APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1991, p. 228–235
0099-2240/91/010228-08$02.00/0

Vol. 57, No. 1

Effects of Toxicity, Aeration, and Reductant Supply on Trichloroethylene Transformation by a Mixed Methanotrophic Culture

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Received 7 May 1990/Accepted 10 September 1990

The trichloroethylene (TCE) transformation rate and capacity of a mixed methanotrophic culture at room temperature were measured to determine the effects of time without methane (resting), use of an alternative energy source (formate), aeration, and toxicity of TCE and its transformation products. The initial specific TCE transformation rate of resting cells was 0.6 mg of TCE per mg of cells per day, and they had a finite TCE transformation capacity of 0.036 mg of TCE per mg of cells. Formate addition resulted in increased initial specific TCE transformation rates (2.1 mg/mg of cells per day) and elevated transformation capacity (0.073 mg of TCE per mg of cells). Significant declines in methane conversion rates following exposure to TCE were observed for both resting and formate-fed cells, suggesting toxic effects caused by TCE or its transformation products. TCE transformation and methane consumption rates of resting cells decreased with time much more rapidly when cells were shaken and aerated than when they remained dormant, suggesting that the transformation ability of methanotrophs is best preserved by storage under anoxic conditions.

Trichloroethylene (TCE) is one of the most frequently detected organic contaminants found in water supplies from groundwater sources (35), is toxic to humans at high exposures, and is a known carcinogen in mice and a suspected carcinogen in humans (6). Under anaerobic conditions, TCE can be biologically reduced to form less halogenated organics (2, 24). However, vinyl chloride, a known human carcinogen, is a common intermediate of the reaction (24, 31). Under aerobic conditions, Wilson and Wilson (36) observed TCE transformation to CO_2 in a soil column that had been exposed to natural gas (74% methane) and concluded that methanotrophic microorganisms were responsible for the TCE transformation. Methanotrophic TCE oxidation has since been confirmed in a number of studies with pure cultures (20, 23, 30). Propane oxidizers (32), ammonia oxidizers (1), and toluene oxidizers (22) have also been reported to carry out TCE oxidation.

Methanotrophic cultures have the potential for high TCE degradation rates and complete transformation to CO_2 and chloride, without the formation of undesirable intermediates (20, 23, 30), after growth on inexpensive substrate (methane). This, coupled with successful field-scale studies for in situ bioremediation of TCE-contaminated groundwater by methanotrophs (26), suggests that further research to better understand factors affecting transformation rates is desirable.

This particular study was conducted at ambient temperature with a mixed methanotrophic culture to explore the effects of time stored in the absence of methane, presence of an alternative energy source, TCE concentration, aeration, and possible toxic effects of TCE and its transformation products on the rate and extent of TCE transformation.

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THEORY

Methanotrophs derive both energy and carbon from the oxidation of methane by the broadly nonspecific enzyme methane monooxygenase (MMO) with NADH or NADPH as an intermediate energy source (5, 19) (Fig. 1).

MMO catalyzes a wide range of oxidative reactions, including the hydroxylation of alkanes, epoxidation of alkenes, and oxidation of ethers, halogenated methanes, and cyclic and aromatic compounds (4, 25). The reaction of interest in this work is the cometabolic or fortuitous epoxidation of TCE, which is catalyzed by MMO in the presence of molecular oxygen and NADH, and the subsequent degradation, which is postulated to occur by hydrolysis and heterotrophic oxidation (20).

Since MMO is responsible for both methane oxidation and TCE epoxidation, methane and TCE are considered to be competitive substrates such that in the presence of both compounds TCE transformation rates are reduced. However, this problem can be avoided by the use of resting methanotrophs (in the absence of methane) which are capable of oxidizing a broad range of organic compounds (3, 15, 28) including TCE (12a, 14, 23, 29, 30).

Methane was used as the sole source of cell carbon and energy for growth of the mixed culture in this study. TCE transformation was then studied with either resting cells alone, that is, in the absence of methane, or in the presence of formate, which can serve as an alternative source of NADH (Fig. 1) but is not used for growth.

The specific transformation rate of a compound is often related to its concentration in solution according to the following modification of Monod kinetics: $-[(dS/dt)/X] = [kS/(K_s + S)]$ where k is the maximum specific rate of transformation (mg of TCE/mg of cells/day), K_s is the half velocity or affinity constant (mg/liter), S is the solution concentration of the transformed compound (mg/liter), and X is the microbial concentration (mg/liter). For TCE transformation by batch resting methanotrophic cultures, X can

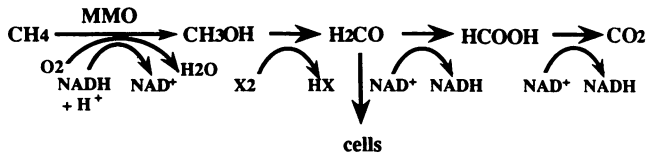

FIG. 1. Oxidation of methane by MMO with NADH or NADPH as energy source (after references 5 and 19).

be considered constant over the course of the transformation; thus, the transformation rate would be a direct function of the TCE concentration.

MATERIALS AND METHODS

Mixed-culture development. A 7.5-liter mixed culture was seeded with effluent from a laboratory column of aquifer material containing an active microbial culture (18a) and was grown in a 10-liter, baffled, continuous-gas-feed chemostat operated with a 9-day detention time, with once-daily cell wasting and liquid medium replacement. The mixed-culture medium contained mineral salts described by Fogel et al. (7), dissolved in deionized water. A mixture of 10% methane in air was continually injected into the reactor bottom at a rate of 280 ml/min, and high-velocity mixing (200 rpm) was maintained to facilitate methane transfer. Steady-state gas concentrations in a submerged Gortex tube (0.6-cm diameter, 10-cm length) indicated methane and oxygen concentrations of 0.1 and 8.0%, respectively, which correspond to liquid concentrations of 0.02 mg of methane and 3.5 mg of oxygen per liter. This indicates that the cell growth was methane limited. The average cell density of 2,500 mg/liter (range, 1,800 to 3,000 mg/liter) and gas effluent of 8.6% methane gave a calculated net growth yield of 0.33 to 0.37 g of cells per g of CH_4 consumed. The mixed culture was salmon pink, with dominant cells being gram negative with diameters in the range of 0.1 to 1.0 μm.

Experimental procedures. (i) Solutions. For the stock TCE solution, approximately 10 ml of TCE (99+% pure ACS reagent; Aldrich Chemicals Co., Milwaukee, Wis.) was added to 160-ml glass bottle containing five glass beads and 120 ml of Milli-Q water and sealed with a Teflon-lined rubber septum and aluminum crimp-top cap. TCE-saturated solution was removed by syringe through the septum, using care to exclude nonaqueous phase TCE.

(ii) Transformation products. The distribution of TCE transformation products was determined by using [^{14}C]TCE and 9-ml glass vials containing three glass beads and sealed with two Teflon-lined septa and a screw cap with a sampling hole. Each vial was weighed, completely filled with medium, amended with a mixture of [^{14}C]TCE and unlabeled TCE by using a Pressure-lok gas-tight syringe, sealed with septa and cap, and reweighed. Some 0.8 ml of freshly harvested cells was injected through the septa with a Multi-fit syringe, while 0.8 ml of medium was simultaneously withdrawn with a second syringe. The vials were incubated in the dark at 20°C and mixed on a 12-rpm vertical bottle rotator. A vial was sacrificed for each analysis.

(iii) TCE transformation and methane consumption studies. Transformation experiments were performed in a 21°C environmental chamber, using 62-ml glass bottles sealed with either Mininert Teflon-lined caps or a set of two 50-mil Teflon-lined septa and inoculated with 20 ml of liquid (mixed-culture medium, cells, or a combination of both). For formate-fed bottles, 1 ml of mixed-culture medium was replaced with 1 ml of a 400 mM sodium formate–Milli-Q water solution to yield a final concentration of 20 mM formate. Corresponding resting cells received 1 ml of pure Milli-Q water. TCE-saturated solution was added by gas-tight syringe through the Mininert valves, and the bottles were vigorously shaken by hand for 10 s before initial headspace samples for TCE were taken. The bottles were then shaken at 200 to 400 rpm (unless otherwise noted) on a Lab-Line circular action shaker table. Since no increase in TCE utilization occurred with shaker speeds varied between 100 and 400 rpm, gas-liquid TCE mass transfer was not rate limiting under the experimental conditions used. Gas samples (200 μl) were withdrawn periodically with a 500-μl Precision-lok gas-tight syringe and 22-gauge side-port needle for TCE analysis. Specific TCE transformation rates reported were determined from the change in total TCE mass, including that in both the liquid and gas phases, divided by the total cell dry mass.

Methane consumption experiments were conducted similarly to the TCE transformation studies. A 3.0-ml portion of methane was injected through the septa with a 5-ml Multi-fit Luer-lok syringe and 26-gauge needle. Bottles which had previously undergone TCE studies were purged for 5 min with a nitrogen gas stream followed by the reintroduction of air into the headspace (hand pumped with a 150-ml syringe without disturbing liquid) prior to sealing and methane injection.

Analytical procedures. (i) Culture density. The culture dry-mass density (milligrams per liter) was determined gravimetrically by adding a specific volume of suspended culture to tared 5.1-cm aluminum foil dishes, evaporating overnight at 105°C, cooling, and comparing weight change with that of medium controls.

(ii) ^{14}C analysis. The uniformly labeled [^{14}C]TCE (4.1 mCi/mmol; Sigma Chemical Co., St. Louis, Mo.) was maintained in aqueous solution in flame-sealed glass ampoules at 4°C. A Tri-Carb 4530 scintillation spectrometer was used for ^{14}C assay, and the specific activity of the labeled compound was determined for each experiment from the ratio of disintegrations per minute to gas chromatograph-measured TCE in equivalent volumes of the sample.

In transformation product studies, the 9-ml vials were centrifuged and the supernatant was filtered (GF/F glass-fiber filters; Whatman International Ltd., Maidstone, England) prior to ^{14}C analysis. Total ^{14}C was determined in 1 ml of filtrate added to a 20-ml glass scintillation vial containing 10 ml of ACS scintillation cocktail (Amersham Co., Arlington Heights, Ill.) amended with 4 drops of 1 N NaOH. The nonpurgeable acid and base fractions were obtained by adding 1 ml of filtrate to 4 drops of 1 N HCl or 1 N NaOH, respectively, stripping with N_2 at 200 ml/min for 10 min, adding 10 ml of scintillation cocktail, and counting. The nonpurgeable acid fraction represented the nonvolatile organics, while the nonpurgeable base fraction included nonvolatile organics plus CO_2. The total minus nonpurgeable base counts gave the volatile fraction (TCE), and the nonpurgeable base minus acid counts gave the CO_2 fraction.

(iii) TCE analysis. TCE concentration was determined from headspace analysis; 200 μl of headspace gas was withdrawn with a Precision-lok gas-tight syringe and analyzed with a Tracor MT-220 gas chromatograph equipped with a 70°C packed column (10% squalene on Chromosorb A/AW), a linearized electron capture detector, and an argon-methane mixture for carrier gas (140 ml/min) and makeup gas (70 ml/min).

Calibration standards were prepared by adding a specific

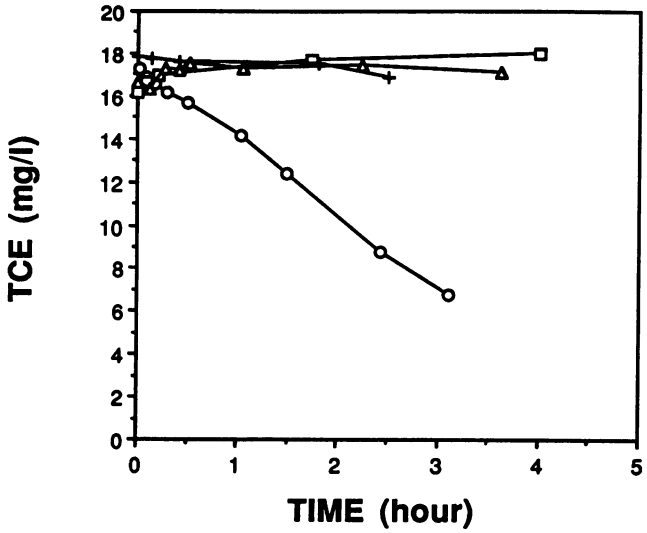

FIG. 2. Comparison of TCE disappearance in active cultures (cells only) with that in three different controls. Symbols: ○, cells only; □, media only; △, acetylene-treated cells; +, autoclaved cells.

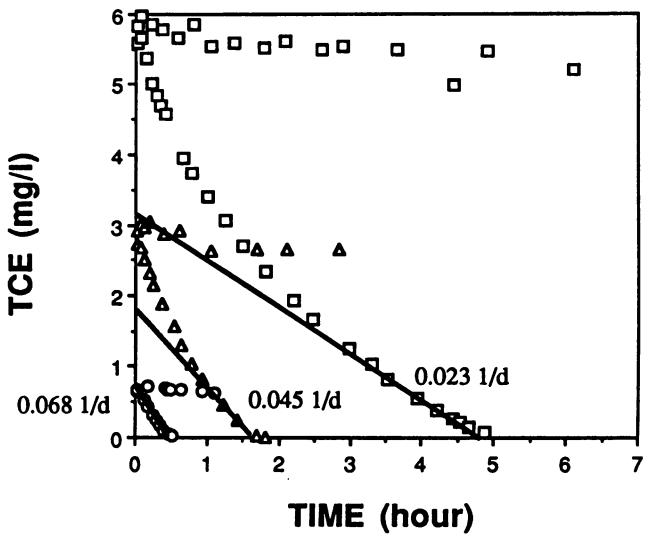

FIG. 3. TCE disappearance at three initial concentrations with controls (data points) and the linearized disappearance rate (lines), respectively, at a TCE solution concentration of 0.5 mg/liter.

mass of TCE to a known volume of methanol contained in a vial sealed with a Teflon-coated silicone septum. Standard was added to bottles containing mixed-culture medium, and bottles were shaken for at least 5 min prior to sampling. Daily calibration curves were prepared from standards at no fewer than three concentration levels within the range of interest, and sample concentration was determined by comparison with the standard curve. A dimensionless Henry's constant of 0.31 for TCE at 21°C (11) was used along with known liquid and gas volumes to compute TCE liquid concentrations or total TCE mass in the bottle.

(iv) Gas analysis. Methane and oxygen concentrations were determined with a 500-μl gas-tight Precision-lok syringe to inject a 200-μl sample of the headspace into a Fisher model 25V Gas Partitioner equipped with a thermal conductivity detector and helium carrier gas (60 ml/min). Certified gas standards were used for proportional calibration.

RESULTS

TCE sorption and controls. To assess whether TCE sorption onto cells would affect mass balances, approximately 17 mg of TCE per liter was added to each of four 62-ml bottles: the first contained 1,860 mg of microbial cells per liter in mixed-culture medium (cells only); the second, medium only; the third, cells autoclaved in medium at 120°C for 20 min; and the fourth, cells in medium with 10% acetylene gas (99.6% purity, A.A. grade; Liquid Carbonics) added to inhibit MMO activity (27). The aqueous TCE concentration with cells only decreased at a constant rate, while that in the three controls remained essentially the same whether or not cells were present, indicating that TCE sorption was insignificant under the experimental conditions used (Fig. 2). Inhibition of TCE transformation by acetylene supported the hypothesis that oxidation was MMO catalyzed. As in all experiments conducted with this culture, no adaptation time was required for the onset of TCE transformation, even though the organisms had experienced no prior TCE exposure.

Transformation products. TCE (15 mg/liter) was added to bottles containing 284 mg of cells per liter. After 21 h, 93% of the ^{14}C was detected in transformation products, 58% as nonvolatile compounds and 35% as CO_2; 5% of the ^{14}C was not recovered, perhaps because of incorporation into cells which was not measured. Additional studies (data not shown) indicated that at lower TCE concentration (1 mg/liter) most of the nonvolatile products were converted into CO_2, resulting in a total $^{14}CO_2$ recovery of >80% of the initial [^{14}C]TCE. Identification of intermediates was not attempted, but the transformation products may be similar to those believed to be formed as intermediates of mammalian TCE transformations including 2,2,2-trichloroacetaldehyde, dichloroacetic acid, glyoxylic acid, formic acid, and carbon monoxide (13, 21).

TCE transformation rate. TCE disappearance was monitored in bottles with 1,100 mg of resting cells per liter and three different initial TCE concentrations, 6, 3, and 0.6 mg/liter (Fig. 3). Based on initial rates of TCE disappearance, no inhibition by TCE was suggested at these levels. However, attempts to model TCE transformation by using Monod kinetics alone were unsuccessful as the rate of TCE disappearance should have been identical in the three bottles at equivalent TCE concentrations. That is, when the concentration of TCE within each bottle reached some chosen value, say 0.5 mg/liter, the specific transformation rates should have been the same; but, as shown in Fig. 3, it is apparent they were not. The transformation rates of 0.068, 0.048, and 0.026 mg of TCE per mg of cells per day, respectively, for the low, medium, and high initial concentration cases when TCE concentration was 0.5 mg/liter indicates that the transformation rate was affected by some other factor such as initial TCE concentration or time. This phenomenon was explored further.

The change in TCE transformation rate with time by resting cells was measured in a bottle which had repeated additions of TCE following its depletion (Fig. 4). The bottle contained 2,300 mg of cells per liter and was repeatedly amended with 550 μg of TCE, rendering a maximum aqueous phase TCE concentration of approximately 15 mg/liter. With each addition, the rate of TCE transformation decreased, and after the fourth addition it ceased, indicating

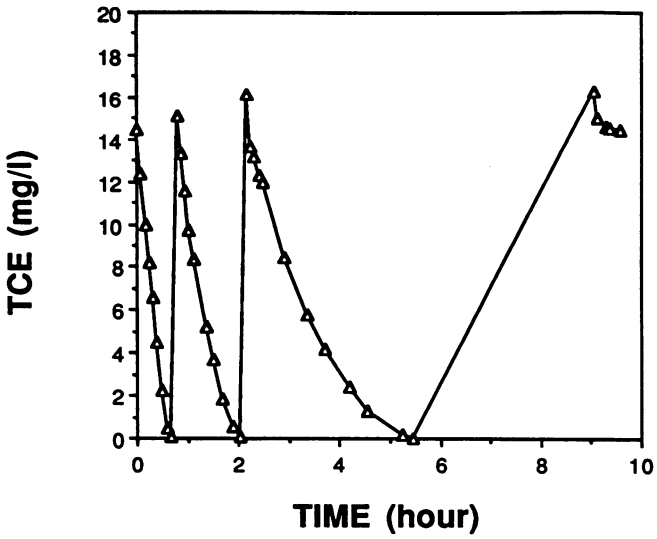

FIG. 4. Results of TCE reinjection to measure total transformation capacity of 46 mg of cells in the absence of methane (0.036 mg of TCE per mg of cells).

that in the absence of methane the resting microbial population has a finite TCE transformation capacity. The capacity from this experiment was 0.036 mg of TCE per mg (dry weight) of cells.

Factors affecting transformation capacity. A series of experiments was performed to explore further the cause for the diminution of transformation rate with time, that is, whether it was related to the quantity of TCE transformed, time in the absence of methane, or other factors. Transformation rates with "fresh" cells (when first harvested from the growth reactor) were compared with rates for harvested cells after sitting undisturbed for 25 h (unshaken), harvested cells that had been shaken at 400 rpm for 25 h in the presence of air,

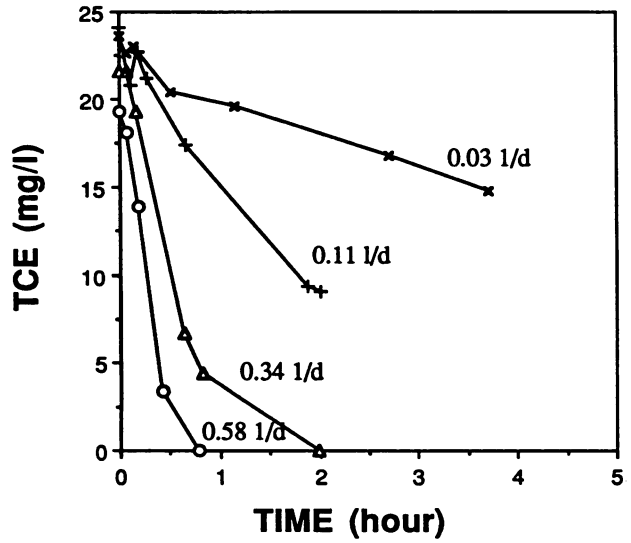

FIG. 5. TCE disappearance with fresh cells and those that had been resting for 24 h under various shaken and unshaken conditions. Initial linearized disappearance rates are also shown. Symbols: ○, fresh; △, unshaken; +, N_2 shaken; ×, air shaken.

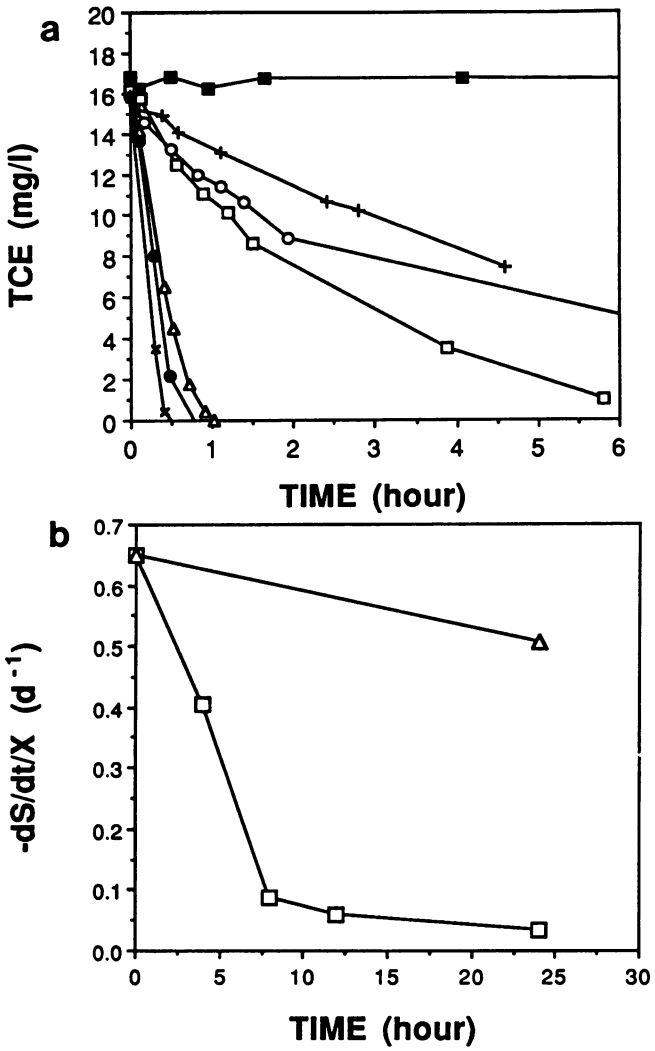

FIG. 6. (a) TCE disappearance by resting cells that had been unshaken for 24 h and those that had been shaken in the presence of air for different periods of time. Symbols: ×, fresh; △, 4 h; □, 8 h; ○, 12 h; +, 24 h; ●, unshaken; ■, control. (b) Change in initial specific TCE transformation rate by resting cultures both unshaken and shaken in air for various time periods. Symbols: □, 200 rpm; △, unshaken.

and harvested cells which had been shaken in a nitrogen atmosphere for 27 h. TCE disappearance with time for fresh cells was faster than for any of the cultures which had been resting for a day (Fig. 5). However, the unshaken cells retained their transformation ability better than cells shaken with N_2, which in turn bested the rate for those shaken in air. While there was air in the unshaken bottle, the oxygen demand by the high cell concentration probably resulted in rapid O_2 depletion in the solution surrounding the cells, perhaps accounting for the retention of transformation capacity noted here. Despite efforts to avoid it, the bottle shaken with nitrogen contained 0.1% oxygen in the gas phase. Thus, it is not clear whether the small amount of oxygen or the shaking per se was responsible for this decreased transformation rate. In any event, the presence of oxygen is of major importance in reducing the transformation rate by resting cells.

TABLE 1. Change in initial specific TCE transformation rate with time of resting cell exposure to various conditions

Experimental condition	Time between rate measurements (h)	Initial specific transformation rate (day^{-1})		$-\Delta$ Rate/Δt (day^{-2})
		First measurement	Second measurement	
Shaken with TCE[a]	0.73	0.835	0.390	14.60
	0.85	0.575	0.289	8.08
	0.92	0.650	0.153	13.00
	0.73	0.602	0.309	9.61
	0.50	1.060	0.420	30.60
Shaken with Air	4.0	0.649	0.406	1.46
	8.0	0.649	0.088	1.68
	12.0	0.649	0.059	1.18
	24.0	0.649	0.034	0.62
Shaken with N_2	27.1	0.575	0.105	0.42
	24.0	0.717	0.226	0.49
Unshaken	25.5	0.575	0.335	0.23
	25.7	0.717	0.433	0.27
	24.0	0.649	0.506	0.14

[a] Added TCE equals 16 to 22 mg/liter.

The decline in TCE disappearance with time (Fig. 6a) and in initial specific TCE transformation rate (Fig. 6b) by resting cells indicate that transformation rates fall off sharply as a function of prior time shaken in the presence of air.

Table 1 contains a summary of the calculated specific TCE transformation rates measured before and after different cell treatments, using results from various experiments. Also included is the rate of decline of the specific transformation rate over the time period between measurements. An average computed rate decline of 15.2 day^{-2} was caused by exposure to TCE. In comparison, the average rate decrease due to shaking with air alone was only 1.2 day^{-2}. Although the decline due to aeration was substantially lower than that due to exposure to TCE, both were significant compared with declines from shaking in the presence of N_2 (0.46 day^{-2}) or no shaking (0.21 day^{-2}).

As a means of examining whether the observed specific TCE transformation rate decline was the result of electron donor depletion, transformation studies were conducted which included the addition of sodium formate to augment the production of reduced NADH (Fig. 1). Comparisons were also made of culture methane consumption rates before and after given treatments to gauge possible toxic effects. The addition of 20 mM sodium formate caused not only an increased initial specific TCE transformation rate (2.1 day^{-1} with formate, 0.6 day^{-1} without) as reported by Oldenhuis et al. (23) and Brusseau et al., (2a), but also an increased transformation capacity (0.073 mg of TCE per mg of cells with formate, 0.036 mg of TCE per mg of cells without) (Fig. 7). While this implies that the supply of reduced NADH played a significant role in increasing both the TCE transformation rate and capacity, a rate decline with formate nevertheless still occurred. To test whether the rate decline resulted from exhaustion of formate, a similar experiment was conducted in which an additional 20 mM formate was added after the TCE transformation rate had markedly slowed (Fig. 8). The additional formate did not significantly stimulate additional TCE transformation. Oxygen in the headspace was found to be 14 to 18% subsequent to the TCE exposure, ensuring that its concentration was not limiting.

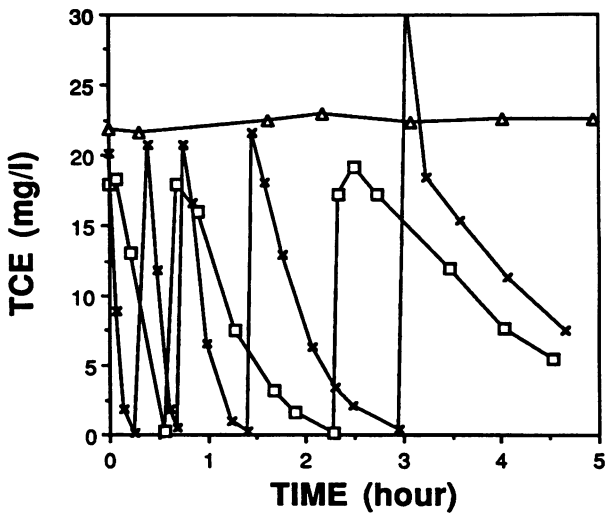

FIG. 7. Comparison of TCE disappearance for cultures with 20 mM formate addition and cultures without formate. Symbols: □, cells; ×, cells plus formate; △, control.

The possible toxic effects from TCE utilization or aeration or both were explored by comparing the rates of methane consumption by freshly harvested cells, cells after TCE utilization with and without formate addition, and cells which had been shaken in an air atmosphere for a similar period with and without formate addition. The decrease in methane consumption rates (computed from initial disappearance data) compared with controls (Table 2) implies that transformation of TCE resulted in a decrease in the cell's ability to consume methane, their primary growth substrate. Hence, TCE utilization appears to have irrevocably harmed the cells. The toxicity effect appears to have been greater, the greater the amount of TCE the cells had transformed. Cells shaken in air also exhibited a diminished methane

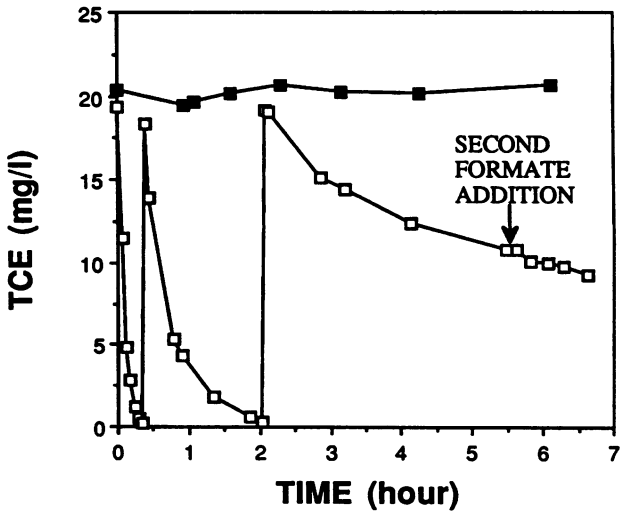

FIG. 8. TCE disappearance in culture with initial 20 mM formate addition followed by a second equivalent formate addition after 5.5 h. Symbols: □, cells plus formate; ■, control.

TABLE 2. Specific rate of methane consumption for fresh cells and after exposure to a range of experimental conditions

Experimental condition	Exposure time (h)	TCE transformed (μg)	Formate addition (mM)	Methane consumption (mg of CH_4/mg of cells per day)
Fresh cells	0	0	0	0.48
	0	0	0	0.54
Shaken with TCE	5.0	2,110	0	0.06
	4.5	1,700	0	0.17
	4.7	2,900	20	0.05
	6.6	1,600	20, 20[a]	0.04
Shaken with air	4.0	0	0	0.30
	4.5	0	0	0.31
	4.3	0	20	0.33
	6.5	0	20	0.32
Unshaken	4.0	0	0	0.45
	6.5	0	0	0.45
	7.0	0	20	0.42

[a] 20 mM formate was added before TCE exposure and an additional 20 mM was added after TCE exposure.

consumption rate regardless of formate addition, although the decrease was less than that found for the cells exposed to TCE.

Transformation of 21 mg of TCE per liter in the presence of formate was measured by using three cell densities (2,500, 1,300, and 670 mg/liter) shaken at 300 rpm. With 670 mg of cells per liter, transformation was also measured at three initial TCE concentrations (2, 4, and 21 mg/liter) with results illustrated in Fig. 9a. The inverse of the initial specific transformation rate for each case is plotted against the inverse of TCE concentration in Fig. 9b. A linear regression of the data for a cell density of 670 mg/liter using the Lineweaver-Burk relation yielded computed parameters of 5.1 mg of TCE per mg of cells per day for maximum specific utilization rate (k) and 7.3 mg/liter for the half-velocity constant (K_s).

Measured specific transformation rates increased inversely with cell density (Fig. 9b), implying that either mixing at 300 rpm was insufficient to overcome localized mass transfer effects (in contradiction to the mass transfer analysis described under Experimental procedures) or the higher proportional formate/cell ratio experienced by the more dilute cells (all reactors contained 20 mM formate) resulted in an elevated initial transformation rate.

Also, it was expected that the dilute cells would be capable of transforming an amount of TCE proportional to that transformed by the denser cultures. However, the measured TCE transformation capacity for the dense cells and dilute cells was 0.033 and 0.025 mg of TCE per mg of cells, respectively. It appears that, mass per mass, the resting cells were capable of transforming a greater amount of TCE when at high densities. Perhaps high cell density allowed a buffering of the toxic effects of the TCE transformation, although other explanations are possible.

DISCUSSION

It has been indicated that pure methanotrophic cultures grown under copper-limited (<0.25 μM) conditions and at elevated temperatures (30°C) catalyze a highly elevated rate of TCE oxidation (23, 30). An important question is whether a mixed culture operating under ambient temperatures would produce similar high rates. The mixed culture used in this study had been grown at 21°C in medium with only 0.075 μM copper (approximately 0.03 μmol per g of cells) and, when freshly harvested, catalyzed a high initial TCE transformation rate of 0.58 to 1.1 mg of TCE per mg of cells per day for resting cells and a significantly increased rate when fed formate (2.0 mg of TCE per mg of cells per day).

A significant observation of this study, as well as that of others (23, 30), was a decline in TCE transformation rate over time by resting cells. A similar decline in cometabolic transformation rates has been observed for reductive dechlorination (9, 10), methanotrophic propylene epoxidation (15, 17), and TCE transformation by ammonia oxidizers (1) and toluene oxidizers (33). Possible explanations for such rate declines include (i) the consumption with time of energy stores needed to produce NADH, the electron donor required to sustain MMO catalyzed reactions (4, 17, 25, 30); or (ii) toxicity from either TCE itself or metabolic intermediates (12a, 30).

Evidence for depletion of energy reserves as a factor in cometabolic rate declines includes the observations that, following diminished cometabolic activity, addition of electron donor resulted in either partial renewal of the oxidative ability of the cells (17) or total regeneration of cometabolic activity (9, 15). In this study, the addition of formate to freshly harvested cells was responsible for increased initial TCE transformation rates and an elevated total transformation capacity, strongly implying that, with resting cells alone, depletion of stored energy reserves was at least a factor in measured TCE transformation rates and capacities. However, the addition of either formate or methane following the exhaustion of TCE transformation potential did not promote renewed MMO-catalyzed oxidations, indicating that toxicity of TCE or its transformation products also was a factor.

Further evidence of toxicity from TCE or its transformation products includes a study by Wackett and Householder (34) on the effect of TCE exposure on the growth of toluene degraders. They found growth inhibition in the presence of TCE, which ceased upon TCE removal, and also that the TCE toxicity noted was counterbalanced by macromolecular synthesis, allowing total recovery of cell activity. Also, in studies with methanotrophs, the total cessation of MMO activity at TCE exposure levels of 6.0 (18) and 7.8 (29) mg/liter has been attributed to TCE toxicity. However, no such acute inhibition was observed here with TCE concentrations even as high as 22 mg/liter; rather, the significant decline in the methane conversion rate with time following transformation of TCE implicates product toxicity as a significant factor affecting the decline in TCE utilization rate with time.

Although it is not conclusive whether the toxic effects noted were from TCE itself or from a product of TCE transformation, studies for mammalian systems have shown that reactive metabolites of cytochrome P-450-mediated TCE oxidations bind irreversibly to DNA and RNA in vitro (13). The reactivity of TCE epoxide has been implicated in the mammalian toxicity of TCE (37). Thus, product toxicity is a distinct possibility for some of the observed TCE transformation rate declines.

A significant observation of this study which is perhaps new is the decline of TCE transformation rate with time of aeration of resting cells. The diminished specific TCE transformation rate (Fig. 6b) and methane consumption rate (Table 2) of cells shaken in air compared with those that

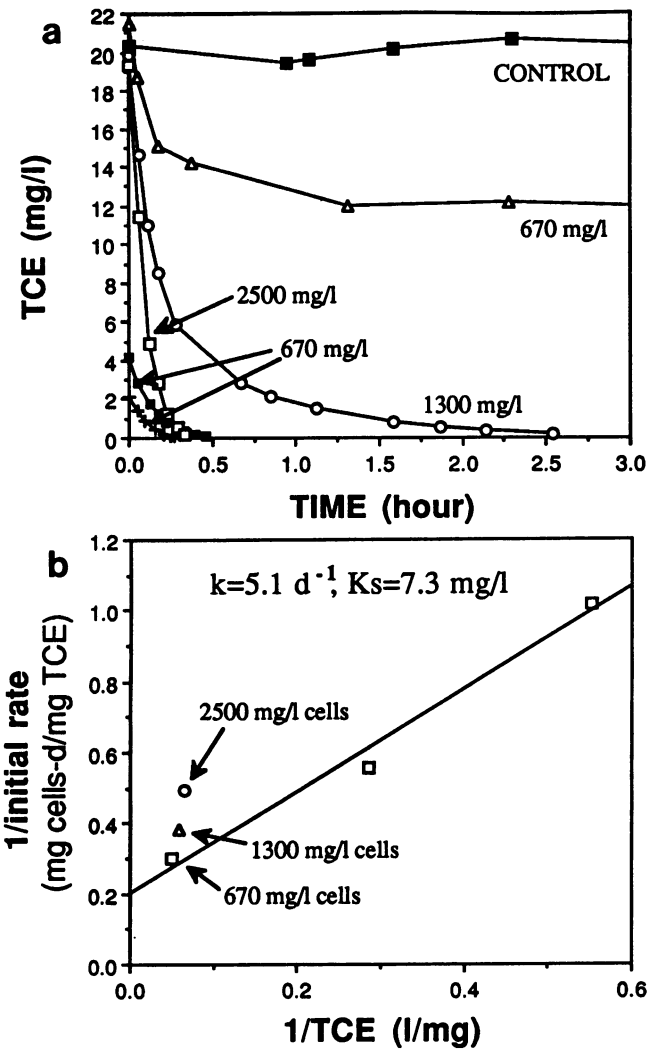

FIG. 9. (a) TCE disappearance with different cell concentrations (670 to 2500 mg/liter) and initial TCE concentrations for formate-fed cells. (b) Plot of inverse initial specific TCE transformation rates against inverse TCE concentration for different cell densities and 20 mM formate addition. Monod parameter estimation from most dilute case (670 mg/liter) only.

were not imply that aeration alone somehow diminished MMO activity, although to a much lesser extent than exposure to TCE. One possible explanation for the observed loss of activity with aeration alone is cell decay through endogenous respiration or predation, both of which would probably require oxygen. However, a decay rate of 1.3 day^{-1} implied by the diminished methane consumption rate with time of aeration is above that generally expected for typical aerobic mixed culture decay (0.1 to 0.25 day^{-1}).

An alternative explanation for the observed aeration effect is based on the suggestion that MMO functions by generating active oxygen species which in turn react with reduced substrate (TCE or methane) to form either epoxidated or hydroxylated products, consuming NADH in the process (8, 16). Aeration promotes the continued generation of MMO/oxygen-activated species, which in the absence of oxidizable substrate can become cytotoxic, a form of suicide. This mechanism would be consistent with the observed rapid decline in methane and initial TCE transformation rates for cells shaken in the presence of oxygen. The preservation of methane and TCE transformation ability in the unshaken cultures would then be explained by the unavailability of oxygen as required for activated species formation.

Due to the described transformation rate decline, the total course over time of the TCE transformation could not be adequately described by Monod kinetics alone. However, initial specific transformation rates measured shortly after the first TCE addition could be expected to be minimally affected by diminished energy stores or product toxicity, suggesting that Monod kinetics might be suitable for describing initial transformation rates of resting cells. On this basis initial TCE disappearance data with formate-fed mixed cultures yielded k of 5.1 mg of TCE per mg of cells per day and K_s of 7.3 mg of TCE per liter. If adjusted for temperature effects (21°C in this study versus 30°C for other studies) and fraction of cell mass represented by active methanotrophs (estimated by methane oxidation rate as about 20% [26a]), these values would be within the range reported by Brusseau et al. (k = 41.7 mg of TCE per mg of cells per day; K_s = 18 mg/liter [2a]) and Oldenhuis et al. (k = 40.5 mg of TCE per mg of cells per day; K_s = 26 mg/liter [23]) for formate-fed pure cultures. Based on the measured net growth yield of 0.35 mg of cells per mg of CH_4 and the measured transformation capacity for the mixed culture used in this study of 0.036 mg of TCE per mg of cells without formate and 0.073 mg of TCE per mg of cells with formate, a transformation yield of 0.013 mg of TCE per mg of CH_4 in the absence of formate and values as high as 0.026 mg of TCE per mg of CH_4 with formate addition were found.

The concept of a finite transformation capacity by resting cells can be an important consideration for the design of a TCE transformation treatment system. Since the observed maximum specific TCE transformation rate was fast (0.6 to 5 day^{-1}), the transformation yield was high (0.026 g of TCE per g of CH_4), and the primary substrate, methane, is reasonably inexpensive, a dual phase reactor, in which methanotrophs are grown on methane in one reactor and then removed to a second reactor where the cometabolic TCE transformation reaction is carried out, may be a practical option. The observed transformation rate decay due to aeration would suggest that the transformation ability of cells could best be preserved between stages by storing and transferring them in the absence of oxygen. As an alternative single-phase design, highly efficient TCE removal could possibly be preserved by alternating additions of formate with methane additions in sufficiently low concentrations to maintain the culture without exerting significant competitive inhibition and TCE-related toxicity.

ACKNOWLEDGMENTS

This research was supported in part by fellowships from the National Science Foundation and the Switzer Foundation and in part by the Office of Research and Development, U.S. Environmental Protection Agency, through the Western Region Hazardous Substance Research Center under agreement R-815738.

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