H_2O$, 10 μg/L $MnSO_4 \cdot H_2O$, 70 μg/L $Zn(NO_3)_2 \cdot 6H_2O$, 10 μg/L $CoCl_2 \cdot H_2O$, 10 μg/L MoO_3, 1 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L $CaCl_2$, 1 g/L KNO_3, 0.1 g/L NH_4Cl, 10 mL of 0.27 g/L $FeCl_3$, and 20 mL of 5% phosphate solution (pH 6.8) was prepared in 250-mL culture bottles. Final media pH was adjusted to 6.8 (Little et al. 1988, Whittenbury et al. 1970). Filtered methane and oxygen were injected when necessary into the bottles with a syringe. Each bottle contained 8 to 8.5% (v/v) methane and atmospheric levels of oxygen and nitrogen in the headspace except as otherwise noted. Each bottle was sealed with a teflon septum, and the bottles were incubated at room temperature (20°C $\pm$ 2°C) for 8 to 14 d. To ensure an airtight seal, modeling compound, sandwiched between parafilm, was used to cover the bottle caps. The culture bottles were shaken inverted, to further guard against gas leakage, on a rotary shaker (Fermentation Design) at 75 rpm. Samples were taken at various intervals for determination of TCE, DCE, methane, and oxygen concentration by GC.

Chemical Analysis

Analysis of TCE and DCE in headspace gas was performed by using a Perkin-Elmer 2000 Gas Chromatograph equipped with an electron capture detector and by using nitrogen as carrier gas. A glass column (0.64 cm diam by 45 cm long) packed with 1% SP-1000 60/80 Carbopack (Supelco) was used, and the oven and injection port temperatures were set at 100°C. The detector temperature was set at 350°C. The retention time of TCE was 2.9 min. Analysis of DCE used the same gas chromatography parameters as described for TCE analysis, except oven temperature was set at 65°C. This gave a DCE retention time of 2.05 min. Liquid DCE standards were used to calibrate the GC daily.

Oxygen and methane concentrations were measured with a Perkin Elmer 3920B Gas Chromatograph equipped with a 6- by 1/8-in. Molecular Sieve 5A column (Supleco) and a thermal conductivity detector. Both the injector and interface temperatures were 150°C. Oven temperature (initial and final) was set at 45°C. Retention times were 0.68, 1.3, and 2.3 min for oxygen, nitrogen, and methane, respectively. Data on

oxygen and methane concentrations are reported as percent (v/v) of headspace gas.

The fate of ^{14}C TCE was followed through radiolabel techniques as described by Little et al. (1988). After incubation with [1,2- ^{14}C] trichloroethene [3.0 mCi/mmol (111 MBq/mmol), Pathfinder Laboratories, St. Louis], the pH of the medium was adjusted to 9.5 to 10, thereby converting CO_2 gas into soluble carbonate ion. A subsample of culture was centrifuged, and the pellet was resuspended in NATE to assess the amount of TCE incorporated into cellular material. Remaining TCE was extracted from the supernatant with hexane. Acid was added to convert the carbonate into CO_2, which was trapped in a vial containing 0.1 N NaOH. Subsamples of the water phase and the trapped $^{14}CO_2$ were then counted by a TriCarb 2000CA liquid scintillation analyzer (Packard, Downers Grove, Illinois).

Comparison of TCE and DCE Degradation Ability Among Cultures

The extent of TCE degradation among the pure cultures 46-1, 68-1, OB3b, and the mixed JS culture was compared at initial TCE concentrations of 414 μg/L and 207 μg/L. Data on concentrations of TCE and DCE added are given here and below as the amount added to the bottles. Comparison of headspace gas to TCE and DCE liquid standards indicated that,under the conditions used in these experiments, approximately 30% of the added TCE and 17% of the DCE partitioned into the gas phase. The 250-mL culture bottles containing the mineral salts media were inoculated with 1.0 mL of log phase starter culture containing about 10^8 cells. There was 8% methane and 18% (v/v) oxygen present in the bottles (the remainder was nitrogen and CO_2), and uninoculated bottles served as controls.

The degradation of DCE (63 mg/L) was compared among the strains 46-1 and OB3b and the consortia JS, DT, and S1. Headspace gas contained 9% (v/v) methane and atmospheric oxygen and nitrogen.

Growth and Degradation in Site Water

The growth of the JS mixed culture was examined at various methane levels after adding NATE elements (to make up 10% of the final volume)

to the site water (90% of final volume). Methane levels were 1.2, 3.5, 5.5., 7.5, and 9.7% (v/v) of headspace. Initial oxygen levels were 18% (v/v) of headspace for all treatments. Initial concentration of TCE in incubation water was about 4.7 mg/L. In a second experiment with site groundwater, the proportion of groundwater was varied by the addition of NATE nutrients and distilled water to yield final concentrations of site water of 22.5, 45, 67.5, and 90%. The purpose of this experiment was to determine the effect of TCE concentration and micronutrient conditions on the growth and TCE degradation. The site groundwater initially contained 12.3 mg/L of DCE and 4.7 mg/L of TCE, and the concentrations in the treatments varied in proportion to the amount of site water added. Initial methane and oxygen concentrations were 8 and 18% (v/v) of headspace gas, respectively.

Effect of DCE on TCE Degradation

A comparison of the ability of DT2 and JS consortia to degrade TCE (0.3 mg/L) with 0, 30, and 63 mg/L of DCE added to the bottles was conducted to determine if DCE inhibited TCE degradation. Initial methane and oxygen concentrations were 8 and 18% (v/v) of headspace gas, respectively. TCE degradation was followed by both radiolabel and GC techniques, and DCE degradation was followed by using GC analysis. The DT2 consortium was incubated for 14 d, while the JS consortium was incubated for 23 d because it grew slower.

Effect of Manganese on TCE Degradation

The effect of manganese on growth parameters (methane and oxygen consumption) and TCE degradation by the mixed cultures JS and DT1 was examined. Concentrations of 0, 10, 50, 100, and 200 μg/L of $MnSO_4 \cdot H_2O$ were used in this experiment, and the initial TCE concentration was 126 μg/L. Bottles were again inoculated with 1 mL of culture, and they contained methane and oxygen concentrations of 6.8 to 7.9% and 18.7 to 19.3% (v/v), respectively.

Effect of Oxygen and Methane on TCE Degradation and Growth of Strain 46-1

The effect of oxygen on TCE degradation by strain 46-1 was examined by varying the concentration of oxygen from 2.2 to 12.3% of the headspace gas. Methane concentration in this experiment was 16% (with a standard deviation of 0.59) of the headspace gas, and TCE concentration was 2.56 μg/mL. The effect of high levels of methane on TCE degradation was examined in an experiment where 15 mL of methane were added to the headspace of two bottles, 25 mL of methane were added to the headspace of two other bottles, and all bottles were inoculated with strain 46-1. In another experiment methane was varied from 0.67 to 16.7% of the headspace gas to determine the effect of methane on the growth rate and the TCE degradation rate. The oxygen concentration was 20% of the headspace gas, and 0.1488 mg of TCE was added to yield a nominal concentration of 1.042 mg/L.

Effect of Phosphorus and Ammonia on Growth and TCE Degradation

The effect of phosphorus concentration on TCE growth and degradation was determined in an experiment where five concentrations of phosphorus (1.4, 3.2, 6.2, 25, and 100 μg/mL) were used. A total of 29.6 μg (final concentration = 0.29 μg/mL) of TCE was added to each bottle. Strain 46-1 was used in these experiments.

In previous experiments the DT mixed culture had shown high rates of TCE degradation, and the effects of NH_4Cl (2.5 g/L and 0.1 g/L) on growth and TCE degradation were examined by using this culture. NATE media was prepared as previously described, and the fate of TCE was followed through radiolabel techniques. Methane and oxygen were 8 and 18% of headspace gas, respectively.

Data Analysis

Analysis of variance with Duncan's multiple range test was used to test for significant treatment differences in total TCE transformation and the transformation to breakdown products in experiments that use the radiolabeled TCE. Other data were analyzed through Lotus 1-2-3 (Lotus Corp.).

RESULTS

Comparison of TCE Degradation Among Cultures

There were no significant ($p = 0.05$) differences ($F = 1.72$, D.F. = 3, 15) in total TCE transformed among the strains, but the radiolabel data indicated that there were significant differences ($F = 18.7$, D.F. = 3, 15) in the proportion of the transformed TCE, which was transformed to CO_2 (Fig. 4A). Duncan's multiple range test indicated that there were significant differences in the conversion patterns between the mixed culture and the pure culture and among the pure cultures. The highest percent conversion of the transformed TCE to CO_2 (68.9%, S.E. = 5.6, n = 4) and lowest conversion to water soluble products (5.6%, S.E. = 2.3, n = 4) was exhibited by the JS mixed culture. Strain 46-1 converted significantly ($p = 0.05$) less of the TCE to cell material (8.4%, S.E. = 0.28, n = 4), and strain 68-1 converted significantly ($p = 0.05$) less to CO_2 (23.4%, S.E. = 2.4, n = 4) than the other pure cultures.

A significantly greater proportion of TCE was transformed ($F = 5.37$, D.F. = 1, 15) at the higher (414 μg/L) initial TCE concentration than at the lower concentration (207 μg/L). This difference is due to cultures 46 and OB. However, the proportion of the transformed TCE converted to CO_2 ($F = 0.48$, D.F. = 1, 15), cell material ($F = 0.08$, D.F. = 1, 15), or water-soluble products ($F = 0.13$, D.F. = 1, 15) was not significantly affected by the TCE concentration.

Strain 68-1 exhibited the fastest rate of methane consumption among the pure cultures, but all the pure cultures used methane and oxygen at a slower rate than did the mixed culture (Fig. 5). The JS mixed culture consumed methane at the fastest rate and consumed all the methane over the course of the experiment. The rate of methane utilization of strains 46-1 and OB3b were almost identical under these conditions, and there was a small amount of methane left at the end of the experiment.

Growth of Cultures Exposed to Site Water

There was substantial growth of mixed cultures in the site water. Although at all concentrations of site water the JS consortia eventually

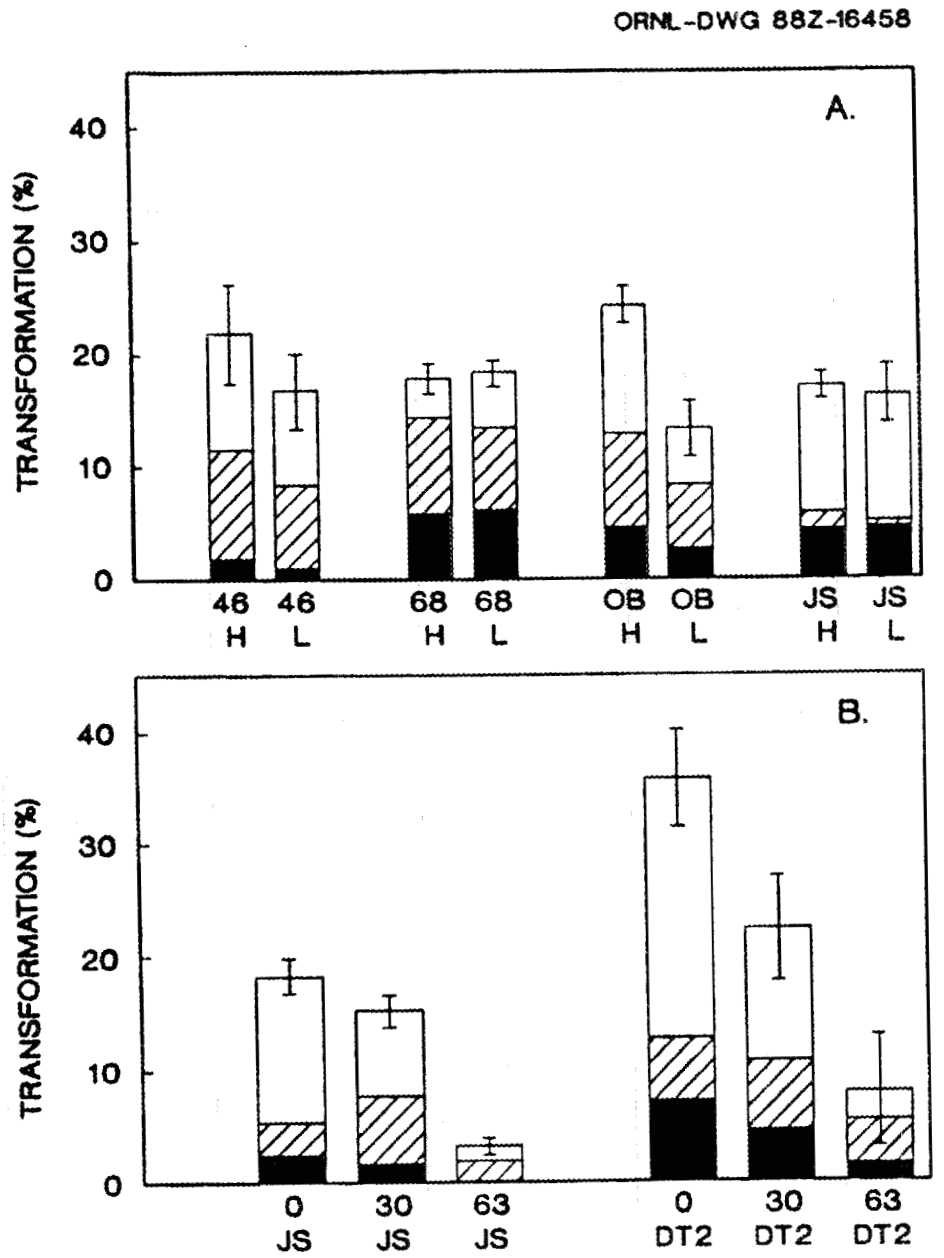

Fig. 4. Distribution of ^{14}C label as percent of TCE (0.3 mg/L) added to microbial cultures. The amount incorporated into cell material is given by the solid bars on the bottom, into water-soluble products by the cross hatched bars in the center, and into CO_2 by the open bars on the top. (A) Comparison of distribution by the 46-1, 68-1, and OB3B cultures and the JS consortia at 4.14 mg/L (H) and 2.07 mg/L (L) of added TCE. (B) Comparison of JS and DT2 consortia with 0, 30, and 63 mg/L added DCE. The range for the total transformation is given by the error bars.

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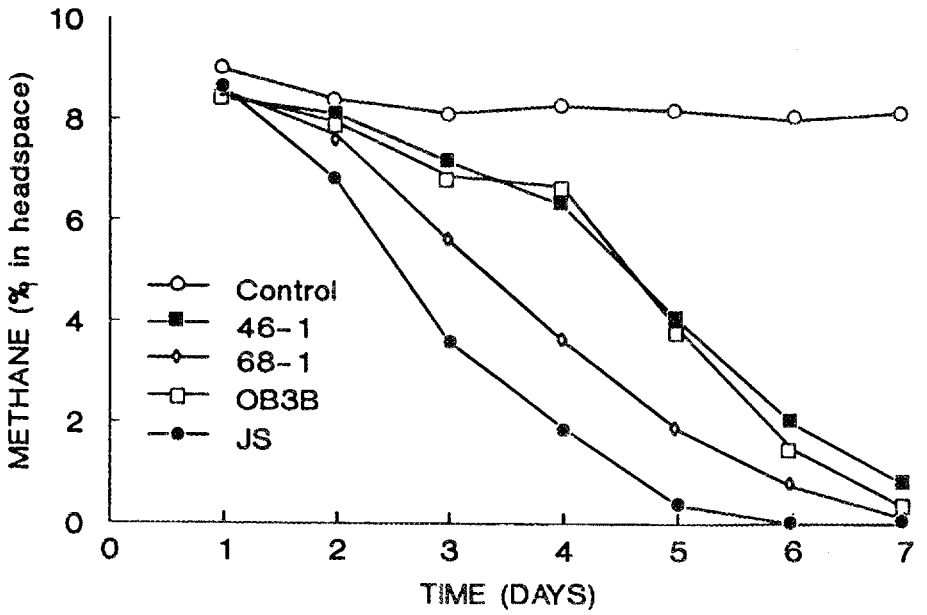

Fig. 5. Utilization of methane in replicate bottles containing uninoculated controls (boxes), strain 46-1 (+), strain 68 (diamonds), OB3b (•), and JS (X) when exposed to TCE.

consumed the same amount of methane and oxygen, consortia growing in the higher concentrations of the site water consumed the methane and oxygen at a slower rate, indicating a decreased growth rate (Fig. 6). The unsterilized treatment without any inoculum contained 25% site water and also displayed methane and oxygen consumption, indicating the presence of methane-utilizing organisms in the site water. The rate of methane and oxygen consumption from day 0 to day 4, however, was not as rapid as in the treatment containing 22.5% site water with added JS mixed culture (Fig. 6). An enrichment (S1) from the site water was used in subsequent experiments.

In the second experiment examining the effect of methane on the growth of methane-utilizing consortia exposed to the site water, the methane was substantially depleted in all treatments after 10 d (Table 5). As in other experiments, oxygen consumption was significantly correlated with the amount of methane consumed ($r^2 = 0.995$, $N = 5$).

Biodegradation of TCE Under Varying DCE Concentrations

Both the DT2 and JS cultures exhibited greater growth, as indicated by methane and oxygen consumption (Table 5), at lower concentrations of DCE. Both consortia also transformed significantly ($F = 19.88$, D.F. = 2, 13) greater amounts of TCE at lower DCE concentrations (Fig. 4). The decrease in the extent of degradation of the TCE was statistically significant (95% level) and was proportional to the concentration of DCE added; the correlation coefficient (r) for the relationship between the percent degradation of TCE, measured by the radiolabel data, and the DCE concentration was -0.92 ($n = 6$) for the DT2 cultures and -0.94 ($n = 6$) for the JS cultures. The DT2 culture was significantly ($F = 12.54$, D.F. = 1, 11) more efficient at degrading the DCE, transforming a mean of 22.3% of the added TCE compared to a mean of 12.4% transformation by the JS mixed culture. The GC data gave similar results for total TCE conversion. The GC data on percent degradation was significantly correlated with the radiolabel data ($r = 0.89$, $p < 0.01$), but it apparently overestimated the degradation. The ^{14}C data indicate that degradation. The mean degradation from the GC data is

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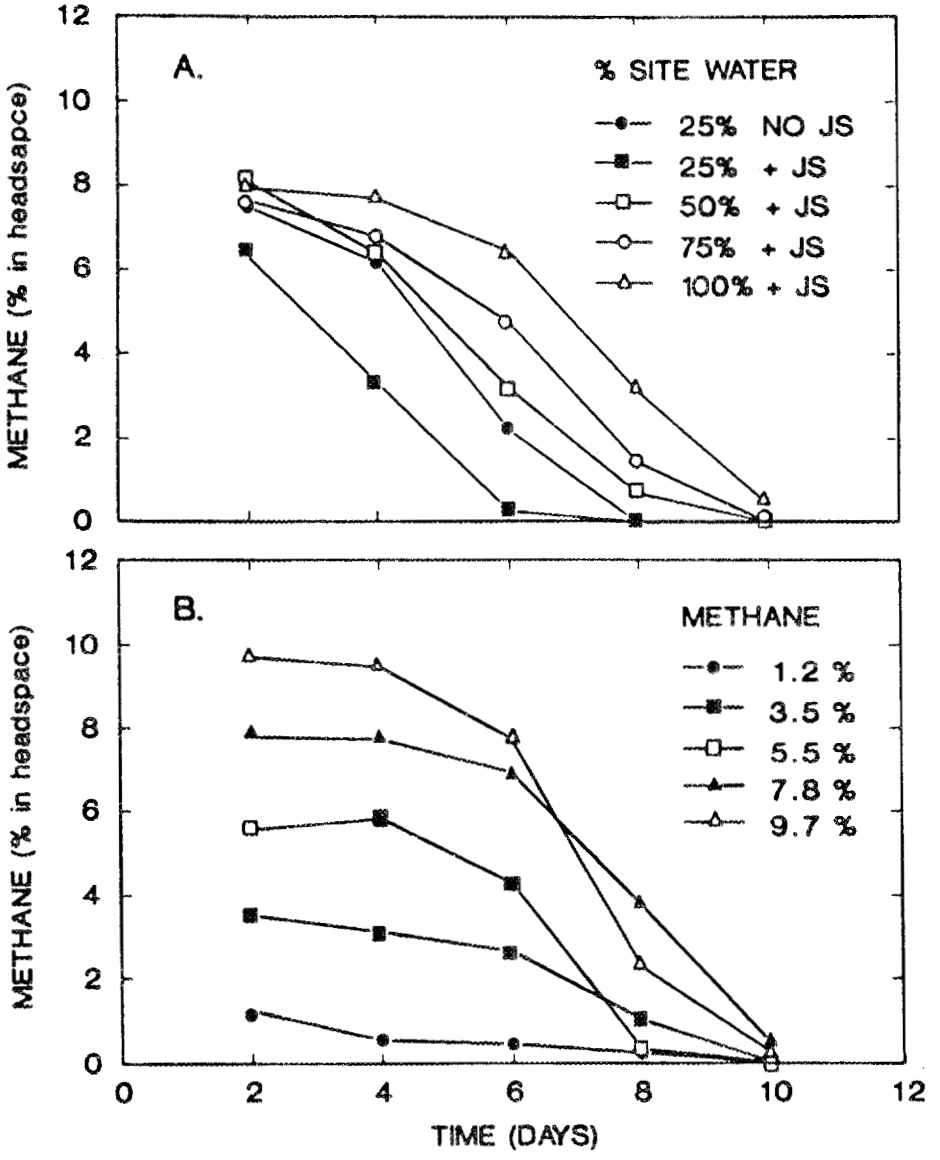

Fig. 6. Methane utilization by the JS consortia growing in site water. (A) Methane utilization with 25% site water (+), 50% site water (diamonds), 75% site water (•), 100% (X) site water, and uninoculated unsterilized 25% site water (boxes). (B) Methane utilization at initial methane concentrations in the headspace of 1.2% (boxes), 3.5% (+), 5.5% (diamonds), 7.8% (triangles), and 9.7% (X).

Table 5. Oxygen and methane remaining (as percent of the headspace) in replicate bottles containing DCE and cultures as well as the uninoculated control

Culture	Treatment	Final gas concentration (%) *			
		Oxygen		Methane	
Control	0 mg/L DCE	18.8	19.0	8.0	8.6
DT-1	0 mg/L DCE	7.0	6.6	0.0	0.0
DT-1	30 mg/L DCE	7.5	9.5	0.0	2.0
DT-1	63 mg/L DCE	14.9	13.6	6.0	5.3
JS	0 mg/L DCE	7.0	6.6	0.0	0.0
JS	30 mg/L DCE	7.5	9.5	1.0	0.1
JS	63 mg/L DCE	14.9	13.6	7.4	3.6

*The least amount of growth is indicated by the highest percent oxygen and methane concentration remaining.

29.4%, and the mean from the radiolabel data is 17.3%. Both consortia converted a high percentage of the TCE to CO_2, and there was no significant difference ($F = 1.73$, D.F. = 1, 11) between the consortia in the proportion of the transformed TCE that was converted to CO_2 (mean = 50.5%).

DCE Degradation

Of the mixed and pure cultures tested, the S1 culture, which originated from the site water, exhibited the fastest rate of DCE degradation (Fig. 7) and methane consumption (Fig. 8). The pattern of oxygen consumption (data not shown) was essentially similar to that for methane consumption. The S1 culture depleted the DCE in 7 d and was the only culture to completely consume all the methane (Fig. 8) during the 10-d experiment. OB3b was the only other culture to completely remove the DCE during the experiment, doing so in 10 d and using less methane than did the S1 culture. In the bottles with the DT2 culture, the concentrations of methane, oxygen, and DCE appeared to be dropping after 10 d, and DCE may have declined to lower levels if the experiment had continued. The JS mixed culture consumed methane rapidly over the first 3 d; however, on the fourth day the rate of methane consumption slowed as did the rate of DCE disappearance. The DCE apparently completely inhibited the growth of strain 46-1; it did not degrade DCE or consume methane under these conditions.

A compound with a retention time of 2.9 min was first observed at day 5 at high levels in the JS and S1 consortia and lower levels in the OB3b and DT2 cultures (Fig. 9). This compound was not observed in the controls or in the bottles containing strain 46-1 (which did not grow or degrade DCE). The JS and S1 consortia had the most rapid initial degradation rates of the TCE (Fig. 8), and as the experiment progressed, the concentration of the unknown compound appeared to decrease (Fig. 9) in these consortia. Although the absolute concentration of this compound cannot be determined in the absence of an authentic standard to determine the gas chromatograph detector response, the concentration of the compound increased in the cultures DT2 and OB3b where DCE degradation increased during the later stages of the experiment. Mass

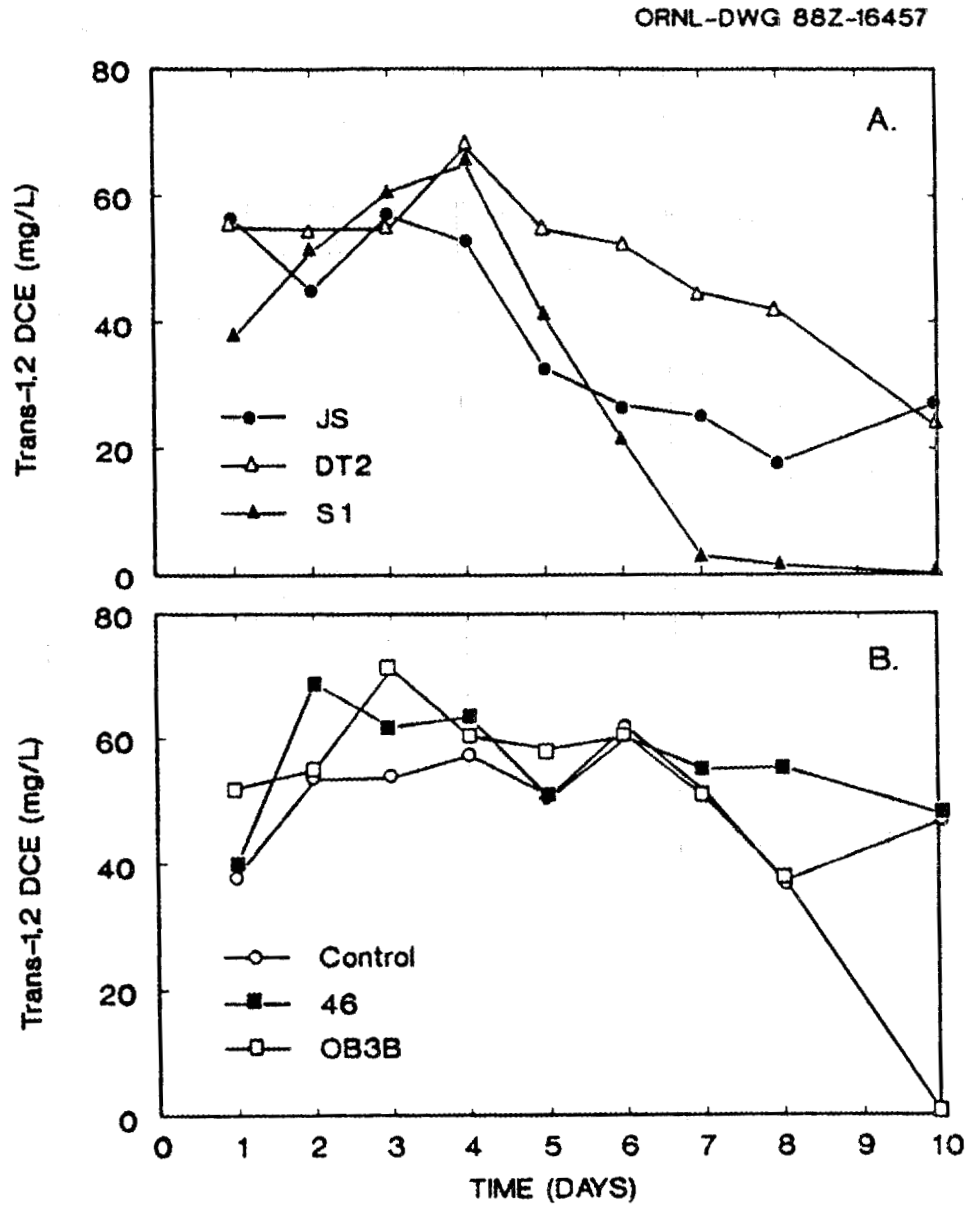

Fig. 7. Disappearance of DCE in replicate bottles containing (A) consortia and (B) pure cultures. Symbols are as follows controls (•), strain 46-1 (+), OB3b (*), JS (boxes), DT1 (X), and S1 (diamonds).

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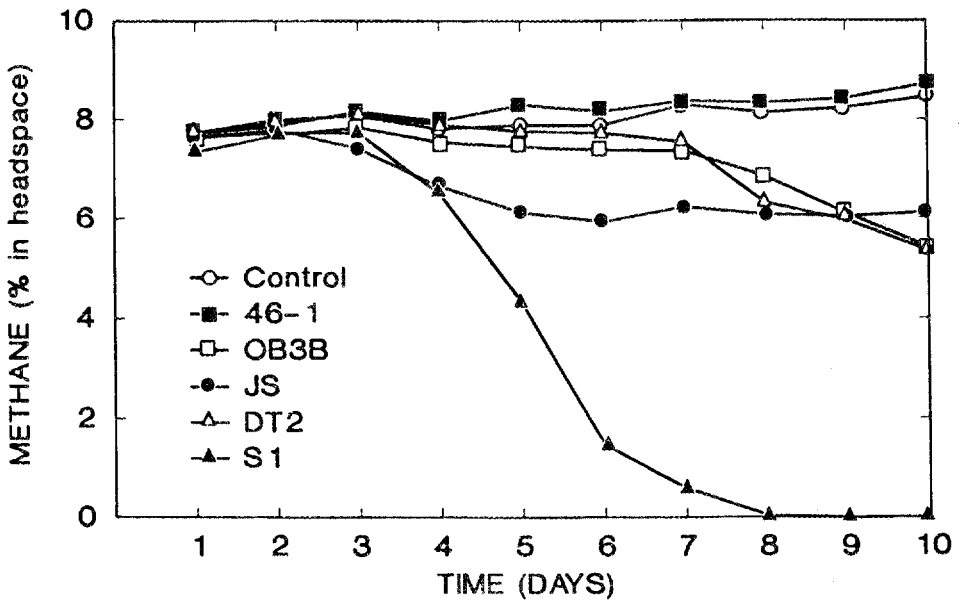

Fig. 8. Methane utilization in bottles containing various cultures exposed to DCE. Symbols as in Fig. 7.

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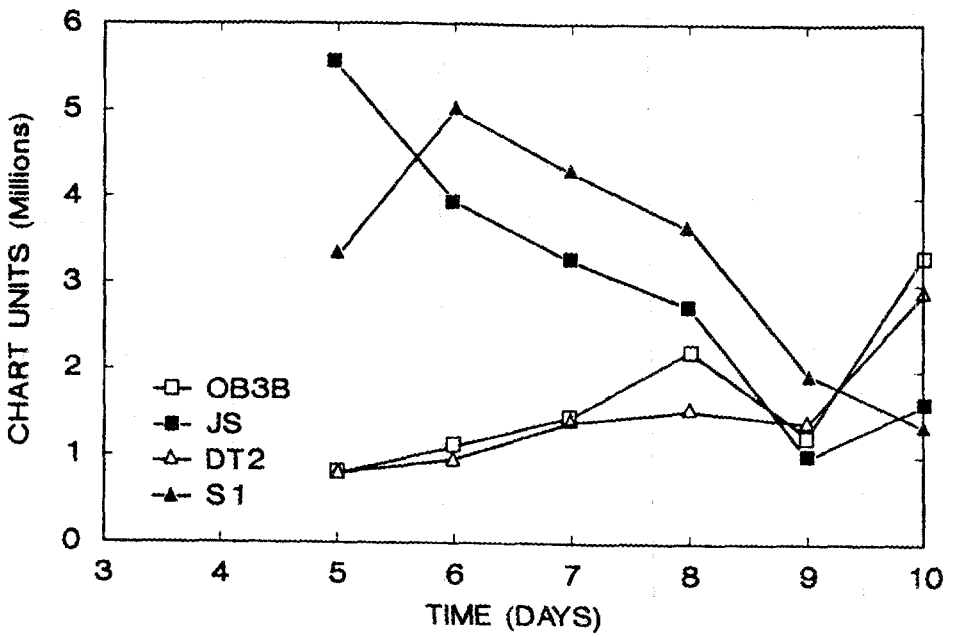

Fig. 9. Unknown compound appearing in replicate bottles containing OB3B (boxes), JS (+), DT1 (diamonds), and S1 (triangles) during DCE degradation. The mean S.D. for the replicate bottles was 0.65 X 10^6 chart units. All readings before day 5 for this compound were 0.

spectroscopy indicates this compound contains 2 carbons, 2 chlorides, and one oxygen atom and has a molecular weight of 112.94 (see Task 1 discussion), which is consistent with a tentative identification as DCE epoxide.

Effect of Manganese on Growth and TCE Degradation

For the mixed JS consortia, the level of TCE degradation after 6 d varied among the manganese treatments (Fig. 10), with the highest transformation of TCE at an intermediate manganese sulfate concentration of 50 μg/L. Although analysis of variance indicates that these differences are not significant ($F = 2.34$; D.F. = 4, 5; $p > 0.05$), further examination of the data indicates that there may be an optimum manganese concentration for TCE transformation. In an analysis of the data that uses a polynomial regression, the DCE squared term is significant at the 94.7% level ($F = 5.39$; D.F. = 1, 7). Since this just fails to meet the 95% criteria for significance, confirmation of this effect (an optimum in TCE transformation in relation to manganese concentration) requires further experimentation with a greater number of replicates. Mineralization to CO_2 accounted for the majority of the transformed TCE, 59.4 $\pm$ 0.58% (mean $\pm$ S.D.). Total water-soluble products represented the smallest proportion of the transformed TCE, 12.4 $\pm$ 4.5% (mean $\pm$ S.D.).

The amount of TCE degradation by the DT1 consortia at 10 and 200 μg/L manganese sulfate was significantly greater ($F = 31.2$, D.F. = 1, 7) than that observed with the JS consortia. Although the total TCE transformation by the two consortia was slightly higher at 10 than at 200 mg/L (Fig. 10) of added manganese sulfate, the effect was not statistically significant. The GC data indicated that noticeable degradation of TCE by the DT1 culture began on the third and fourth days and continued to day 10 at both 10 and 200 μg/L of manganese. There were no significant differences ($p > 0.05$) in the distribution of transformation products between the two consortia.

The amount of oxygen and methane consumption by the two consortia was similar, but the DT1 culture consumed slightly less oxygen. Oxygen consumption by the JS culture did not differ as a response to the

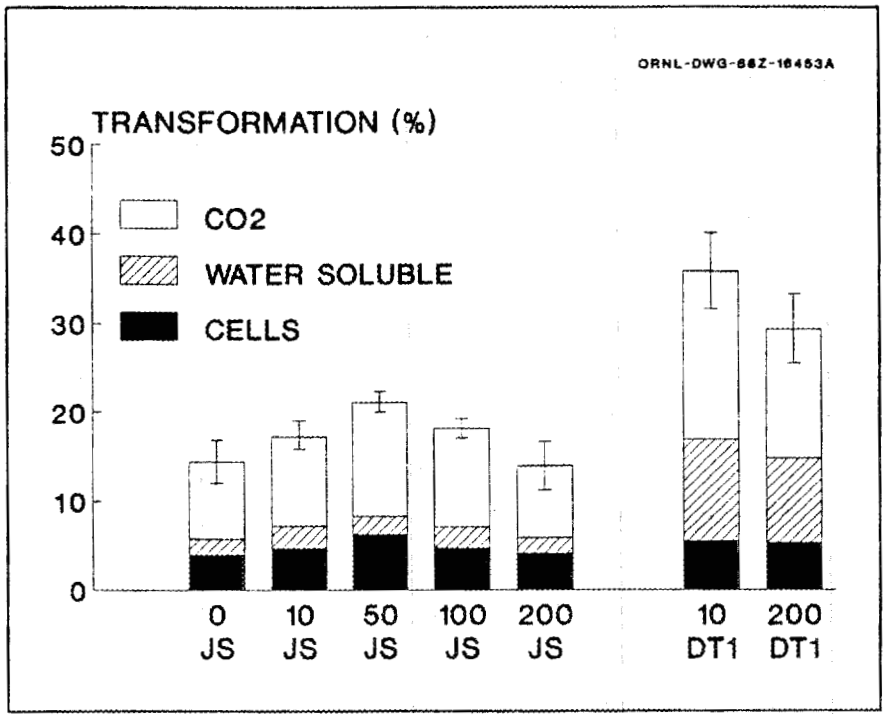

Fig. 10. Distribution of ^{14}C label as percent of added TCE (0.3 mg/L) by the JS and DT1 consortia as a function of $MnSO_4 \cdot {}_2O$ concentration.

manganese; the final concentration of oxygen in the headspace gas was 6.00% (S.D. = 0.79%). Nonsterile controls with no added culture exhibited some oxygen loss, but there was no methane consumption or TCE degradation. The mean final headspace oxygen concentration in the bottles containing the DT1 culture was 7.6% (S.D. = 0.27%). There was no detectable methane in the JS consortia at the end of the experiment, and there was a mean of 0.3% (S.D. = 0.2%) methane in the headspace of the bottles containing the DT1 consortia at the end of the experiment.

Effect pf Methane and Oxygen on Growth and TCE Degradation

Both methane and oxygen affected the growth rate, and the effect of methane and oxygen has been modeled by using Michaelis-Menten kinetics. These bacteria are apparently well adapted to low oxygen and methane concentrations. The calculated half-saturation constant for oxygen was low (1.2 mg/L), and the half-saturation constant for methane was even lower (0.071 mg/L). Even an addition of 1 mL of methane to the 150 mL headspace, giving a dissolved methane concentration of 0.109 mg/L, resulted in a growth rate that was over half the maximum growth rate.

In the ranges tested in these initial experiments, the TCE degradation rate was relatively unaffected by changes in initial oxygen and methane concentration except at the highest methane concentrations tested. In the oxygen experiment TCE degradation in all treatments appeared to be depressed, perhaps due to the high methane concentration added to ensure oxygen limitation. The mean rate of TCE degradation at the six oxygen concentrations from 2.2% of the headspace to 12.3% of the headspace was 0.0034 μg/d $\pm$ 0.0008 (mean $\pm$ S.E.). The results of the methane variation experiment were similar except for the lowest and highest methane concentrations tested. At the lowest methane concentration (0.67% of the headspace gas), there was no measurable degradation of the TCE, probably due to the limited growth observed. Direct microscopic counts indicated that there was much less growth in this treatment than in the other treatments, including the next lowest which contained five times as much methane. The mean degradation rate for the other five treatments in this methane experiment was 2.1 $\pm$ 0.41%

per day. The degradation rate at 3.3% methane, 1.4% ± 0.49% per day, was somewhat lower than at concentrations of 6.6 to 16.7 mg/L.

In the second methane experiment the effect of methane on the TCE degradation rate was apparent at relatively high levels of methane. Twenty-nine percent of the added TCE was transformed by strain 46-1 with 15 mL of added methane (10% to CO_2), and only 16% of the TCE was transformed with 25 mL of added methane (6% to CO_2).

Effect of Methanol on Growth and TCE Degradation

Methanol appeared to reduce the TCE toxicity to some extent, allowing growth at somewhat higher TCE levels. With the pure culture 46-1, bacteria yields were greatest at 1 mg/L TCE. No growth was observed at 30 mg/L TCE. Total TCE conversion was 13.5, 12.5, 6.4, 1.9, and 0.4% for 1, 3, 8, 18, and 30 mg/L TCE, respectively. In the experiment with the S1 mixed culture, bottles containing methane (8.9×10^{-4} mol) converted 32.1 to 36.3% of the TCE, while bottles containing methanol (11.9×10^{-4} mol) converted 3.6 to 3.8%. Similar results were found with the DT consortia. No water-soluble products were formed by cultures using methanol. Additionally, the cultures were visibly different. The cultures grown on methane contained orange-colored pigments, whereas the cultures grown on methanol were unpigmented.

Effects of Ammonia on TCE Degradation

Increased levels of ammonia inhibited TCE degradation by the DT1 consortia. At the highest concentration of NH_4Cl (2.5 g/L), 22.7 to 24.4% of the TCE was degraded. At a lower concentration (0.1 g/L NH_4Cl), more of the TCE (53.7 to 48.0%) was degraded. No degradation occurred in culture bottles without methane.

Effect of Phosphorus on TCE Degradation and Growth of Methanotrophs

Variations in phosphorus content affected the growth rate of the organism but not the rate of TCE degradation. The methane and oxygen consumption data indicate that the variations in phosphorus content affected the growth rate of the organism (Fig. 11) with higher levels of

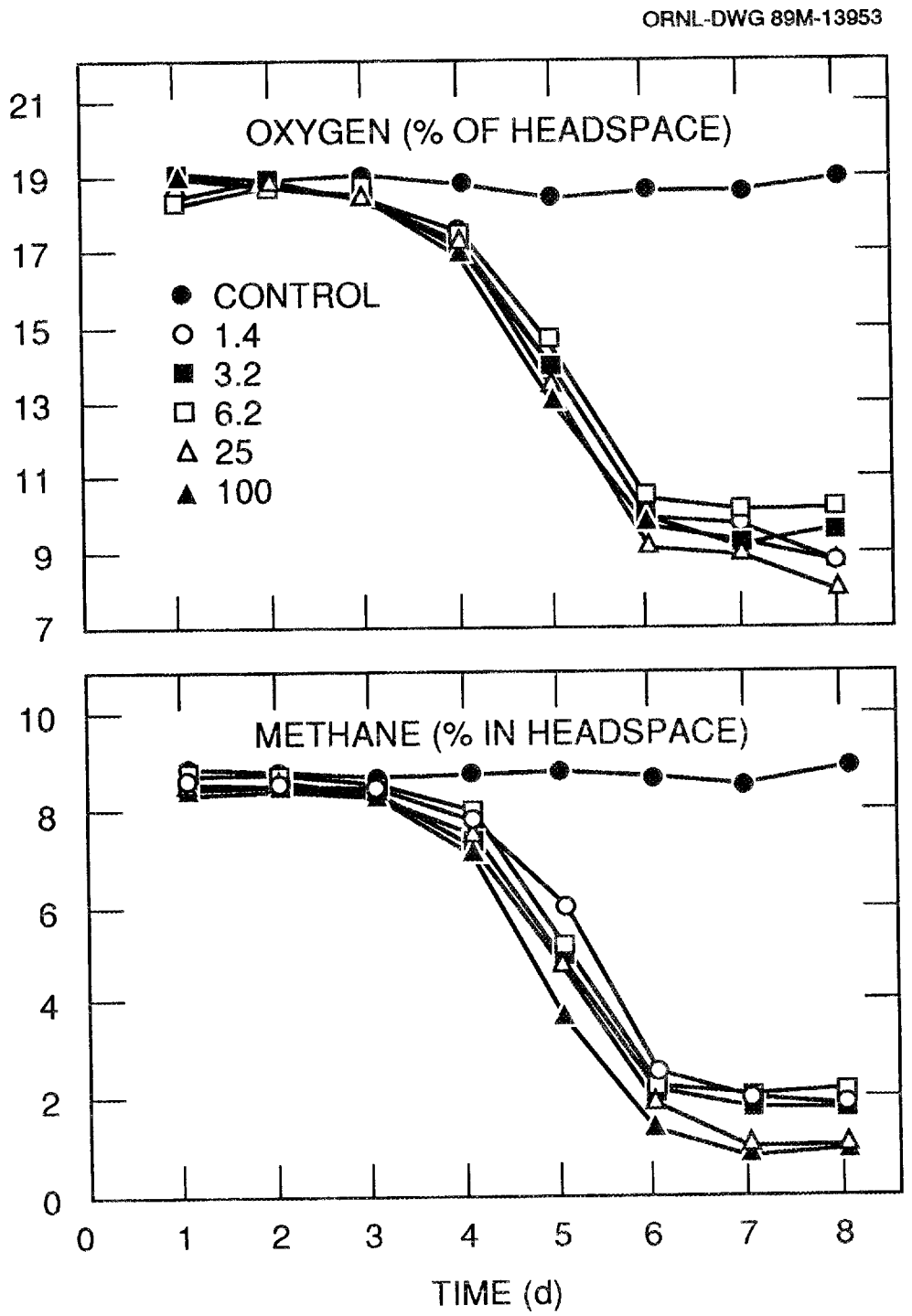

Fig. 11. Methane and oxygen consumption by strain 46-1 at 1.4 (+), 3.2 (*), 6.2 (open box), 25 (X), and 100 (open diamond) μg/ml of phosphorus and the uninoculated control (•).

phosphorus leading to higher growth rates. There was no significant relationship between the PO_4 concentration and the TCE remaining at the end of the experiment ($r = 0.36$, $N = 5$). Thus, the extent of TCE degradation was not significantly affected by phosphorus concentration.

DISCUSSION

The extent of TCE loss and the high degree of conversion of TCE to CO_2 observed with the DT1, DT2, and the S1 consortia make these prime candidates for subsequent tests in the bioreactor. The S1 culture exhibited higher rates of both TCE degradation (Fig. 4) and DCE degradation (Fig. 7) than did the JS bioreactor culture, and the DT1 (Fig. 8) and DT2 (Table 5) cultures exhibited higher rates of TCE degradation than the JS bioreactor culture.

The rapid rate of growth and extensive TCE degradation exhibited by the S1 culture may be due to its recent isolation and consequent high biological diversity. This S1 culture was obtained from contaminated groundwater immediately prior to these experiments. Newly isolated cultures have been reported to degrade TCE more effectively, although it is not reproducible after several transfers (Fliermans et al. 1988). However, recent isolation is not the only key to rapid TCE degradation. The highest rates of TCE degradation were obtained with the DT cultures that were obtained from a site on the Oak Ridge reservation over one year prior to these experiments. These consortia contain both amoeba and bacteria and utilize methane. The JS culture has been tested in a bioreactor, and it exhibits a greater degree of TCE degradation in the bioreactor than it does in batch culture (Strandberg, personal communication). The other consortia may also show similar improvements in TCE degradation in bioreactors where constant supplies of methane and nutrients are added and the population is maintained in a more active state.

Degradation of TCE by pure methanotrophic cultures appears from these results to be a fairly general phenomena. Thus, the usefulness of particular cultures in remediation may be related as much to their tolerance to chlorinated alkenes as to their ability to degrade them. Under the conditions in these experiments with low-to-moderate methane

levels (<20% in the headspace), TCE degradation has been found in every isolate tested (Fig. 4). However, there were differences among the cultures in their tolerances for chlorinated alkenes. Pure strain OB3b had a greater tolerance for chlorinated alkenes than did strain 46-1. OB3b grew and degraded DCE and transformed 95% of it (Table 3) at DCE concentrations of 63 mg/L, but the same concentration completely inhibited the growth of strain 46-1.

Although it was shown that DCE can inhibit TCE degradation, the levels of DCE required are so high that degradation of the TCE in the site water should not be inhibited by the presence of the DCE. If, as previously hypothesized, TCE degradation is initiated by an epoxide mechanism (Little et al. 1988), DCE, which is a structurally similar compound, should be degraded in a similar manner (Colby et al. 1977) and thus may compete for the same enzyme sites. In the experiment varying the proportion of site water in the media, it was anticipated that growth would be best at the lowest concentration of site water due to the inhibitory effects of TCE on growth, and this was the case. However, despite the high TCE concentrations, consortia were capable of growth at all concentrations of site water. Further experiments indicated that DCE could inhibit the degradation of the TCE (Fig. 4), but even with the very high ratios of DCE to TCE in these experiments there was still TCE degradation observed. Thus, it is evident that the presence of DCE does inhibit TCE degradation. However, it is unclear whether this effect is due to DCE retarding growth or DCE competing with TCE for the same active site or a combination thereof. The ratio of DCE to TCE in the site water used in the experiments was on the order of 2 to 1; thus, DCE probably was not critical in inhibiting TCE degradation in the site water. It appears that consortia maintained in bioreactors are capable of degrading TCE in the presence of DCE when they are present in equal proportions (see Task 1 discussion). However, at some of the other wells at the site, the ratio can be much higher, in the range of 100 to 1. Based on the batch culture work presented here, it is possible that the presence of DCE at these ratios in contaminated water may require modification of bioreactor operation so that both the TCE and DCE are degraded to acceptable levels.

Previous reports of DCE degradation have indicated that DCE epoxide can be formed (Jansen et al. 1987), and the results of this study are consistent with this finding. A peak appeared in the GC scans in treatments where DCE was being degraded, and the peak was tentatively identified by mass spectroscopy as DCE epoxide.

Methane utilization exhibited in bottles containing uninoculated, diluted site water (Fig. 6) indicates that there was a population of methane-utilizing bacteria present in the site water, which was able to effectively grow under relatively high concentrations of DCE and TCE. The mixed culture (S1) obtained from the site is able to degrade DCE and TCE at relatively high rates. Isolates of methane-utilizing bacteria from this mixed culture have been made but have not been extensively tested for strain purity.

As with pure culture, alterations in the manganese concentration of the media affected the rate of TCE degradation of the two consortia tested, JS and DT1. Manganese is a cofactor of some oxygenase enzymes (Grady 1984), and altering its concentration may affect the specificity of the methane monooxygenase. Growth of consortia was not noticeably affected by the changes in manganese concentration, but there was an apparent optimum manganese concentration for TCE degradation. For the JS culture, TCE degradation increased as manganese concentration increased from 0 to 50 μg/L, but degradation decreased beyond 50 μg/L. Thus, alterations in the available cofactors may be a promising avenue for increasing the rate of degradation of chlorinated hydrocarbons by methanotrophs in bioreactors.

The methane and oxygen concentrations resulting in maximum growth of strain 46-1 were fairly low compared to saturated concentrations of these gases, and high levels of methane decreased the TCE degradation rate. This finding supports the idea that maintaining a low methane level in the feed water for a bioreactor will result in greater degradation of TCE.

Methanol apparently stimulated growth of populations that do not degrade TCE. Methanol was not observed to increase the extent of TCE degradation in pure cultures, and in mixed cultures methanol decreased the extent of TCE degradation. In mixed cultures methylotrophs lacking

the methane monooxygenase enzyme are apparently stimulated by the methanol addition. In pure methanotrophic cultures TCE degradation was observed with methanol addition, probably because the methane monooxygenase is constitutive. However, there was no improvement in the extent of TCE degradation with the methanol. Thus, despite the apparent reduction in TCE toxicity with methanol due to the negative effect of methanol on TCE degradation in mixed cultures, it would not be useful in a mixed culture bioreactor.

As with methanol addition, addition of ammonia did not improve TCE degradation; rather, it decreased the extent of TCE degradation. However, it is not certain that the cultures tested contained ammonia oxidizers. Thus, this line of research could be continued by culturing microbial consortia containing ammonia oxidizers and by testing the TCE degradation capabilities of pure cultures of ammonia oxidizers. However, at this time the best strategy for bioreactor operation is to use nitrate as a nitrogen source rather than ammonia, although testing of other ammonia concentrations may show a lower concentration.

Phosphorus could be used to control the growth of the consortia in the bioreactor. The data indicated that phosphorus concentration affected the growth rate of the bacteria but not the extent of TCE degradation. Thus, in a bioreactor where the maximum amount of TCE degradation is desired with the minimum increase in biomass, phosphorus could be used as a control agent. The lowest phosphorus concentration used in the experiment (1.4 μg/L) is still fairly high compared to natural waters, but since the extent of TCE degradation was not reduced at this level, further reductions are still possible.

CONCLUSIONS

The results of this research are summarized in Table 6 where the effect of various parameters is shown on the growth rate of methanotrophs and TCE degradation rate. There are several strategies that should be followed to optimize TCE degradation in bioreactors. Selection of consortia resistant to the toxic effects of chlorinated hydrocarbons and the identification of appropriate micronutrient conditions, specifically manganese concentration, are two factors that

Table 6. Summary of experimental results showing the effect of various parameters on the growth rate of the methane-utilizing bacteria and on the rate of TCE degradation

Parameter	Effect on growth	Effect on TCE degradation
Methane	Yes	Inhibits at very high concentration
Oxygen	Yes	No apparent effect on rate
Site water	Inhibits growth	Inhibits degradation
DCE	Inhibits growth	Inhibits at high concentration
TCE	Inhibits growth	Apparent 0-order kinetics
Manganese	No (range tested)	Rate may change with concentration
Phosphorus	Yes	No effect
Ammonia	Not measured	Inhibits TCE degradation
Methanol	Reduces TCE toxicity	Reduces rate
Culture	Yes	Large differences in rates among cultures

this research has identified as being important in efforts to increase the utility of consortia in bioreactors. The addition of methanol or ammonia will tend to decrease bioreactor efficiency rather than to increase it, and phosphorus concentration may be utilized to control the growth of the consortia without affecting the TCE degradation rate. The presence of high levels of other chlorinated alkenes such as DCE may reduce the rate of TCE degradation, but the importance of this effect will depend on the specific composition of the contaminated water.

TASK 4
PRELIMINARY ENGINEERING EVALUATION

INTRODUCTION

A design for a 1-gal/min pilot plant and conceptual designs for larger full-scale plants of several sizes have been prepared, and cost estimates have been made. These designs are based on the performance of the laboratory-scale bioreactor system and assumptions about the site and the groundwater to be treated, as summarized below.

Pilot Plant

A schematic of the 1-gal/min pilot plant is shown in Fig. 12. It consists of two bioreactor columns in series, each constructed from 2-ft-diam PVC pipe. The packing is a PVC material. This pilot plant could be skid mounted and transported to the field site for testing or rack mounted at the site.

The list of equipment and estimated costs are shown in Table 7. The total equipment and construction costs are estimated to be approximately $20,000. The total 6-month project cost to construct the pilot plant, install it at a site, and carry out a 3-month minimal test campaign is estimated to be approximately $180,000.

Full-Scale Plants

A conceptual design for a full-scale plant is shown in Fig. 13, and the list of major equipment is given in Table 8. The design is patterned after conventional trickle-filter processes for wastewater treatment. The first-order rate constant for TCE degradation is taken to be 0.01 min^{-1}, based on laboratory studies. The TCE concentration in the influent water is chosen to be 1 mg/L, and the effluent concentration is specified to be 0.05 mg/L. The gas phase is 4% methane in air, and the nutrient addition is based on laboratory experience. The effluent is to be discharged directly to a local sewage treatment plant. It is anticipated that there will be some stripping of TCE into the gas phase, and thus an activated carbon system is specified for off-gas cleanup. However, stripping may actually be quite minimal with proper optimal operation of the bioreactor.

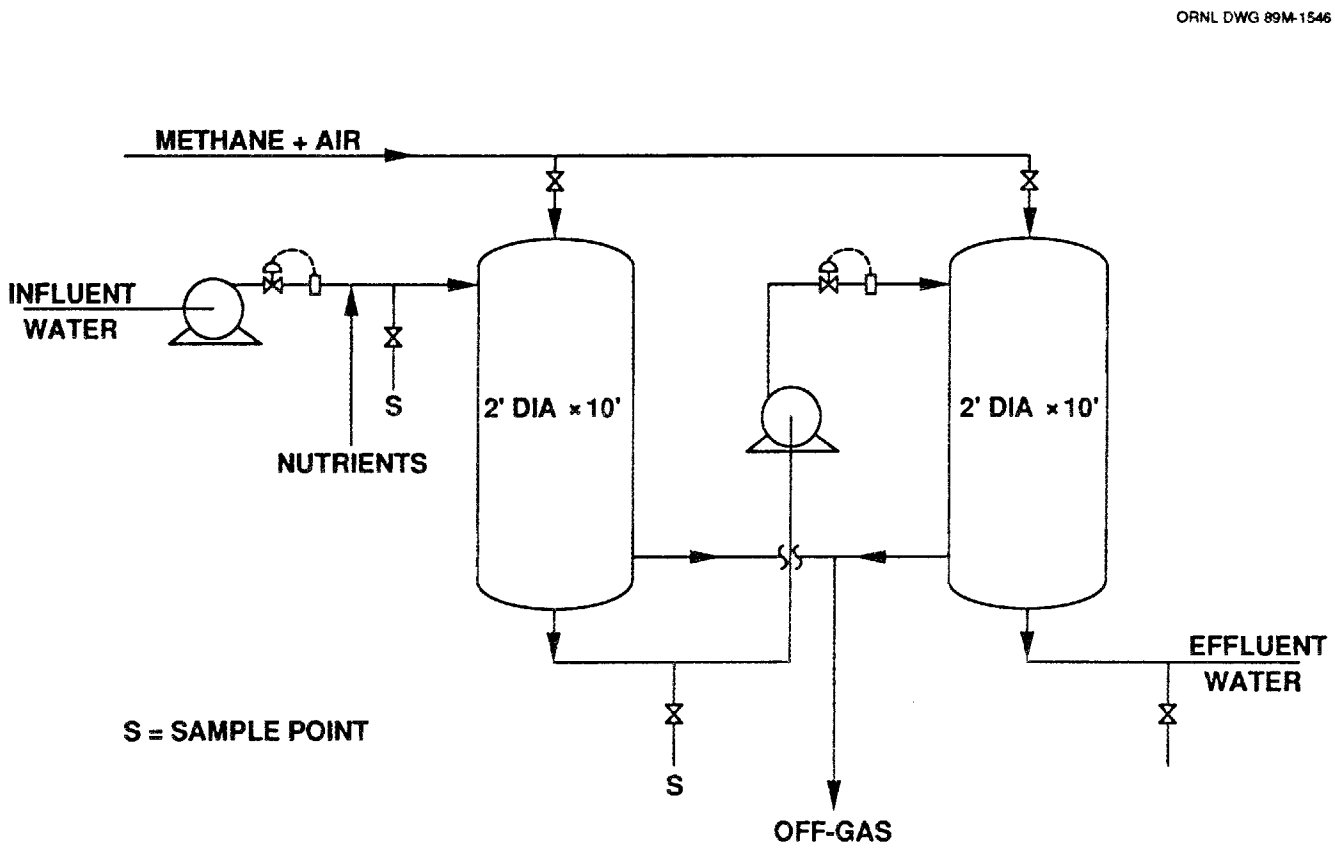

Fig. 12. Schematic diagram of 1-gal/min pilot plant for bioremediation of TCE-contaminated groundwater.

Table 7. Equipment list for pilot plant

Equipment	Cost (dollars)
PVC pipe, 1 in. sch 40 (water), 100 ft	35
PVC pipe, 1/2 in. sch 40 (gas), 50 ft	18
Valves, ball, 7 each	140
Metering valve/controller, 2 each	2,000
PVC pipe, 2 ft sch 40 (columns), 20 ft	920
Packing media, PVC Flexipac, 62 ft^3	930
Pump, 2 each	800
Gas supply system	2,000
Skid/pipe support	2,000
Total equipment	8,843
Fabrication, 200 work-hours	10,000
Total cost	19,000

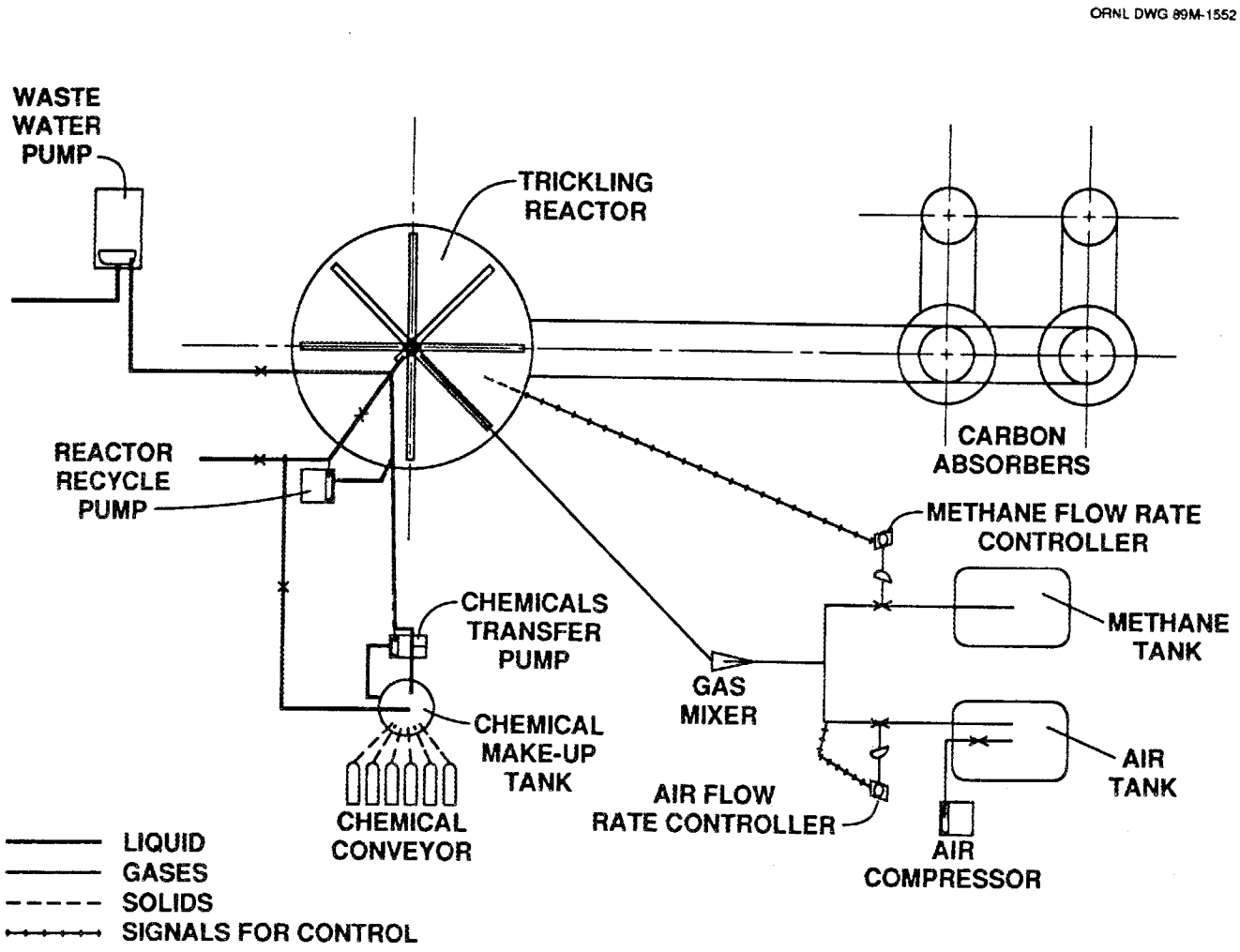

Fig. 13. Conceptual schematic of full-scale plant for bioremediation of TCE-contaminated groundwater.

Table 8. Estimated capital equipment costs for plants of various sizes (dollars)

Item	Plant size (gal/min)				
	50	100	50	200	700
Groundwater pump	1,168	1,516	1,733	1,980	7,516
Recycle pump	1,168	1,516	1,733	1,980	7,516
Reactor and packing	26,642	49,465	73,873	93,040	307,332
[size = gal]	[15,000]	[28,000]	[42,000]	[56,000]	[180,000]
[height = diameter, ft]	[9]	[16]	[20]	[23]	[34]
Chemicals pump	1,168	1,516	1,722	1,980	7,516
Chemicals tank and handling	3,256	3,256	4,480	4,480	6,162
Carbon adsorbers (two)	11,408	15,696	18,912	21,592	33,312
Methane tank	12,632	12,632	12,632	12,632	12,632
Air tank	7,083	7,083	11,333	11,333	11,333
Air compressor	2,947	2,947	5,263	5,263	10,526
Flow controller - air	(not costed separately)				
Flow controller - methane	(not costed separately)				
Gas mixer	(not costed separately)				
Chemicals conveyors	(not costed separately - see item 5)				
Totals (round numbers)	68,000	96,000	132,000	154,000	404,000
Add instruments and controls (15%), taxes (3%), and freight (5%)	84,456	119,232	163,944	191,268	501,768
Add installation costs: foundations and support (5%), erection and handling (50%), electrical (8%), piping (2%), painting (2%)	141,041	199,117	273,786	319,417	837,952
Add indirect costs, as % of equipment: engineering supervision (25%), construction fee (10%), startup (2%), contingency (10%)					
Total capital cost	180,735	255,156	350,840	409,313	1,073,782

Five plant sizes were costed: 50, 100, 150, 200, and 700 gal/min. The latter flow rate is equivalent to 1 million gal/d, which is comparable to small municipal wastewater treatment plants. The smaller plant sizes may be representative of typical groundwater treatment requirements. The reactor volume and a characteristic linear dimension for each size of trickle filter is given in Table 8, assuming that the tank diameter and height are equal. (This number is given to illustrate the physical size of the tank; the actual design would not necessarily have an aspect ratio of unity.) The larger reactor sizes would be fabricated on-site by using standard procedures, while the smaller reactors could be constructed from off-the-shelf tanks. Alternatively, the larger systems could be constructed from multiple smaller modules.

No buildings are specified for enclosure of equipment nor for administration and laboratory and maintenance functions. It is assumed that these latter functions are incorporated into existing facilities at the site.

The operating costs are summarized in Tables 9 and 10. The approach is based on EPA recommendations (EPA 1978) and is probably conservative.

The chemicals for nutrients are similar to the nutrients used in the laboratory studies. The economic impact is considerable if this level is necessary. However, the actual requirements will likely be site specific, and further laboratory studies are needed to define the minimal requirements. The activated carbon requirement is about $0.06/1000 gal with no regeneration and no charges for disposal. Optimal reactor design and operation may lead to a lower requirement for activated carbon.

SUMMARY

From these preliminary evaluations it appears that a trickle-filter plant to treat contaminated groundwater could be constructed with conventional equipment and procedures. Capital and operating costs may be comparable to those for conventional biological treatment of industrial and municipal wastewaters (depending on nutrient requirements). However, further laboratory data are needed on nutrient

Table 9. Estimated operating costs for various plant sizes (dollars/year)

Item	Plant size (gal/min)				
	50	100	50	200	700
O&M labor	35,000	35,000	40,000	40,000	45,000
Overhead (60%)	21,000	21,000	24,000	24,000	27,000
Electricity ($0.06/kWh)	6,220	9,330	12,440	15,550	18,660
Taxes, insurance, administration (4%)	7,230	10,206	14,033	16,372	42,951
Capital recovery (10% interest)	28,917	40,825	5,6134	65,490	171,805
Total, dollars/year (no chemicals)	98,367	116,361	146,607	161,412	305,416
Total, dollars/1000 gal (no chemicals)	3.75	2.21	1.86	1.54	0.84
Add chemicals ($0.20/1000 gal)	3.95	2.41	2.06	1.74	1.04

Table 10. Estimated operating costs for various plant sizes (dollars/1000 gal)

Item	Plant size (gal/min)				
	50	100	150	200	700
O&M labor + overhead (60%)	2.13	1.06	0.81	0.61	0.20
Electricity ($0.06/kWh)	0.24	0.18	0.16	0.15	0.05
Taxes, insurance, administration (4%)	0.28	0.19	0.18	0.16	0.12
Capital recovery (10% interest)	1.10	0.78	0.71	0.62	0.47
Chemicals ($0.20/1000 gal)	0.20	0.20	0.20	0.20	0.20
Total	3.95	2.41	2.06	1.74	1.04

requirements, stripping, and reaction kinetics at low TCE concentrations, and a pilot plant study is needed to verify and demonstrate the scale-up parameters.

CONCLUSIONS

1. Reduction of TCE in a synthetic groundwater was demonstrated in a continuous-flow, trickle-filter bioreactor that used methanotrophic bacteria.

2. With influent concentrations to the bioreactor of 1 mg/L for both TCE and DCE, approximately 50% of the TCE and 90% of the DCE were degraded in a single pass. Liquid recycle increased the degradation further.

3. The presence of other chlorinated hydrocarbons, such as DCE, may reduce the rate of TCE degradation, but this reduction is dependent upon the specific composition of the contaminated water. The other chlorinated hydrocarbons may degrade faster than the TCE. It is not clear if DCE inhibited the degradation of TCE or was preferentially degraded by the microorganisms.

4. The use of a mixed microbial consortium performs better than a pure strain. Indigenous microorganisms from the groundwater were used successfully in batch shake-flash tests to degrade TCE. This implies that future developmental work should concentrate on indigenous microorganisms.

5. The indigenous microorganisms degraded DCE and consumed methane in batch shake-flash tests at the fastest rate of all other cultures.

6. The batch shake-flash tests indicate that there is an optimum concentration of manganese for TCE degradation.

7. Increased concentrations of ammonia inhibited TCE degradation.

8. Phosphorus concentration did not affect the extent of TCE degradation.

9. The consortia tested in the batch shake-flash tests were well-adapted to TCE reduction at low methane concentrations (relative to saturated concentrations), and high methane concentrations decreased TCE degradation rates.

10. Methanol addition reduced the rate of TCE degradation.

11. A pilot plant utilizing a trickle-filter bioreactor should be operated to further develop and demonstrate the technology.

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