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Methanol Suppression of Trichloroethylene Degradation by *Methylosinus trichosporium* (OB3b) and Methane-Oxidizing Mixed Cultures

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ABSTRACT

The effect of methanol on trichloroethylene (TCE) degradation by mixed and pure methylotrophic cultures was examined in batch culture experiments. Methanol was found to relieve growth inhibition of *Methylosinus trichosporium* (OB3b) at high (14 mg/L) TCE concentrations. Degradation of TCE was determined by both radiolabeling and gas chromatography techniques. When cultures were grown on methanol over 10 to 14 d with 0.3 mg/L TCE, OB3b degraded 16.89±0.82% (mean±SD) of the TCE, and a mixed culture (DT type II) degraded 4.55±0.11%. Mixed culture (JS type I) degraded 4.34±0.06% of the TCE. When grown on methane with 0.3 mg/L TCE, 32.93±2.01% of the TCE was degraded by OB3b, whereas the JS culture degraded 24.3±1.38% of the TCE, and the DT culture degraded 34.3±2.97% of the TCE. The addition of methanol to cultures grown on methane reduced TCE degradation to 16.21±1.17% for OB3b and to 5.08±0.56% for JS. Although methanol reduces the toxicity of TCE to the cultures, biodegradation of TCE cannot be sustained in methanol-grown cultures. Since high TCE concentrations appear to inhibit methane uptake and growth, we suggest the primary toxicity of TCE is directed towards the methane monooxygenase.

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Index Entries: Biodegradation; trichloroethylene; methanotroph; methanol; methane.

INTRODUCTION

Owing to inadequate disposal methods, trichlorethylene (TCE) has become a common pollutant of groundwater (*1*), but several remediation methods have emerged. They include chemical extraction, ozonolysis, and biodegradation. Aerobic biodegradation can be carried out by methanotrophs (*2*). Its methane monooxygenase (MMO) apparently initiates mineralization of TCE by forming its epoxide (*2*). The epoxide then spontaneously breaks down to glyoxylic acid, dichloroacetic acid, and CO_2. Our research focuses on understanding the processes of TCE degradation by methanotrophs.

Whereas many researchers have demonstrated methanol suppression of methane uptake (*3–6*), others have maintained that the MMO remains active during methanol oxidation (*7,8*). Janssen (*9*) recently showed that a methanotrophic strain grew on methanol and that this constitutive MMO strain degraded *trans*-1,2-dichloroethylene as rapidly when grown on methanol as when grown on methane, but this strain has since been lost. However, Fogel (*10*) showed TCE degradation was inhibited 50% when methanol was added to a methane-utilizing mixed culture, but offered no explanation. To clarify the effects of methanol, we designed experiments to compare its ability to stimulate TCE degradation with that of methane.

MATERIALS AND METHODS

Organisms

Two mixed cultures and one pure culture were used in the experiments. A mixed methane-oxidizing culture (JS) was obtained from a bioreactor operated by G. W. Strandberg (Chemical Technology Division, Oak Ridge National Laboratory). This culture originated from enrichments of TCE-contaminated groundwater (*2*). Another mixed culture (DT), consisting of methylotrophs and amoebae, was obtained from R. Tyndall (Health Physics Division, Oak Ridge National Laboratory). Phospholipid analysis of the JS and DT mixed cultures indicates that they consist primarily of type I and type II methylotrophs, respectively (Eng, unpublished data). A pure culture, *Methylosinus trichosporium* (OB3b), was obtained from M. Lidstrom, California Institute of Technology. The culturing methods have been described earlier (*2*) and will be only briefly described here.

All methylotrophic cultures were maintained on mineral salts plates in a desiccator jar (20–22°C) with 20–30% methane and atmospheric con-

centrations of oxygen (20% v/v) in the headspace. Starter cultures were generated by sterile loop inoculation of nutrient and trace element (NATE) medium (2,5) from agar plates. Methane (9–10% v/v) was added to the headspace to stimulate growth. Strain purity was assured by the absence of a growth on NATE plus yeast extract (0.2 g/L) agar plates (15 g/L) without methane.

Culture Growth Conditions

All cultures were grown in 250-mL septum bottles containing 100 mL of sterilized NATE medium and atmospheric concentrations of oxygen. A 1-mL aliquot of starter culture (log phase) was added by sterile syringe. Filter-sterilized methane gas was injected where noted to obtain a 9–10% (v/v) (or 890 μmol/L) atmosphere. Methanol was added where noted to a final concentration of 1.187 mmol/L. Finally, radiolabeled TCE (15 mCi/mmol, Sigma, St. Louis, MO purity >98%) was added with reagent grade TCE to obtain the final concentrations of either 14 mg/L or 0.3 mg/L.

Several precautions were taken to minimize leakage and influx of gases. The bottles were capped with Teflon-lined silicone septa (Superlo). Also the bottle cap was covered with modeling compound sandwiched between two sheets of parafilm. The culture bottles were then inverted on a rotary shaker (75 rmp) at 22°C for the duration of the experiment.

Experimental Design

TCE degradation was examined in two pure and two mixed culture experiments by comparing (Table 1) growth and TCE degradation among cultures grown on methanol, methane, and combined methane and methanol. To correct for any toxic effects of TCE on growth, controls without TCE were grown simultaneously in pure culture experiments. Sterile controls containing methane, methanol, and TCE corrected for any background activity not caused by biodegradation.

The effect of TCE degradation by pure culture OB3b was examined at two TCE concentrations. The initial cell concentration was 6×10^5 cells/mL with 14 mg/L TCE in the first experiment. After 13 d, the treatments were sacrificed for radiolabel analysis. The initial cell concentration was 6.58×10^5 cells/mL with 0.3 mg/L TCE in the second experiment. This experiment ended after 10 d. Triplicate bottles were used for each treatment.

The effect of growth substrate(s) on TCE degradation was also examined in two experiments using the JS culture with 0.3 and 14 mg/L TCE, respectively. The first JS experiment ended after 12 d, whereas the second ended after 10 d. No attempt was made to quantify biomass in the first experiment, but growth was followed by OD_{600} in the second experiment. Duplicate treatments were used for the first experiment, and triplicates were used for the second.

Table 1
Conditions for Experiments, Including Culture Used, TCE Concentration, Growth Substrates (E = methane, L = methanol, B = both), and Length of Experiment in Days

Culture	TCE concentration (mg/L)	Growth substrate (s)	Time (days)
OB3b	14	E, L, B*	13
OB3b	0.3	E, L, B*	10
JS	14	E, L, B*	10
JS	0.3	E, L, B	12
DT	0.3	E, L	14

*Treatments without TCE also tested.

The effect of methanol on TCE degradation was compared to that of methane using the DT culture. The duplicate treatments contained 0.3 mg/L TCE and were sacrificed after 14 d.

Cell Counting Method

The acridine orange staining/UV florescence method was used to determine cell numbers (*11*). Subsamples of culture (0.5 mL) were removed by sterile syringe. After acridine orange staining, the culture was filtered onto a black polycarbonate membrane (25 mm in diameter, 0.2 μm pore size) (Nucleopore Corporation, Pleasanton, CA). After this process, cells were counted by using a Nikon type 104 microscope equipped with UV illumination.

Oxygen and Methane Analysis

Oxygen and methane concentrations were measured simultaneously with a Perkin Elmer 3920 gas chromatograph (GC). The GC was equipped with a Molecular Sieve 5A (Applied Science Laboratory) column (6 ft × 1/8 in.) and thermal conductivity detector. Helium (50 mL/min) was used as carrier gas and the column was held at 45°C. Cultures containing only methanol (without methane) had a 2% higher initial oxygen concentration than other treatments, owing to the compression by methane.

TCE Analysis

TCE degradation was determined both by gas chromatography and ^{14}C techniques. Aliquots (24 μL) of headspace gas were injected into a

Perkin Elmer 2000 gas chromatograph equipped with a electron capture detector and (2 ft × 1/8 in.) 1% SP 1000 60/80 Carbopack B (Supelco) packed glass column. Nitrogen (40 mL/min) was used as the carrier gas, and the column was held at 120°C.

The fate of ^{14}C TCE was determined by fractionation of ^{14}C-labeled products into CO_2, cell-bound products, and water-soluble compounds (2). The medium was first adjusted to pH 9–9.5 to convert radiolabeled CO_2 into carbonic acid. A subsample (50 mL) was then centrifuged (2500 rpm, 30 min, 10°C) to pellet the cells (International PR-2 centrifuge, #253 rotor, Needham Heights, MA). The cells were washed twice in NATE medium before counting. Hexane was added to the supernatant fraction to extract any untransformed ^{14}C TCE. The water phase was then acidified to release CO_2, which was trapped in a vial containing 0.1 N NaOH. The remaining ^{14}C in the water phase and in the CO_2 traps was counted by liquid scintillation spectrometry (TriCarb 2000 Model). Sterile control counts have been subtracted from all radiolabel results.

Statistical Analysis

Results were analyzed with the analysis of variance procedure (ANOVA) of SAS software run on an IBM XT computer. All significant differences are statistically significant at the 95% confidence level.

RESULTS

M. trichosporium (OB3b)—High TCE

In experiments with 14 mg/L TCE, methanol appeared to reduce the inhibitory effects of TCE on growth of OB3b. In cultures grown on methane in the presence of the TCE, there was significant inhibition ($p < 0.05$) of the growth (compared to controls without TCE), but cultures grown on methanol showed increased growth over treatments with methane and TCE. The cultures with methane and methanol exhibited a similar pattern of growth (Figs. 1a, 1b, and 1c.)

In general, oxygen consumption (Table 2) correlated with the increase in cell numbers of OB3b. Methane-grown treatments without TCE exhibited the greatest extent of oxygen uptake, final headspace concentration of $4.14 \pm 1.69\%$. With TCE, the uptake of oxygen was reduced (final concentration of $14.3 \pm 0.94\%$). All treatments containing methanol, with and without TCE, showed similar patterns of oxygen uptake and greater oxygen consumption than methane-grown cultures with TCE, but oxygen uptake was slower (final concentration of 5 to 9%) than for treatments with methane and no TCE.

Although methanol reduced the extent of methane consumption in treatments containing both substrates, TCE also had an inhibitory effect on methane uptake by OB3b. In the absence of TCE, methane was con-

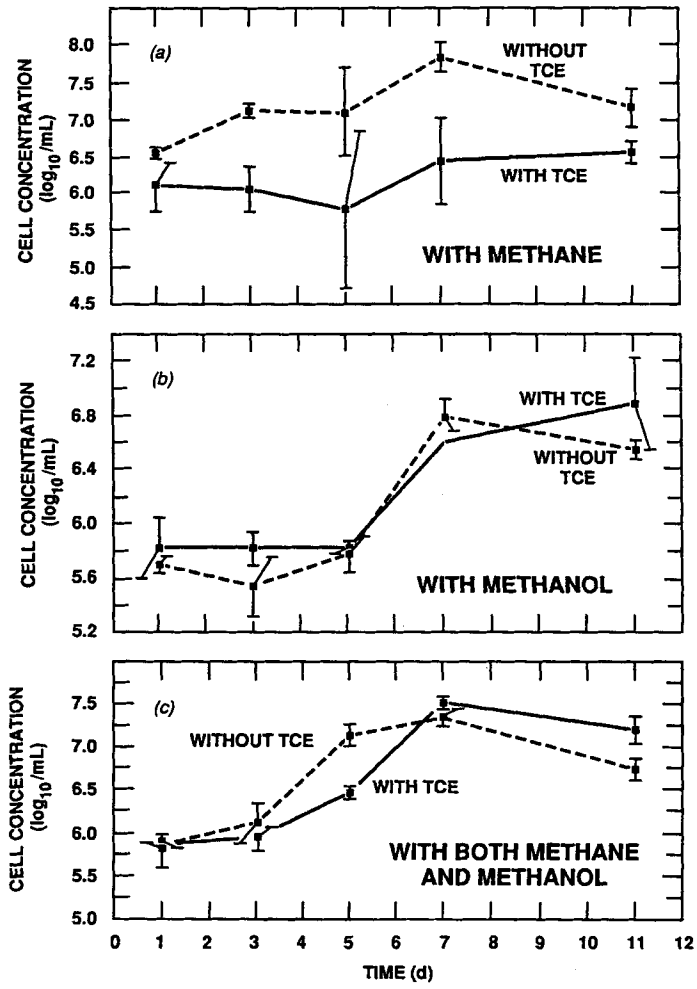

Fig. 1. Growth of OB3b (log_{10} cells·mL^{-1}) on (a) methane, (b) methanol, and (c) methane plus methanol. Treatments are with 14.0 mg/L TCE and without TCE.

sumed to below detectable concentrations in the methane-only treatments at the end of 13 d, but where methanol was added, methane uptake was not as extensive (final 5.32±0.6% methane headspace). When TCE was present, the methane-only treatments contained a 7.56±0.64% methane headspace; similarly, the methane and methanol contained 7.07±0.83% methane (Table 2).

The extent of TCE degradation by OB3b did not differ among the three treatments, and was relatively low. The total degradation by statistical analysis (mean±SD) for methane was 7.17±1.7%, for methanol, 6.83±0.34%, and for both substrates, 5.9±0.31% (Table 3).

Table 2
Final Gas Headspace Gas Concentrations (% mean ± S.D.)
for Methylosinus trichosporium (OB3b)

Treatment	Gas	14 mg/L TCE Mean ± S.D.		Without TCE Mean ± S.D.		0.3 mg/L TCE Mean ± S.D.		Without TCE Mean ± S.D.	
CH_4	CH_4	7.56	0.64	0	0	0	0	0	0
	O_2	14.3	0.94	4.14	1.69	7.34	0.62	6.70	1.13
CH_3OH	O_2	8.89	3.10	6.47	1.61	9.19	1.91	12.63	2.29
Both	CH_4	7.07	0.83	5.32	0.6	4.40	0.28	5.04	1.02
	O_2	5.3	0.28	6.74	0.32	6.57	0.26	6.30	1.67
Sterile	CH_4	9.08	0.11	NT*	NT*	8.57	0.26	NT*	NT*
Control	O_2	18.10	0.05	NT*	NT*	18.91	0.08	NT*	NT*

*NT = Not tested.

Table 3
Percent of TCE Degradation in Cultures Grown on Methane, on Methanol, or on Both

Culture	Initial TCE (mg/L)	Percent TCE Degradation					
		CH_4		CH_3OH		Both	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
OB3b	14	7.17	1.7	6.83	0.34	5.9	0.31
OB3b	0.3	32.9	2.01	16.8	0.82	16.2	1.17
JS	14	3.20	0.67	2.76	0.97	4.09	1.00
JS	0.3	24.3	1.38	4.34	0.06	5.08	0.55
DT	0.3	34.3	2.97	4.55	0.11	NT*	NT*

*NT = Not tested.

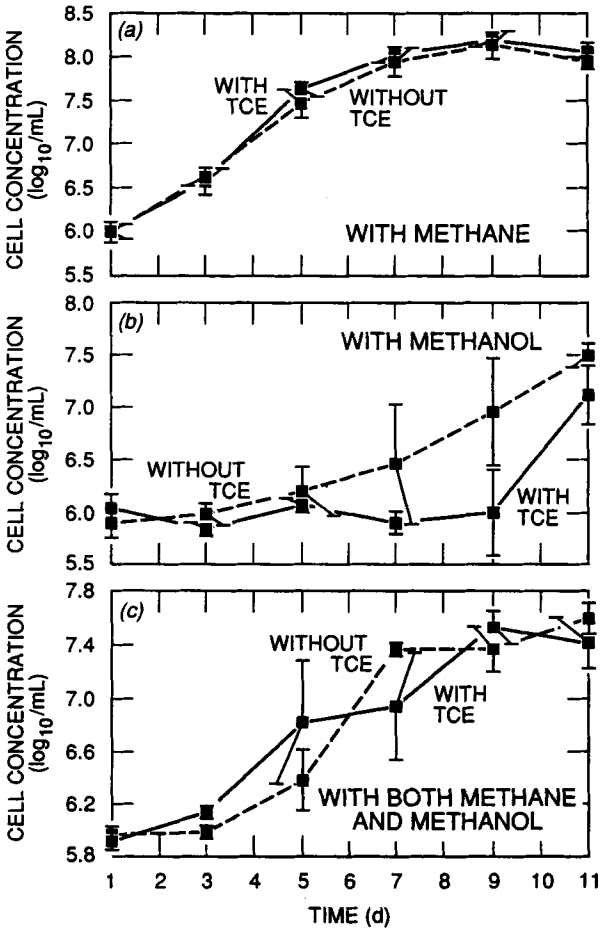

Fig. 2. Growth of OB3b (log_{10} cells·mL^{-1}) on (a) methane, (b) methanol, and (c) methane plus methanol. Treatments are with 0.3 mg/L TCE and without TCE.

M. trichosporium (OB3b)—Low TCE

TCE at 0.3 mg/L does not significantly inhibit the growth of OB3b cultures containing both methane and methanol (Fig. 2). Although the growth of the methane-only stimulated treatments was more consistent among the replicates than the methanol-stimulated treatments, final cell yield was not significantly affected by TCE in any of the treatments (Fig. 2).

Methanol inhibited methane uptake at this TCE level, but TCE itself did not. All of the methane was consumed in the methane-only treatments, but in the combined methane and methanol treatments, 4.4 to 5% of the methane remained in the headspace (Table 2) at the end of the experiment.

Oxygen consumption was significantly reduced by the presence of methanol. At the end of 10 d, treatments containing TCE had a 1 to 3%

higher oxygen concentration than treatments without TCE (Table 2). Oxygen uptake was greatest in the methane and the combined methane and methanol treatment (final concentration 6.3 to 7.3%). However, the methanol treatments with or without TCE consumed little oxygen (final concentration 9 to 12%).

TCE degradation was greater in this experiment, with 0.3 mg/L TCE, than in the experiment described above, with 14 mg/L TCE, and the methanol inhibition of the degradation was clearly evident. In the treatment containing only methane, 32.9±2.01% of the TCE was degraded, and in the treatments with methanol, about 16 to 17% of the TCE was degraded (Table 3).

JS Mixed Culture—High TCE

Treatments with or without 14 mg/L TCE containing methane and methanol had 2.29% oxygen remaining, but TCE inhibited oxygen uptake in the other treatments (Table 4). The biomass at day 6 was lower in all treatments with TCE than in the corresponding treatments without TCE (Fig. 3).

Without TCE, the methane and methanol treatments had 9.44±0.39 and 4.08±0.21% (mean±SD) oxygen remaining, respectively. The methanol treatment with TCE had 9.54±4.2% oxygen remaining, which is lower than 16.62±0.89% found in the methane treatment with TCE (Table 4).

In these high-TCE treatments, methanol allowed greater methane uptake in treatments with or without TCE. Methane remaining in treatments with TCE was 7.35±1.15%, but 0.0% in treatments without TCE. In the presence of methanol and TCE, methane remaining was only 3.53±1.49%; without the TCE, 2.79±0.81% methane remained (Table 4). Less than 5% of the TCE was degraded in any treatment (Table 3).

JS Mixed Culture—Low TCE

All treatments with and without 0.3 mg/L TCE showed considerable oxygen uptake. The combined methane and methanol treatment showed the greatest uptake (final concentration of 2.3±0.1%). The methanol-stimulated treatments had a final oxygen concentration of 3.75±0.64%, whereas the methane-only treatments had a 4.6±0.1% oxygen concentration (Table 4).

As in other experiments, methane uptake was reduced in the presence of methanol. Whereas no methane was detectable in the methane-only treatments, the combined methane and methanol treatments had 7.35± 2.07% methane remaining at the end of the experiment (Table 4).

The JS culture exhibited significant degradation (23–25%) only when grown on methane. Of the ^{14}C TCE in the cells grown on methane, 6.82–6.84% was found as cell-bound products, and 16.5–18.5% was transformed to CO_2. None of the radiolabel was found as water-soluble products. Less

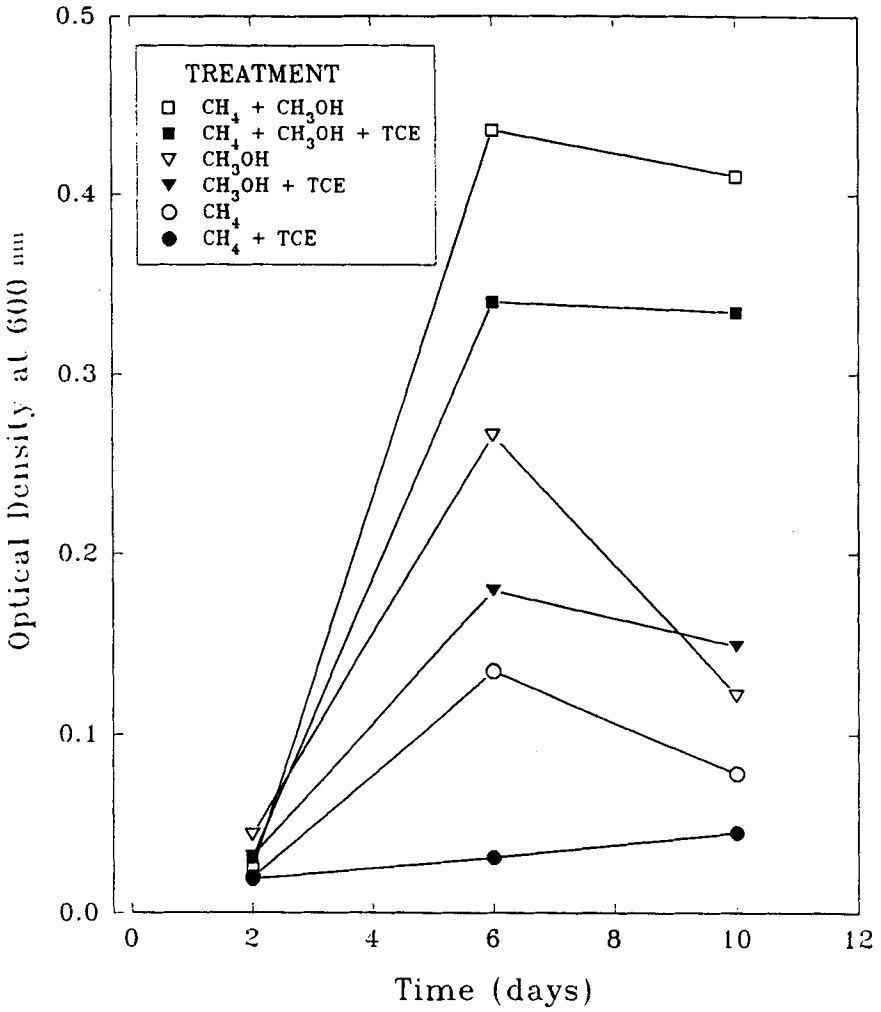

Fig. 3. Growth of JS mixed culture (OD_{600}). Treatments with 14 mg/L are labeled as follows: methane (solid circle), methanol (solid triangle), or methane and methanol (solid box). Treatments without TCE are labeled as follows: methane (open circle), methanol (open triangle), methane and methanol (open box).

than 6% of the initial TCE was degraded in the methanol and in the combined methanol and methane treatments (Table 3).

DT Mixed Culture—Low TCE

As in experiments with the JS culture and OB3b, methane proved to be a better growth substrate for TCE degradation than methanol. The DT cultures grown on methane degraded 34.3±2.97% of the TCE, but the methanol treatments showed less than 5% degradation (Table 3).

Table 4
Final Headspace Gas Concentrations (%) for Mixed Cultures JS and DT

		14 mg/L TCE (JS)		Without TCE (JS)		0.03 mg/L TCE (JS)		0.03 mg/L TCE (DT)	
Treatment	Gas	Mean ± S.D.		Mean ± S.D.		Mean ± S.D.		Mean ± S.D.	
CH_4	CH_4	7.35	1.15	0	0	0	0	0	0
	O_2	16.62	0.89	9.44	0.39	4.6	0.1	5.74	0.01
CH_3OH	O_2	9.54	4.20	4.08	0.21	3.75	0.635	4.85	0.35
Both	CH_4	3.53	1.49	2.79	0.81	7.35	2.07	NT*	NT*
	O_2	2.29	0.02	2.25	0.02	2.3	0.108	NT*	NT*
Sterile	CH_4	8.57	0.26	NT	NT*	9.95	0.01	NT*	NT*
Control	O_2	18.91	0.08	NT	NT*	17.93	0.24	20.3	0.67

*NT = Not tested.

DISCUSSION

Methanol appears to reduce the TCE degradation by both the pure and mixed cultures. There are two possible mechanisms by which this may occur. Methanol may act as a competitive inhibitor of both TCE degradation and methane oxidation. Second, methanol may repress the expression or function of the MMO.

Dalton (*12*) has shown that methanol can also be oxidized by the MMO enzyme; thus, there could be competitive effects. In addition, Best (*7*) showed that cultures of *M. trichosporium* maintained on methanol for 9 mo retained both epoxidizing and methane-hydroxylating activity. Since TCE is also oxidized by the MMO of *M. trichosporium* (*13*), the rate of oxidation should also depend on methane and/or methanol concentrations. Whereas methane uptake was observed in treatments containing methanol, the uptake was significantly lower than in methane-only treatments. The exception was in the treatments with 14 mg/L TCE, where significant inhibition of methane uptake was observed in pure cultures. In the mixed cultures, the greater methane uptake could be explained by the increase of biomass (Fig. 3), which may have raised the basal levels of MMO activ-

ity. Many researchers (*3–6*) have also observed decreased methane oxidation in the presence of methanol. But earlier results also showed that the concentration of the soluble MMO enzyme decreases during growth on methanol (*14*).

Our data indicate that the primary toxicity of TCE may stem from its interaction with the MMO as suggested from work with liver monooxygenases (*15–19*). Methanol could relieve the toxic effects of TCE on growth by allowing growth of the cultures not expressing the TCE-sensitive MMO. This hypothesis, toxicity resulting from TCE interactions with MMO, would explain why TCE at 14 mg/L did not affect the growth pattern of methanol-stimulated cultures but severely inhibited the growth of culture grown on methane. This hypothesis would also imply that TCE degradation should not be expressed for long in the absence of methane since the cells would need to constantly make new MMO as it interacts with the TCE, and this synthesis would be suppressed by methanol.

A basic principle of cometabolism is that the cometabolite (TCE) will not support growth, and many researchers already have confirmed this finding. Whereas Fogel (*10*) proposed that the breakdown intermediates from TCE degradation are metabolized only by heterotrophs, we have found considerable amounts of ^{14}C bound to cell material. Thus, we suggest that methanotrophic incorporation of ^{14}C can occur through several routes. Radiolabeled CO_2 may be incorporated by the phosphoenolpyruvate carboxylase. This enzyme is part of the serine assimilation pathway of type II methanotrophs. However, this does not explain the amount of ^{14}C incorporation in strain 46-1, a type I methanotroph (2) of glyoxylic acid, which is one of the water soluble products of TCE degradation that was identified by Little (*2*). This compound could participate in glyoxylic cycle, an anapleurotic pathway.

Some of the ^{14}C incorporation may be related to binding of a radiolabeled metabolite (TCE) to the MMO. Many scientists have shown covalent binding of TCE, 1,1-dichloroethylene, and vinyl chloride to liver monooxygenases (*19–21*). Their experiments indicate that covalent binding is caused by the epoxide, the reactive intermediate formed. This mechanism may also be occurring in methanotrophs.

Although we showed that methylotrophic cultures grown on methanol are able to survive at higher concentrations of TCE than cultures grown on methane, the reduction in TCE degradation precludes the use of methanol as a substrate in bioreactors. However, if a culture with a constitutive MMO was again isolated, the use of methanol as a growth substrate might allow for TCE degradation by this strain at very high TCE levels.

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