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Treatment of Chlorinated Solvents by Nitrogen-Fixing and Nitrate-Supplied Methane Oxidizers in Columns Packed with Unsaturated Porous Media

KUNG-HUI CHU AND
LISA ALVAREZ-COHEN*

Department of Civil and Environmental Engineering, University of California, Berkeley, California 94720-1710

This study compares the feasibility of employing nitrogen-fixing and nitrate-supplied methane-oxidizing cultures grown in unsaturated porous media to degrade *cis*-1,2-dichloroethylene (cDCE) and trichloroethylene (TCE) in gas streams. Both nitrate-supplied and nitrogen-fixing columns (supplied with 10% CH_4 and 10% O_2) degraded TCE completely at a gaseous concentration of 0.7 mg/L for 8−10 days. However, when columns were supplied with 4% CH_4 and 10% O_2, nitrate-supplied columns were not able to recover after degrading TCE at a gaseous concentration of 0.13 mg/L for 7 days. In contrast, nitrogen-fixing columns recovered after degrading 0.13−0.4 mg/L TCE for 3−10 days and were capable of repeatedly degrading TCE at gaseous concentrations of 0.03−0.14 mg/L during long-term intermittent operation (lasting from 38 to 84 days) that was punctuated by appropriate microbial recovery periods (7−9 days). Both nitrate-supplied and nitrogen-fixing columns were capable of degrading cDCE at concentrations of 0.7−1.0 mg/L for 5−10 days, but only the nitrogen-fixing columns recovered from cDCE exposure. The operating period for columns treating a mixture of TCE and cDCE was significantly shorter than that for treatment of TCE or cDCE alone. Several operating curves (percent chlorinated solvent removal versus mass of chlorinated solvent loaded per mass of methane consumed) were developed to facilitate comparisons between operating conditions and to aid in predicting chlorinated solvent removals in such systems. Nitrogen-fixing columns consistently outperformed nitrate-supplied columns, and columns inoculated with a mixed culture outperformed those inoculated with *Methylosinus trichosporium* OB3b for TCE removal but not for cDCE removal.

Introduction

The presence of volatile organic contaminants in the vadose zone has been recognized in recent years to be a potential long-term threat to groundwater quality. Soil vapor extraction (SVE) is commonly used for vadose zone remediation. However, this process is often hindered by mass transfer limitations and may result in the production of large quantities of dilute off-gases containing volatile contaminants (*1*). To avoid the emission of contaminants into the atmosphere, such off-gases are commonly passed through activated carbon units or subjected to thermal destruction that can be complicated and costly (*2*). Therefore, effective contaminant destruction approaches such as in-situ vadose zone bioremediation or above-ground bioreactors for the treatment of off-gases from soil vapor extraction and air-stripping would be highly beneficial.

Volatile contaminants of particular concern for subsurface remediation include chlorinated solvents such as *cis*-1,2-dichloroethylene (cDCE) and trichloroethylene (TCE), which are frequently detected groundwater contaminants and are listed as priority pollutants. A number of studies have shown that TCE and cDCE can be quickly degraded to harmless products such as chloride ions and carbon dioxide by a variety of naturally occurring microorganisms, including methane-oxidizing bacteria (methanotrophs). Methanotrophic cultures that produce methane monooxygenase (MMO) enzymes are capable of cometabolically oxidizing a wide range of groundwater contaminants including aliphatic, aromatic, and halogenated hydrocarbons (*3*−*5*).

Methanotrophic populations in a subsurface aquifer were stimulated in a field demonstration of enhanced in situ bioremediation for the treatment of chlorinated solvents at Savannah River (*6, 7*). At this site, nitrogen and phosphate were added to the stimulation zone since it was determined that methanotrophic growth was limited by the availability of nutrients. In fact, some methane-oxidizing bacteria are known to fix molecular nitrogen as their sole nitrogen source (*8*−*10*). The type of methanotroph that fixes nitrogen grows optimally at reduced oxygen partial pressures (4−10%) and produces a form of MMO (soluble MMO) that exhibits high activity with respect to the oxidation of chlorinated aliphatics. In fixed-nitrogen limited subsurface environments, naturally abundant nitrogen gas could potentially serve as the primary nitrogen source for methanotrophic growth, giving nitrogen-fixing methanotrophs a selective enrichment advantage.

Chlorinated solvent degradation by methanotrophs has been shown to cause product toxicity, resulting in decreased methane and TCE oxidation rates and cell inactivation (*11*−*16*). This product toxicity has been quantified by means of a transformation capacity (T_c) that is defined as the mass of chlorinated solvent degraded per mass of cells inactivated (*17*). Our previous batch studies with methane-oxidizing bacteria grown using different nitrogen sources showed that nitrogen-fixing methanotrophs exhibit enhanced TCE degradation abilities over nitrate- and ammonia-supplied cells, including higher TCE oxidation rates, higher TCE transformation capacities, and lower TCE product toxicities (*18, 19*). These results suggested that nitrogen-fixing methanotrophs may be promising candidates for biotreatment of chlorinated solvent laden off-gases and/or in-situ vadose zone bioremediation.

The goal of this study was to examine the feasibility of employing nitrogen-fixing methane oxidizing cultures grown in unsaturated porous media columns to degrade TCE and cDCE. A series of short- and long-term degradation tests for TCE and cDCE were conducted using active populations of methanotrophs grown in columns under nitrate-supplied and nitrogen-fixing conditions. Data collected from these experiments were used to develop a series of operating curves to define optimal operating conditions for contaminant degradation in such systems.

Materials and Methods

Chemicals. TCE (99+% pure ACS reagent), tetrachloroethylene (PCE, 99+% pure ACS reagent), and cDCE (98% pure ACS reagent) were purchased from Aldrich Chemicals Co.,

* Corresponding author phone: (510)643-5969; fax: (510)642-7483; e-mail: alvarez@ce.berkeley.edu.

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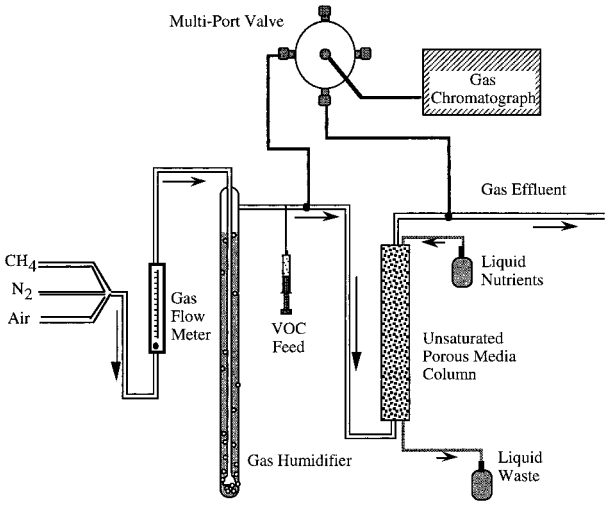

FIGURE 1. Schematic diagram of one of four parallel unsaturated biofilm column reactors.

Milwaukee, WI. Chloride ionic strength adjuster (CISA) solution, containing 1 M HNO_3 and 0.1 M $NaBrO_3$ (Fisher Science Co., Fair Lawn, NJ), was prepared for use in chloride ions measurements.

Unsaturated Porous Media Columns. Column experiments were conducted in continuous gas-flow columns packed with porous media that consisted of either: glass beads (6 mm diameter), silicalite pellets (3 mm diameter, consisting of synthetic silicon dioxide crystals and binders), or diatomaceous earth pellets (DEP, 5 mm diameter × 3−10 mm long, Celite Biocatalyst Carrier R-635, Celite Corp., Lompoc, CA). All attachment materials were washed with deionized water and dried at 550 °C overnight to remove any organic matter prior to use. Figure 1 shows a schematic diagram of one of four parallel continuous gas-flow columns used in this study. Columns were emptied, washed, and repacked with fresh attachment materials when new experiments were initiated. The main body of each column consisted of a glass tube (5 cm i.d. × 30 cm long) capped with threaded Teflon plugs. Stainless steel tubing (1/8 in. diameter) and fittings were used to minimize chemical reactions or sorption during the experiments. At the bottom of each column, a layer of glass beads 2−3 cm in height (11 mm diameter, purchased from VWR, Bridgeport, NJ) was used to prevent clogging and to help with gas distribution. The influent gas line was attached directly to the bottom of the column. Gas flow rates were controlled by variable constant differential gas flow controllers (model VCD 1000 purchased from Porter Instrument Company, Inc., Hatfield, PA). A gas flow bubble meter was used to calibrate gas flow rates before and after each liquid flushing (i.e., every 2−3 weeks). The empty bed volume was 589 cm^3, and the empty bed retention time was 58.9 min. The influent and effluent gas lines were connected to a 16-port, electronically actuated multi-function sampling valve (Valco Instruments Co., Houston, TX) with a 20-μL gas sample loop for automated gas chromatograph (GC) sampling of organic solvents. Chlorinated solvents were also measured manually as were CH_4, O_2, N_2, and CO_2 in influent and effluent gas streams.

Microbial Inoculation. Before microbial inoculation, the packed columns were filled with nitrate mineral salts (NMS) or nitrogen-free mineral salts (NFMS) medium for 2 h and then drained by gravity. A mixed chemostat culture of methanotrophs (*18*) or *Methylosinus trichosporium* OB3b (ATCC 35070, referred to below as OB3b) were used as microbial inocula. The columns were inoculated by filling with cell suspension (~3.7 g/L for mixed chemostat culture, ~1.0 g/L for OB3b) for 90 min and then draining by gravity.

TABLE 1. Development of Microbial Populations in Unsaturated Biofilm Column Reactors

column label	culture inoculum	N source	influent gas	time to reach steady state[a] (day)
1a	mixed	NO_3^-	10% CH_4 20% O_2	35
1b[b]	mixed	N_2	4% CH_4 10% O_2	60[c]
1c[d]	mixed	NO_3^-	4% CH_4 10% O_2	4[c]
2	mixed	NO_3^-	10% CH_4 10% O_2	7
3a	mixed	N_2	10% CH_4 4% O_2	5
3b[b]	mixed	N_2	10% CH_4 10% O_2	6[c]
3c[d]	mixed	N_2	4% CH_4 10% O_2	6[c]
4	mixed	N_2	4% CH_4 10% O_2	17
5a, 5b[e]	mixed	NO_3^-	4% CH_4 10% O_2	5
6	OB3b	N_2	4% CH_4 10% O_2	45

[a] Steady-state was assumed when the effluent concentrations of CH_4, O_2, and CO_2 stabilized. [b] Change of operating conditions for column a. [c] Time required for culture to reach a new steady-state from previous operating conditions. [d] Change of operating conditions for column b. [e] Similar results were observed in a repeated experiment.

The inoculated columns were supplied with an upflow gas stream composed of CH_4, O_2, and N_2 at a flow rate of 10 mL/min at 20−23 °C. On the basis of our previous studies (*18*, *19*), the influent O_2 was maintained at 10−20% for nitrate-supplied cultures and at 4−10% for nitrogen-fixing cultures in order to maximize cell growth. Influent CH_4 was initially maintained at 10% and later changed to 4% in air to remain below potential explosive levels. Desiccating conditions within the columns were avoided by bubbling the influent gas mixture through a water column prior to entering the biological columns. Micronutrients were added, and cellular waste products were removed every 2−3 weeks by flooding columns with NMS or NFMS medium for 90 min before draining by gravity. Microbial growth within the columns was monitored by daily measurements of influent and effluent gas compositions and by measuring cell densities in the flushing liquid.

Chlorinated Solvent Degradation Tests. After a stable attached microbial population was established, chlorinated solvent (TCE and/or cDCE) vapor was introduced into columns along with the feed gases. Chlorinated solvents were diffused into the influent gas stream using a 5-mL gas-tight Pressure-Lok Dynatech-Precision syringe (Alltech Co.) or a 3-mL serum bottle containing 1 mL of pure chlorinated solvent (TCE and/or cDCE) attached to the influent gas flow tube upstream from the columns. The chlorinated solvent concentrations in influent and effluent gas were measured by GC headspace analysis.

Chlorinated solvent degradation tests were conducted with a range of influent concentrations, from 0.03 to 0.7 mg/L of TCE and from 0.7 to 1 mg/L of cDCE. Short-term TCE degradation tests were conducted for 2−10 days. Each of the chlorinated solvent experiments was initiated after the columns reached steady-state conditions as measured by constant effluent gas compositions (see Table 1). The changes in influent and effluent gas compositions in terms of CH_4, O_2, N_2, and CO_2 were monitored throughout the experiment. Solvent degradation tests were followed by recovery periods during which the columns were initially flushed with nutrient media (NMS or NFMS) and then fed methane and oxygen

without solvents. Recovery period lengths were determined by the amount of time required for cultures to resume their original levels of CH_4 uptake and CO_2 production. Long-term TCE degradation tests were performed for 38−84 days and included repetitive periods of chlorinated solvent exposure alternating with recovery periods.

During degradation tests, the CO_2 resulting from TCE degradation was removed by the continuously flowing gas stream; however, the chloride ions were retained and accumulated in the column until the next nutrient flushing (i.e., the recovery period). Since NMS and NFMS are chloride-free media, any chloride ions detected in the waste nutrient flushing liquid were assumed to be from dechlorination of the chlorinated solvents. Mass balances on the chlorine were conducted based upon the amount of chlorinated solvent within the influent and effluent gases and the chloride ions released during nutrient flushing.

Analytical Methods

Gas and Organic Solvent Analysis. Compositions of the influent and effluent gases of the columns (CH_4, O_2, N_2, and CO_2) were measured by withdrawing 0.25 mL of gas samples from the sampling ports with a 0.5-mL gas-tight Pressure-Lok Dynatech-Precision syringe (Alltech Co.) and injecting it into a Hewlett-Packard HP 5890 series II gas chromatograph (GC) equipped with a CTR1 column (Alltech Co.) (*18, 19*).

Organics within the column influent and effluent streams were measured either by manual sampling using a Hamilton CR-700-20 constant rate gas-tight syringe (Hamilton Co., Reno, NV) or by automated sampling with a 20-μL gas sample loop. The 20- or 50-μL headspace samples were analyzed on a Hewlett-Packard HP 5890A GC as described previously (*18, 19*).

Chloride Ion (Cl^-) Measurements. Chloride ion concentrations were measured using a chloride ion electrode (model 94-17B, Orion Res. Inc., Boston, MA) with a sensitivity range from 0.01 to 10 mM. Cells were first removed from liquid samples by filtering through a 0.47-μm pore membrane. Five milliliters of supernatant and 5 mL of CISA solution (containing 1 M HNO_3 and 0.1 M $NaBrO_3$) were added to a 26-mL vial. The mixture was allowed to react for 3 min before measurement. Standard solutions were prepared by dissolving a range of known amounts of KCl (0.01−10 mM) in filtered (0.47-μm pore membrane) liquid nutrient media (NSM or NFSM). Fresh CISA and standard solutions were prepared for each calibration. Duplicate vials were used for samples and standard solutions.

Results

Growth of Methane-Oxidizing Cultures in Unsaturated Porous Media. Three different microbial attachment materials [glass beads, silicalite pellets, and diatomaceous earth pellets (DEP)] were tested for the ability to support stable cultures of nitrate-supplied and nitrogen-fixing methane oxidizers in batch tests [25% water saturation by volume (*20*)] and in column tests. Regardless of nitrogen source, glass beads and silicalite pellets were unable to support stable methane-oxidizing populations under the conditions tested. However, steady and active methanotrophic cultures were able to grow on DEP under both nitrate-supplied and nitrogen-fixing conditions. Therefore, DEP packing was used in the columns for all experiments in this study.

The operating conditions and the periods for biofilm development in the unsaturated columns are listed in Table 1. The time required for the microbial populations to reach steady-state conditions was defined as the time required for effluent CH_4, O_2, and CO_2 concentrations to stabilize. In general, longer periods of time were required for nitrogen-fixing cultures to develop in the unsaturated columns than for nitrate-supplied cultures under similar influent gas conditions (columns 4, 5a, and 5b). The influent oxygen partial pressures also affected the time to reach steady state. In the nitrate-supplied columns, a much longer development period was observed when influent oxygen was supplied at 20% O_2 (column 1a) than at 10% O_2 (columns 2, 5a, and 5b). Oxygen was observed to have similar effects in nitrogen-fixing columns (column 3a, 4% O_2; columns 4 and 6, 10% O_2). The column inoculated with OB3b (column 6) required a much longer time (45 days) to stabilize than a similar column inoculated with the mixed chemostat culture (column 4, 17 days).

Both nitrate-supplied and nitrogen-fixing cultures were able to develop stable populations in the columns supplied with CH_4 concentrations that were below the lower explosive limit in air (4% CH_4). During the first 5 days after inoculation of a nitrate-supplied column with the mixed chemostat culture, effluent methane and oxygen concentrations declined rapidly while the production of carbon dioxide increased. Then, the effluent methane, oxygen, and carbon dioxide concentrations leveled off for 10 days. From the 15th day until the 21st day when nutrient media flushing was applied, methane and oxygen consumption slowly decreased. After nutrient media flushing, effluent gas concentrations reverted to previous levels and the cycle repeated. The decreased consumption of methane and oxygen prior to nutrient flushing events may be due to the accumulation of cell wastes, the buildup of excess biomass, or the depletion of nutrients. Similar patterns were also observed for nitrogen-fixing columns.

An interesting observation was that the column that was initially operated under nitrate-supplied conditions (column 1a, Table 1) was able to fix molecular nitrogen when the supply of nitrate was discontinued (column 1b, Table 1); however, the culture required 60 days to re-attain steady state. This may have been partly due to the decrease in influent CH_4 gas that was implemented concurrent with the elimination of nitrate.

TCE Degradation Tests. Experiments were conducted to examine the potential abiotic losses of chlorinated solvents in both uninoculated and inoculated columns. Continuous TCE vapor (0.09 mg/L) was injected into a column packed with wetted DEP to test for abiotic losses of chlorinated solvents in uninoculated columns. Effluent TCE concentrations rapidly merged with influent TCE concentrations, demonstrating no significant abiotic uptake of TCE by the columns. Similarly, PCE vapor (0.062 mg/L) was injected into a column with a stable population of nitrogen-fixing OB3b. PCE was chosen for this test because it is not degraded by methanotrophs. No significant loss of PCE was observed in the column.

Once stable populations of methane oxidizers were established in the columns, short-term TCE degradation tests were performed (Table 2). Tests A and B were conducted in nitrate-supplied and nitrogen-fixing columns (with 10% methane influent) receiving influent TCE gas concentrations of 0.7 mg/L for 10 and 8 days, respectively. Effluent TCE concentrations were close to zero throughout the experiments. These experiments were terminated when decreased methane and oxygen uptakes (60% less than before TCE exposure) signaled the need for a recovery period. Although the methane uptake activity of both columns decreased during TCE degradation, the activity resumed original levels following nutrient flushing and 2 days of recovery in the absence of TCE.

For columns operated with lower methane influent (4%), both nitrate-supplied and nitrogen-fixing cultures were capable of degrading TCE at gaseous concentrations ranging from 0.1 to 0.4 mg/L (Table 2, tests C−G; Figure 2). However, a column containing the nitrate-supplied mixed culture (test C) was not able to recover after degrading 0.13 mg/L TCE for

TABLE 2. Summary of Short-Term Degradation Tests of TCE, cDCE, and TCE + cDCE in Unsaturated Biofilm Column Reactors

test label	column label from Table 1	culture	N source	influent gas	av gaseous solvent inf (mg/L)	solvent removal[a] (%)	solvent exposure time [breakthrough[b]] (day)	Cl^- ion recovery[c] (%)	recovery period[d] (day)
A	2	mixed	NO_3^-	10% CH_4 10% O_2	0.7 (TCE)	100 (TCE)	10 [−]	NM[e]	2
B	3	mixed	N_2	10% CH_4 10% O_2	0.7 (TCE)	100 (TCE)	8 [−]	NM	2
C	5	mixed	NO_3^-	4% CH_4 10% O_2	0.13 (TCE)	92 (TCE)	7 [5]	103	no recovery after 36 days
D	1c	mixed	NO_3^- [f]	4% CH_4 10% O_2	0.17 (TCE)	60 (TCE)	5 [1]	103	30
E1	3c	mixed	N_2	4% CH_4 10% O_2	0.40 (TCE)	89 (TCE)	3 [2]	NM	24
E2	3c	mixed	N_2	4% CH_4 10% O_2	0.20 (TCE)	99 (TCE)	6 [5]	NM	14
E3	3c	mixed	N_2	4% CH_4 10% O_2	0.12 (TCE)	100 (TCE)	9 [−]	NM	10
F1	1b	mixed	N_2	4% CH_4 10% O_2	0.37 (TCE)	89 (TCE)	2 [1]	NM	22
F2	1b	mixed	N_2	4% CH_4 10% O_2	0.21 (TCE)	90 (TCE)	6 [3]	NM	12
F3	1b	mixed	N_2	4% CH_4 10% O_2	0.11 (TCE)	100 (TCE)	9 [−]	NM	11
G	6	OB3b	N_2	4% CH_4 10% O_2	0.13 (TCE)	96 (TCE)	10 [−]	97	27
J	5a	mixed	NO_3^-	4% CH_4 10% O_2	0.83 (cDCE)	90 (cDCE)	5 [4]	85	no recovery after 64 days
K1	4	mixed	N_2	4% CH_4 10% O_2	0.73 (cDCE)	95 (cDCE)	10 [9]	88	22
K2	3c	mixed	N_2	4% CH_4 10% O_2	0.92 (cDCE)	97 (cDCE)	6 [5]	90	25
L	6	OB3b	N_2	4% CH_4 10% O_2	0.98 (cDCE)	98 (cDCE)	7 [6]	91	18
M	4	mixed	N_2	4% CH_4 10% O_2	0.14 (cDCE) 0.11 (TCE)	89 (cDCE) 81 (TCE)	4 [2] [3]	90	44

[a] Percent removal for each experiment was calculated as (mass of solvent removed during the experiment/total mass of solvent loaded) × 100%. [b] Time prior to effluent breakthrough of ≥10% influent solvent concentration. [−] indicates that there was no breakthough during the exposure time. [c] Recovery of chloride ions was based on 3 mol of chloride ions produced/mol of TCE removed or 2 mol of chloride ions produced/mol of cDCE removed. [d] Recovery period is defined as the time required for cultures to resume their original levels of CH_4 uptake and CO_2 production following nutrient flushing. No solvent was added during the recovery period. [e] NM, not measured. [f] NO_3^--supplied column was flushed with nitrogen-free nutrient medium following solvent exposure.

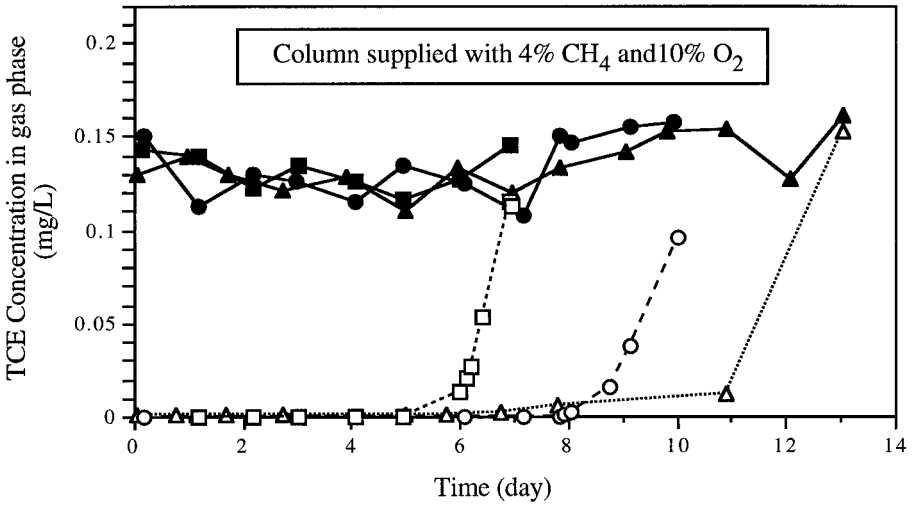

FIGURE 2. Short-term TCE degradation tests in nitrogen-fixing (N_2) and nitrate-supplied (NO_3^-) columns (tests C, E3, and G in Table 2). (■) NO_3^-, mixed (Inf); (▲) N_2, mixed (Inf); (●) N_2, OB3b (Inf); (□) NO_3^-, mixed (Eff); (△) N_2, mixed (Eff); (○) N_2, OB3b (Eff).

7 days (Figure 3a). As TCE exposure time increased, methane and oxygen uptakes declined rapidly, and carbon dioxide production diminished until all detectable biological activity in the column ceased. Following nutrient flushing, no methane uptake or CO_2 production was detected in the column even after 36 days of recovery. Interestingly, a similar nitrate-supplied column was able to recover from 5 days of comparable TCE loading (0.17 mg/L) after a 30-day recovery

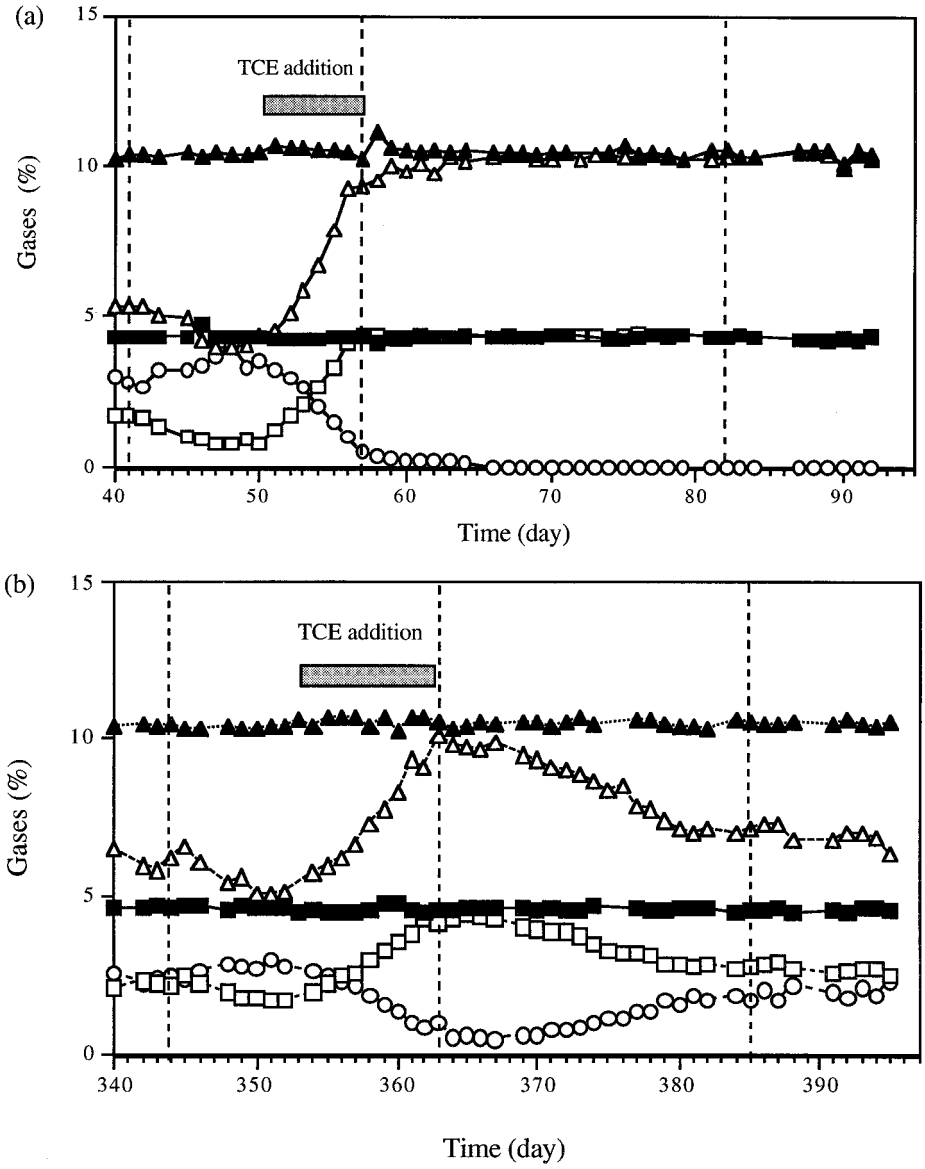

FIGURE 3. Changes in daily gases before and after short-term TCE degradation tests in (a) nitrate-supplied column (test C in Table 2) and (b) nitrogen-fixing OB3b column (test G in Table 2). (■) CH_4 (Inf); (▲) O_2 (Inf); (□) CH_4 (Eff); (△) O_2 (Eff); (○) CO_2 (Eff).

period when the column was flushed with a nitrogen-free liquid nutrient medium following solvent exposure (test D). An additional nitrate-supplied column operating under identical conditions was unable to recover within 90 days after exposure to TCE influent that fluctuated between 0.1 and 0.4 mg/L for 13 days with a 24-h spike to 0.8 mg/L on day 10 (data not shown).

In contrast to the nitrate-supplied columns, the columns containing nitrogen-fixing cultures were able to recover methane uptake activity within 10−27 days after degrading TCE at similar concentrations for 9−10 days (tests E3, F3, and G) and recovered in 12−24 days after exposure to higher concentrations of TCE (0.2−0.4 mg/L) for 2−6 days (tests E1, E2, F1, and F2). The longest recovery period for a nitrogen-fixing column (27 days, test G) was required for the column inoculated with the OB3b cells (Figure 3b). Mass balances based on chloride ions collected in flushing liquid confirmed the complete biodegradation of removed chlorinated solvents in tests C, D, and G.

Because of the poor recovery record of nitrate-supplied columns receiving low methane influent (4% CH_4) during the short-term TCE degradation tests, no long-term TCE degradation tests were conducted with nitrate-supplied columns. Also, no long-term TCE degradation tests were conducted in columns supplied with high influent methane (10% CH_4) due to concerns over potential explosive hazards. In long-term TCE degradation tests, nitrogen-fixing columns (with 4% CH_4) were repeatedly supplied with TCE vapors of 0.03−0.14 mg/L for 9−14 days and then allowed to recover for 7−9 days (Figure 4a; Table 3). In each case, columns achieved 88−100% TCE removal throughout the tests. The column that was converted from nitrate-supplied to nitrogen-fixing conditions (column 1b) showed similar degradation patterns to the column originally grown with nitrogen-fixing cultures (column 3c) in both short-term (tests F1−F3 and E1−E3 in Table 2) and long-term TCE degradation tests (tests H1−2 and I1−2 in Table 3). Figure 4b shows the repeated column recovery following TCE exposure as measured by daily gas compositions during long-term TCE tests in this column (Test H1).

cDCE Degradation Tests. Short-term cDCE degradation experiments were also performed with nitrogen-fixing and nitrate-supplied columns (Table 2). Columns inoculated with either the mixed methane-oxidizing culture or pure OB3b

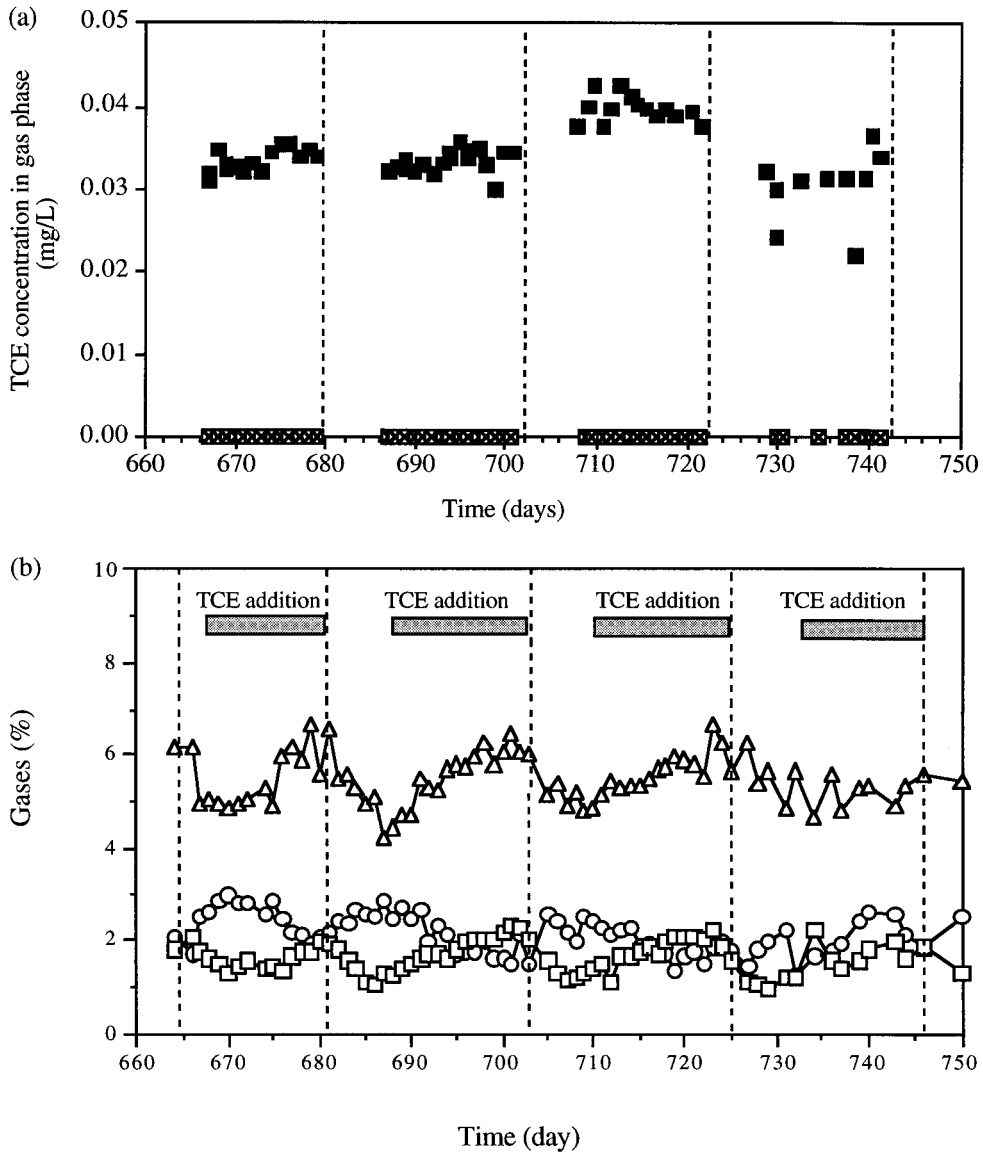

FIGURE 4. (a) TCE concentrations and (b) daily gas compositions during a long-term TCE degradation test with a nitrogen-fixing column inoculated with the mixed culture receiving 4% CH_4 and 10% O_2 (test H1 in Table 3). This column was converted from nitrate-supplied to nitrogen-fixing conditions on day 525. Dashed lines indicate when the column was flushed with medium and allowed to recover for 7−8 days. (■) TCE (Inf); (× inside a box) TCE (Eff); (□) CH_4 (Eff); (△) O_2 (Eff); (○) CO_2 (Eff).

cells were able to degrade cDCE at gaseous concentrations of 0.7−1 mg/L. The nitrate-supplied column had an earlier breakthrough of cDCE than either of the nitrogen-fixing columns (Figure 5), and the nitrogen-fixing column inoculated with pure OB3b cells exhibited the most prolonged cDCE degradation prior to breakthrough at the high cDCE concentration. The nitrate-supplied column was completely inactivated following cDCE breakthrough and was not able to recover methane-oxidizing activity even after exposure to methane for 64 days (test J in Table 2), while both of the nitrogen-fixing columns recovered completely within 18−25 days (tests K1−2 and L in Table 2).

TCE + cDCE Degradation Test. An additional experiment was conducted to examine if a mixture of TCE and cDCE (0.1 and 0.13 mg/L, respectively) could be effectively removed in a nitrogen-fixing column (Table 2). Both TCE and cDCE were completely degraded during the first 2 days, after which a partial breakthrough of cDCE (to 0.05 mg/L) and a complete breakthrough of TCE (to 0.1 mg/L) occurred. Although the culture was severely inactivated and exhibited diminished methane and oxygen uptake following exposure to the mixture of chlorinated solvents, the culture recovered full methane-oxidizing activity after nutrient media flushing and exposure to methane and oxygen for 44 days.

Operating Curves. Data collected from the both long- and short-term degradation tests were used to develop a family of operating curves that plotted chlorinated solvent removal (%) against the operational transformation yield, defined as the mass of chlorinated solvent loaded per mass of methane consumed within the columns (Figures 6 and 7). The total solvent loading was calculated by multiplying the solvent flow rate (mass of solvent per time) by the operation time. The total mass of methane consumed for each degradation test was calculated from the summation of daily methane consumption (the difference between influent and effluent daily methane concentrations) immediately following the previous liquid medium flushing to the last day of chlorinated solvent degradation. Points A−C in Figures 6 and 7 indicate the maximum values of operational TCE or cDCE transformation yields that can be applied while still maintaining >99% solvent removal during intermittent operation. Points marked as D−F, at the *x*-intercept of the operating curves in Figures 6 and 7, reflect the maximum theoretical solvent transformation yields of the columns and,

TABLE 3. Summary of Long-Term Degradation Tests of TCE in Unsaturated Biofilm Column Reactors

test label	column label from Table 1	culture	N source	influent gas	av gaseous TCE inf (mg/L)	repetitive additions of TCE	TCE removal[a] (%)	TCE exposure time[b] [breakthrough[c]] (day)	Cl^- ion recovery[d] (%)
H1	1b	mixed	N_2	4% CH_4 10% O_2	0.03−0.04	4 times	100	13−14 days of TCE addition followed by 7−8 days of recovery [−, −, −, −]	95−105
H2	1b	mixed	N_2	4% CH_4 10% O_2	0.11−0.14	2 times	88−100	9−13 days of TCE addition followed by 8−9 days of recovery [10, −]	95−105
I1	3c	mixed	N_2	4% CH_4 10% O_2	0.03−0.04	4 times	100	13−14 days of TCE addition followed by 7−8 days of recovery [−, −, −, −]	95−105
I2	3c	mixed	N_2	4% CH_4 10% O_2	0.11−0.14	4 times	88−100	9−14 days of TCE addition followed by 8−9 days of recovery [6, 8, −, −]	95−105

[a] Percent removal for each experiment was calculated as (mass of TCE removed during the experiment/total mass of TCE loaded) × 100%. [b] Recovery period was defined as the time required for cultures to resume their original levels of CH_4 uptake and CO_2 production. No TCE was added during the recovery period. TCE was repeatedly degraded after recovery periods. [c] Time prior to effluent breakthrough of ≥10% influent TCE concentration. [−] indicates that there was no breakthough during the exposure time. [d] Recovery of chloride ions was based on 3 mol of chloride ions produced/mol of TCE removed.

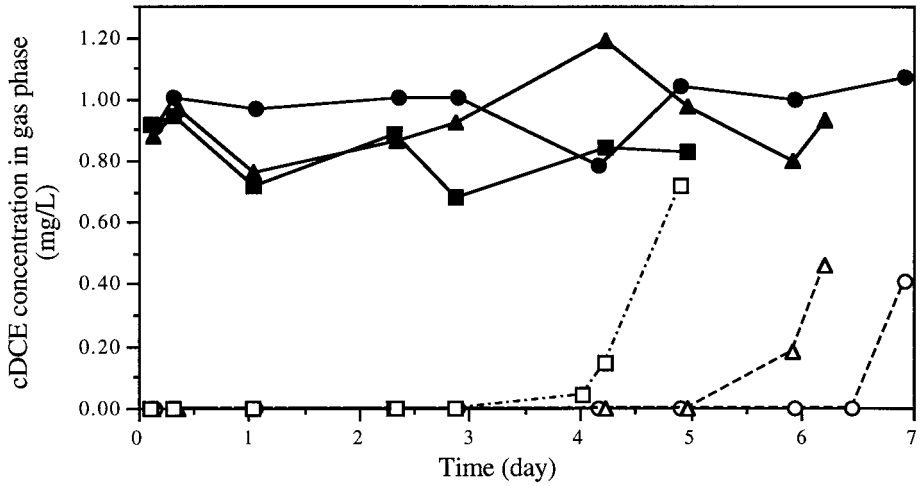

FIGURE 5. Short-term cDCE degradation tests in columns with nitrate-supplied and nitrogen-fixing mixed or OB3b cultures fed 4% CH_4 and 10% O_2 (tests J, K2, and L in Table 2). (■) NO_3^-, mixed (Inf); (▲) N_2, mixed (Inf); (●) N_2, OB3b (Inf); (□) NO_3^-, mixed (Eff); (△) N_2, mixed (Eff); (○) N_2, OB3b (Eff).

hence, the solvent loadings at which the microbial activity within the columns would be expected to cease entirely. Significantly higher operational TCE transformation yields were observed for nitrogen-fixing cultures than for the nitrate-supplied mixed cultures (Figure 6). A similar trend but with smaller resolution between column performance is shown for cDCE in Figure 7. The operational transformation yields for cDCE were significantly higher than those for TCE in all cases. Nitrogen-fixing columns inoculated with the mixed culture outperformed OB3b inoculated columns for TCE removal but not for cDCE removal.

Discussion

Although a number of studies have focused on utilizing saturated, liquid-phase biofilm reactors for cometabolic oxidation of chlorinated aliphatics (*21*−*25*), little work has been conducted using gas-phase bioreactors (*26*−*28*). Wilson et al. (*28*) used a gas-phase bioreactor for the cometabolic degradation of TCE and 1,1,1-trichloroethane (TCA) by *n*-butane utilizing bacteria in an airstream. In their study, the mixed cultures cometabolically degraded 90 μg/L TCE and 200 μg/L TCA with 80−90% removals. Removals decreased to 20% for TCE and 10−15% for TCA as the concentrations were increased to 770 μg/L TCE and 990 μg/L TCA. Speitel and McLay (*27*) reported 20−80% removal of 300−720 μg/L TCE and 800−1000 μg/L 1,2-dichloroethane in columns inoculated with methanotrophic bacteria and showed that chlorinated solvent removal decreased over time (the duration of each experiment was less than 25 days). Ensley and Kurisko (*26*) achieved 90−95% removal of 300−4000 μg/L TCE in gas-lift reactors containing aromatic degrading pure cultures that operated for up to 2 weeks. Each of these studies reported problems in sustaining long-term chlorinated solvent degradation. Decreased reactor performance was attributed to product toxicity from chlorinated solvent degradation and/or competitive inhibition between primary and cometabolic substrates. Product toxicity caused by the aerobic degradation of chlorinated solvents has been observed to result in cell damage and decreased degradation activity with a wide range of cultures, including both alkane and aromatic oxidizers (*11*−*16, 19, 29*−*31*).

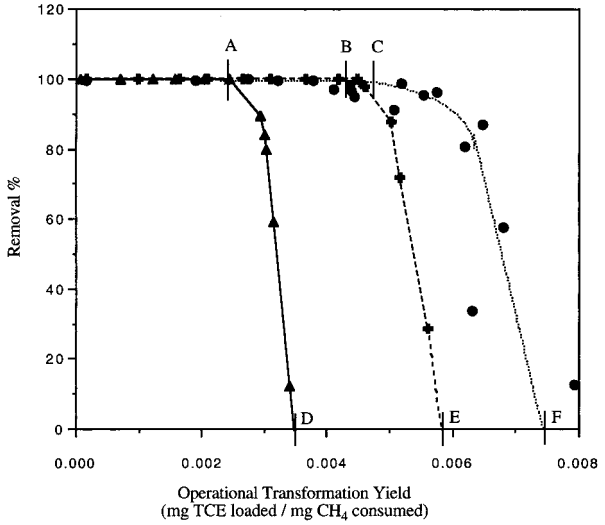

FIGURE 6. Percent TCE removal plotted against operational transformation yields in unsaturated columns. Points A−C represent the maximum transformation yields for >99% TCE degradation with values of 0.0024, 0.0044, and 0.0048 mg of TCE loaded/mg of CH_4 consumed, respectively. Points D−F show the maximum theoretical transformation yields for each of operating conditions with values of 0.0034, 0.0058, and 0.0074 mg of TCE loaded/mg of CH_4 consumed, respectively. (▲) NO_3^-, mixed; (●) N_2, mixed; (solid cross) N_2, OB3b.

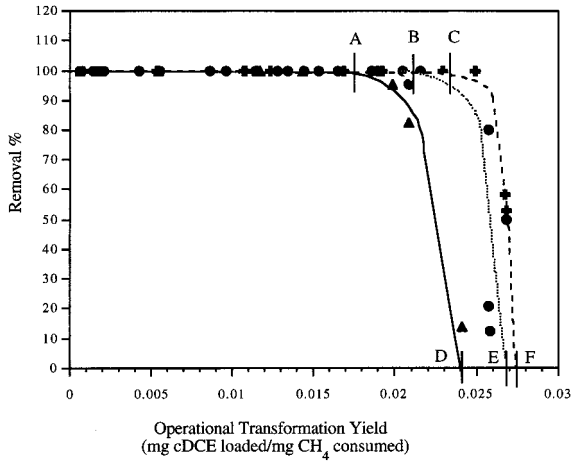

FIGURE 7. Percent cDCE removal plotted against operational transformation yields in unsaturated columns. Points A−C represent the maximum transformation yields for >99% cDCE degradation with values of 0.0175, 0.0211, and 0.0233 mg of cDCE loaded/mg of CH_4 consumed, respectively. Points D−F show the maximum theoretical transformation yields for each of operating conditions with values of 0.024, 0.027, and 0.0275 mg of cDCE loaded/mg of CH_4 consumed, respectively. (▲) NO_3^-, mixed; (●) N_2, mixed; (solid cross) N_2, OB3b.

This study demonstrates the feasibility of growing nitrogen-fixing methanotrophs for sustained cometabolic degradation of chlorinated solvents in intermittently operated unsaturated columns. The time required to achieve a pseudo-steady-state of microbial populations in the unsaturated columns was found to be affected by the influent methane concentrations, the supplied nitrogen source, the influent oxygen concentrations, and the type of microbial inocula. For example, when columns were supplied with 4% CH_4 rather than 10%, a longer time was required for nitrogen-fixing cultures than that for nitrate-supplied cells to develop. These observations are consistent with our previous reports for mixed and pure methane-oxidizing cultures grown in liquid media (*18, 19*). It was not surprising that a much longer development time was observed for the column inoculated with the pure OB3b cells since a lower inoculating concentration was used and this culture does not grow well on solid surfaces (*32*). Furthermore, high initial oxygen partial pressures slowed biofilm development of both nitrate-supplied and nitrogen-fixing columns, suggesting that low oxygen pressures are favored to promote methanotrophic biofilm development. This suggestion is logical since sMMO enzymes are known to be oxygen-sensitive and a number of studies have reported toxic effects due to aeration with methane-oxidizing cultures (*11, 13, 33−35*).

The supplied nitrogen source had an observable effect not only on biofilm development but also on chlorinated solvent degradation in the unsaturated columns. Nitrate-supplied columns were not able to recover from TCE or cDCE product toxicity as effectively as nitrogen-fixing columns, suggesting that higher resistance to chlorinated solvent product toxicity was obtained when cells fixed molecular nitrogen. In addition, the consistent TCE degradation demonstrated by the nitrogen-fixing columns during the long-term experiments suggests the potential stability of such systems for long-term solvent treatment. The superior performance of the nitrogen-fixing columns was consistent with results of our previous studies with mixed and pure cultures (*18, 19*). These results suggest that strategies for enhancing in-situ methanotrophic bioremediation of chlorinated solvents may be more successful if fixed-nitrogen sources are not provided.

Since many solvent wastes contain a mixture of contaminants at low concentrations, it is not practical to overlook the effects of solvent mixtures on degradation. In this study, a shorter operating period and longer recovery period were required for treatment of TCE and cDCE mixtures than for treatment of either solvent alone, suggesting that the toxic effect of the mixture is greater than the cumulative effects of the individual components. These results are in contrast to a previous study conducted with suspended cultures of methanotrophs that found the toxic effects of solvent mixtures to be cumulative and predictable from knowledge of mixture components (*36*). The reason for the diminished column performance in the presence of this binary mixture is uncertain. Clearly, more research on the treatment of chlorinated solvent mixtures is required in order to interpret these discrepancies.

Although methane is a competitive inhibitor during the cometabolic degradation of chlorinated solvents by methanotrophs, significant TCE and cDCE removals were observed in both nitrate-supplied and nitrogen-fixing columns even though they were continuously supplied with methane. In fact, TCE removal in columns supplied with 10% methane was generally greater than in columns supplied with 4% methane. This suggests that the amount of methane provided under the tested conditions was not sufficient to cause significant inhibition during chlorinated solvent degradation. Furthermore, the continuous supply of low methane concentrations during chlorinated solvent treatment might actually enhance degradation in the system by providing the reducing energy to fuel the cometabolic reaction (*37*).

It is likely that the active biomass concentration within the columns was not uniform and varied spatially along the column length. Since the measurement of active biomass in such a system may be subject to significant uncertainty, a methane consumption-based parameter, defined here as the operational transformation yield (mass of chlorinated solvent loaded per mass of methane consumed), rather than a biomass-based parameter, such as the transformation capacity (T_c, mass of chlorinated solvent degraded per mass of cells inactivated) (*17*), was adapted to quantify column

performance in this study. Similar to T_c, the value of the operational transformation yield would be expected to be a function of chlorinated solvent product toxicity. Plots of the operational transformation yield against solvent removal are useful for comparing column performance and for estimating the range of solvent loadings that can be successfully treated within these methanotrophic columns. To maintain sustained chlorinated solvent removal in columns such as this, they should be operated at solvent loadings that result in operational transformation yields below those measured for declining chlorinated solvent removals, and sufficient microbial recovery periods during which the solvent loadings are eliminated must be applied.

In fact, the concept of operational transformation yield is equivalent to the previously introduced calculation of the minimum substrate concentration required per mass of chlorinated solvent degraded for sustained microbial growth during chlorinated solvent degradation (*37*−*39*). As shown in Figures 6 and 7, the operational transformation yields for >99% cDCE removal are 5−7-fold higher than those for TCE, suggesting that cDCE exerts less product toxicity than TCE. This is consistent with previous observations that cDCE transformation capacities for suspended cultures are significantly higher than TCE transformation capacities (*40*, *41*).

When chlorinated solvent removal approaches zero (*x*-intercepts of curves in Figures 6 and 7), the resultant operational transformation yields represent the theoretical maximum and can be related to the transformation yield (T_y), a value that characterizes the maximum amount of chlorinated solvent that can be degraded prior to complete inactivation of the biomass per mass of methane consumed to grow the biomass. In this study, the maximum transformation yields for TCE and cDCE estimated from the operational curves (points D−F in Figures 6 and 7) were much lower (2−to 20-fold lower for TCE and 2−15-fold lower for cDCE) than those reported for suspended cultures (*11*, *15*, *36*, *40*−*42*) and for saturated biofilm systems (*23*, *24*, *43*). The lower values of operational transformation yields for TCE and cDCE observed in this study might be due to pH changes along the length of the columns, accumulation of waste products within the columns, oxygen limitation within the biofilms, or some unknown factors that resulted in diminished degradation capabilities in unsaturated porous media. Some of these adverse effects may be minimized by more frequent liquid flushing of the unsaturated columns.

In this study, we tested the feasibility of using unsaturated porous media columns containing methanotrophs for the treatment of chlorinated solvents. We found that nitrogen-fixing columns outperformed nitrate-supplied columns and that inoculation with a mixed community was more successful than inoculation with a pure culture for TCE degradation but not for cDCE degradation. Sustained chlorinated solvent removal was possible when columns were operated with continuous gaseous substrates (methane and oxygen) and intermittent chlorinated solvent loadings alternating with recovery periods. It should be emphasized that recovery periods that involved a brief nutrient media flush followed by a period of gaseous substrate supply in the absence of chlorinated solvent load was essential for sustained chlorinated solvent removal in these systems. These results are valuable for enhancing subsurface bioremediation of chlorinated solvents, for improving the design of bioreactors for the treatment of SVE and air-stripping off-gases, and for increasing our understanding of the influence of nitrogen sources on microbial degradation processes.

Acknowledgments

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