APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1991, p. 1770–1776
0099-2240/91/061770-07$02.00/0
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Vol. 57, No. 6

Inhibition of Trichloroethylene Oxidation by the Transformation Intermediate Carbon Monoxide

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Received 12 February 1991/Accepted 8 April 1991

Inhibition of trichloroethylene (TCE) oxidation by the transformation intermediate carbon monoxide (CO) was evaluated with the aquifer methanotroph *Methylomonas* sp. strain MM2. CO was a TCE transformation intermediate. During TCE oxidation, approximately 9 mol% of the TCE was transformed to CO. CO was oxidized by *Methylomonas* sp. strain MM2, and when formate was provided as an electron donor, the CO oxidation rate doubled. The rate of CO oxidation without formate was 4.6 liter mg (dry weight)$^{-1}$ day^{-1}, and the rate with formate was 10.2 liter mg (dry weight)$^{-1}$ day^{-1}. CO inhibited TCE oxidation, both by exerting a demand for reductant and through competitive inhibition. The K_i for CO inhibition of TCE oxidation, 4.2 μM, was much less than the K_i for methane inhibition of TCE oxidation, 116 μM. CO also inhibited methane oxidation, and the degree of inhibition increased with increasing CO concentration. When CO was present, formate amendment was necessary for methane oxidation to occur and both substrates were simultaneously oxidized. CO at a concentration greater than that used in the inhibition studies was not toxic to *Methylomonas* sp. strain MM2.

An important consideration in the development of biological methods for remediation of sites polluted with xenobiotics is the effect of transformation intermediates and products on the transformation process. Compounds that are inhibitory to the active microorganisms may be produced. Carbon monoxide (CO) had been proposed to be an intermediate in the aerobic transformation of trichloroethylene (TCE) by methanotrophs (20, 31). This was based on the findings of Henschler et al. (24) and Miller and Guengerich (33), who evaluated the nonenzymatic hydrolysis of TCE epoxide, which was proposed to be the immediate product of the methane monooxygenase (MMO)-catalyzed oxidation of TCE (20, 31). In aqueous solution, TCE epoxide was shown to decompose to CO and formate as a result of C-C fission, as well as to several acid products, including glyoxylic acid and dichloroacetic acid (24, 33). The relative ratio of the products was pH dependent, with CO and formate predominating under neutral and basic aqueous conditions (33). Fox et al. (13) recently demonstrated with purified MMO from *Methylosinus trichosporium* OB3b that TCE epoxide is indeed formed from the MMO-catalyzed oxidation of TCE and that CO is one of the transformation products.

Methanotrophs are among the microorganisms capable of oxidizing CO to carbon dioxide (2, 17, 29). CO oxidation has been demonstrated in whole-cell suspensions and cell extracts of several strains, including *M. trichosporium* OB3b (26), *Methylomonas albus* BG8 (26), *Methylomonas methanica* (10, 11), and *Methylococcus capsulatus* (Bath) (43–45). Ferenci first proposed that CO was oxidized by MMO (10), and this was subsequently confirmed by studies with purified MMO from *M. trichosporium* OB3b (48), *M. capsulatus* (Bath) (6), and *Methylobacterium* sp. strain CRL-26 (37). Inhibition of whole-cell methane oxidation (9–11, 43) and bromomethane oxidation (5) by CO, as well as competitive inhibition of CO oxidation by ethane (11), has been demonstrated. CO oxidation required an exogenous electron donor (6, 10, 11, 43, 45), which is consistent with MMO-catalyzed oxidations that, unlike the oxidation of methane (25), do not lead to subsequent transformations that regenerate the reductant. Since TCE and CO are both oxidized by MMO, it is likely that CO would also inhibit TCE oxidation by competing for the enzyme as well as by exhausting the cell of available reductant.

A field study evaluating methane-stimulated in situ biodegradation of chlorinated ethenes was conducted at a site located at the Moffett Naval Air Station (Mountain View, California) (38, 40, 41). In conjunction with this study, we have enriched and isolated TCE-transforming methanotrophic mixed and pure cultures from the Moffett Field groundwater aquifer (21–23). We previously described the effect that mineral medium formulation, reductant supply, and TCE oxidation toxicity had on TCE transformation by these cultures. In this paper we report the production of CO during TCE oxidation and the inhibition of TCE oxidation and methane oxidation by CO in *Methylomonas* sp. strain MM2.

(Preliminary reports of this study have been published [18, 19]).

MATERIALS AND METHODS

Organism and culture conditions. *Methylomonas* sp. strain MM2, isolated previously in our laboratory (21, 22), was grown in Whittenbury mineral medium (51) to mid-exponential growth phase (0.4 to 0.9 g of cells [dry weight] liter^{-1}) in continuously stirred reactors under a continuous stream of 30 to 35% (vol/vol) methane (99.3% [vol/vol] pure; Liquid Carbonic Specialty Gas Corp., San Carlos, Calif.) in air at room temperature (21 to 23°C). The reactors were inoculated from cultures grown at room temperature in desiccators filled with 30 to 40% (vol/vol) methane in air on mineral medium-Noble agar plates (15 g of Noble agar liter^{-1}, Difco Laboratories, Detroit, Mich. prepared with a modification of Fogel mineral medium [12, 22]). Cultures were tested for contamination by streaking on one-fourth-strength tryptone-

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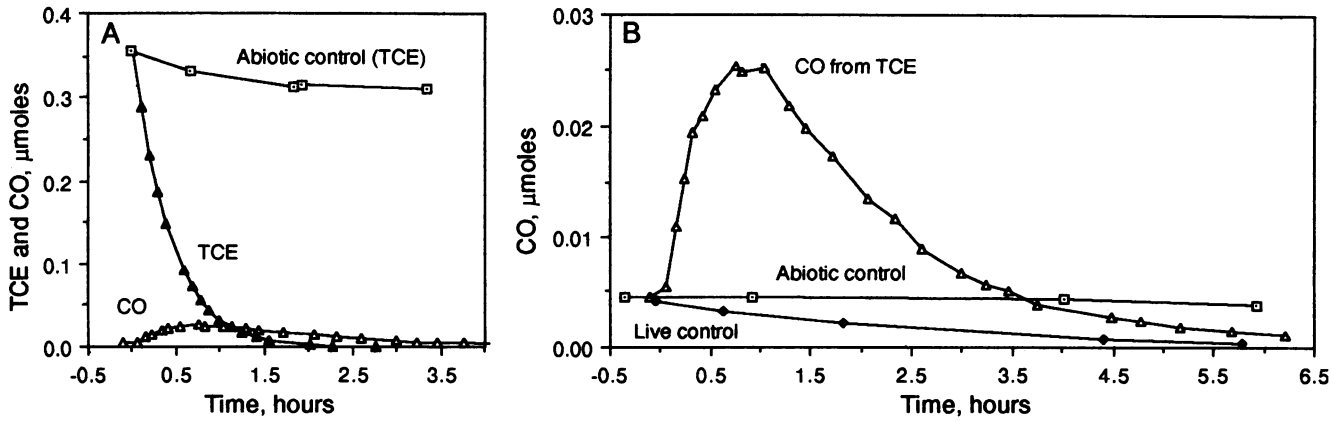

FIG. 1. (A) Production of CO during TCE oxidation and subsequent oxidation of the CO by *Methylomonas* sp. strain MM2. (B) Expanded view of the CO data from panel A. Total micromoles of TCE and CO in each bottle are plotted against time in hours. The aqueous TCE concentration at 0.35 µmol was 2.3 µM. The aqueous CO concentration at 0.025 µmol was 0.004 µM. The cell density was 0.11 g of cells (dry weight) liter^{-1}. The abiotic control contained sterile mineral medium and TCE. The live control was a replicate subculture of *Methylomonas* sp. strain MM2 but received no TCE or other amendments.

glucose plates (6 g of tryptone-glucose extract agar [Difco] and 11.25 g of Noble agar liter^{-1}) incubated in air and on mineral medium-Noble agar plates incubated in the methane-filled desiccators. For all experiments, subcultures were removed from the reactor, diluted with Whittenbury mineral medium to a volume of 100 ml, and transferred to 250-ml bottles containing 150-ml headspace. The bottles were incubated upside down on a rotary shaker in a 21°C environmental chamber as described previously (22). Formate, when provided as an exogenous electron donor, was added as 2 mM sodium formate (Fisher Scientific Co., Fair Lawn, N.J.). Methane or any other growth substrate was not provided unless otherwise noted.

Analytical procedures. TCE oxidation was monitored by measuring headspace concentrations by gas chromatography as described previously (22). Methane, oxygen, carbon dioxide, and nitrogen concentrations were determined by headspace analysis on a gas partitioner (Fisher-Hamilton model 25 V; Fisher Scientific, Pittsburgh, Pa.) equipped with a packed column (45/60 molecular sieve; Supelco Inc., Bellefonte, Penn.), a thermal conductivity detector, and an integrator (model 4270; Spectra-Physics, Sunnyvale, Calif.) as described previously (22). CO oxidation and production were monitored by measuring headspace concentrations of CO on a reduction gas detector (model RGD2; Trace Analytical, Inc., Menlo Park, Calif.) with air as carrier gas (breathing air; Liquid Carbonic Corp., Oakland, Calif.) and on the gas partitioner. The reduction gas detector was sufficiently sensitive for quantification of trace CO in the air (0.7 ppm), but injection of greater than approximately 2×10^{-10} mol of CO (approximately 500 ppm in a 10-µl injection) exceeded the linear range of the instrument. The lower limit for quantification on the gas partitioner was approximately 2×10^{-7} mol of CO (approximately 10,000 ppm in a 400-µl injection). Consequently, since CO concentrations between 0.05 and 1% (vol/vol) of headspace were difficult to quantify, they were avoided by way of experimental design. When it was necessary to quantify CO in that range, the CO was diluted before analysis by injecting 100-µl subsamples of the headspace to be analyzed into sealed, gas-tight bottles of known volume filled with air. Subsequently 100 µl of headspace from the dilution bottles were injected into the reduction gas detector. For quantifying the gases, calibration curves with a linear response were prepared using calibration gases (Scotty Specialty Gases, Fremont, Calif.; Alltech Associates, Inc., Deerfield, Ill.). Aqueous concentrations were calculated using unitless Henry's constants of 40.8 at 21°C for CO and 25.6 at 21°C for methane (47). CO was provided for the oxidation and inhibition studies by injecting 0.1 to 15 ml of either 99.3% (vol/vol) CO (Scotty) or one of the CO calibration gases into the bottle, depending on the CO concentration desired. Calibration gas (10% [vol/vol] acetylene in nitrogen; Alltech) was used to evaluate acetylene inhibition of CO and methane oxidation with acetylene at 3% (vol/vol) of headspace. Cell biomass was determined on a dry weight basis using 0.2-µm-pore-size Supor filters (Gelman Sciences Inc., Ann Arbor, Mich.) as described previously (22). CFU were enumerated by dilution plating on plates made from Fogel mineral medium (12, 22) and Noble agar. Monod kinetics for substrate degradation were used to model the oxidation of TCE and CO and to determine the pseudo-first-order rate coefficient $k' = k/K_s$ (liter milligram^{-1} day^{-1}) as previously described (22, 23). When TCE oxidation occurred in the presence of methane or CO, the competitive inhibition model for substrate degradation (14) was used, yielding a pseudo-first-order rate coefficient $k' = k/(K_s[1 + I/K_i])$ (liter milligram^{-1} day^{-1}), where k is the maximum rate coefficient, K_s is the half-saturation coefficient, I is the concentration of the inhibitor, and K_i is the inhibition coefficient. (Note that when $I = 0$, $k/(K_s[1 + I/K_i])$ reduces to k/K_s.)

RESULTS

Carbon monoxide from TCE oxidation. As TCE was oxidized by *Methylomonas* sp. strain MM2, CO was produced and subsequently oxidized (Fig. 1A). An expanded view of the CO data is provided in Fig. 1B. No formate was added in this experiment. The CO present in the control bottles came from the air in the laboratory, which contains 0.7 ppm CO. The CO disappeared in the two bottles containing the live cultures, but not in the abiotic control. Since CO was being simultaneously oxidized as it was produced, the percentage of TCE that was transformed to CO cannot be determined directly from the data presented in Fig. 1. The percentage of TCE transformed to CO, Δ, was estimated using a graphical

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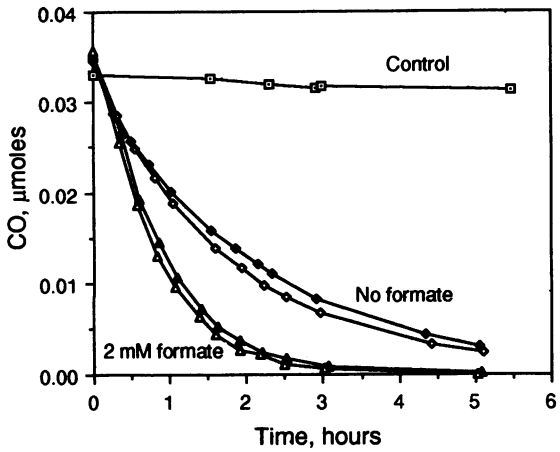

FIG. 2. CO oxidation by replicate subcultures of *Methylomonas* sp. strain MM2. Amendment with 2 mM formate increased the CO oxidation rate. Total micromoles of CO in the bottles are plotted against time in hours. The aqueous concentration of CO at 0.035 µmol was 0.006 µM. The cell density was 0.16 g of cells (dry weight) liter^{-1}. The control was a sterile mineral medium blank.

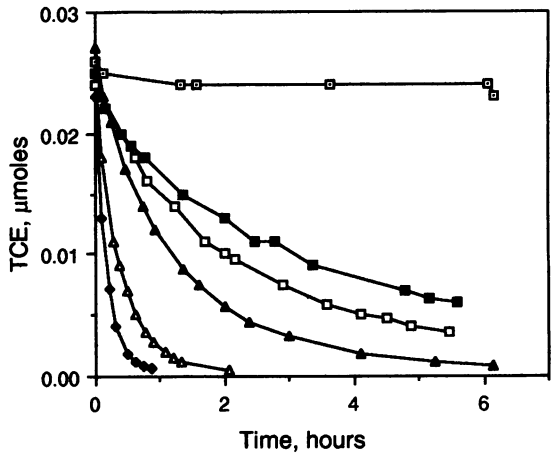

FIG. 3. Competitive inhibition of TCE oxidation by CO. Total micromoles of TCE in the bottles are plotted against time in hours. The aqueous TCE concentration at 0.025 µmol was 0.17 µM. The cell densities were 0.18 to 0.21 g of cells (dry weight) liter^{-1}. Bottles were amended with 2 mM formate. Symbols: ◆, no CO; △, 6.7 µM CO; ▲, 25.6 µM CO; ■, 53.5 µM CO; □, 57.3 µM CO; ⧈, control (sterile mineral medium blank).

solution. At 1 h of incubation, where three data points represent the peak of the CO curve (Fig. 1B), the slope, and consequently the derivative, is 0, so the CO formation rate equals the CO oxidation rate. By setting the CO oxidation rate equal to Δ times the instantaneous TCE oxidation rate, using the competitive inhibition model for CO oxidation (14), and assuming that the CO concentration (S) was much less than the half-saturation coefficient for CO (K_s), the following equation is obtained: $(k)(XV_1)(S)/(K_s)(1 + I/K_i) = \Delta$ (instantaneous TCE oxidation rate). The instantaneous TCE oxidation rate at 1 h was 2 µmol day^{-1}. The mass of cells in the bottle (XV_1) was 10 mg, the CO concentration (S) was 0.004 µmol liter^{-1}, the TCE concentration was 0.2 µmol liter^{-1}, and the inhibition coefficient for TCE (K_i) that was used was 3.9 µmol liter^{-1} (the half-saturation coefficient [K_s] previously determined for *Methylomonas* sp. strain MM2 grown on Whittenbury mineral medium [22]). For (k/K_s), 4.6 liter mg^{-1} day^{-1}, the rate for CO oxidation without formate added (Fig. 2), was used. Solving for Δ yields a value of 0.088. Approximately 9 mol% of the TCE was converted to CO.

CO oxidation rates. Formate addition doubled the CO oxidation rate (Fig. 2). The pseudo-first-order CO oxidation rate without formate amendment was 4.6 ± 0.4 liter mg^{-1} day^{-1}, and with 2 mM formate added, it was 10.2 ± 0.9 liter mg^{-1} day^{-1}. The correlation coefficients describing the fit of the data to the kinetic model were 0.98 to 1.00, indicating that the data were adequately modeled by Monod kinetics and that the assumptions inherent in the use of the pseudo-first-order rate model were correct. Acetylene completely inhibited CO oxidation and methane oxidation (data not shown).

Inhibition of TCE oxidation. CO inhibited TCE oxidation by *Methylomonas* sp. strain MM2, and the rate of TCE oxidation decreased with increasing CO concentration (Fig. 3). The inhibition coefficient (K_i) was calculated from the respective CO concentration and the TCE oxidation rate k' (Table 1). The K_i for CO was 4.2 ± 1.7 µM. Methane also inhibited TCE oxidation, decreasing TCE oxidation rates as the methane concentration was increased (Fig. 4). The K_i for methane was 116 ± 13 µM (Table 2). For both experiments, the correlation coefficients describing the fit of the data to the model ranged from 0.98 to 1.00, indicating that the data were adequately modeled by the pseudo-first-order competitive inhibition model. Formate was added in both experiments. In a similiar experiment in which formate was not added, CO at 7, 20, and 33 µM resulted in complete inhibition of TCE oxidation at all three CO concentrations, and no evidence of TCE oxidation was observed during the 3 days the bottles were monitored (data not shown).

Inhibition of methane oxidation by CO. CO inhibited methane oxidation, and the extent of inhibition increased as the CO concentration increased (Fig. 5). The CO and methane were simultaneously oxidized; however, in all cases CO disappeared before methane did, as exemplified in Fig. 6 for 25 µM CO. A similar behavior was observed for all concentrations used. In the bottles containing both methane and CO, no significant methane oxidation occurred until formate was added (Fig. 5 and 6). In a similar experiment (data not shown), formate was provided at the same time the methane and CO were added, and there was no lag in the methane utilization.

CO toxicity. Replicate bottles of identical subcultures were incubated for 17 h with formate, with CO (bottles A and B), and without CO (bottles C and D). After incubation, CFU were enumerated, then the CO was removed, and the

TABLE 1. Calculation of K_i for CO inhibition of TCE oxidation

CO concn (µM) (I)	TCE oxidation rate (k' [liter mg^{-1} day^{-1}])[a]	Inhibition coefficient[b] (K_i [µM])
0	0.86	
6.7	0.43	6.7
25.6	0.11	3.7
53.5	0.04	2.7
57.3	0.05	3.9

[a] Pseudo-first-order rate coefficient $k' = k/(K_s[1 + I/K_i])$.
[b] The mean inhibition coefficient was 4.2 ± 1.7 µM.

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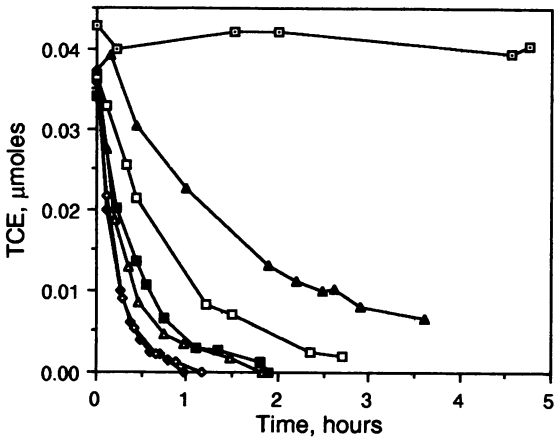

FIG. 4. Competitive inhibition of TCE oxidation by methane. Total micromoles of TCE in the bottles are plotted against time in hours. The aqueous TCE concentration at 0.035 µmol was 0.23 µM. The cell densities were 0.12 to 0.15 g of cells (dry weight) liter^{-1}. Bottles were amended with 2 mM formate. Symbols: ◆, no methane, replicate 1; ◇, no methane, replicate 2; △, 111 µM methane; ■, 225 µM methane; □, 479 µM methane; ▲, 998 µM methane; ⊡, control (sterile mineral medium blank).

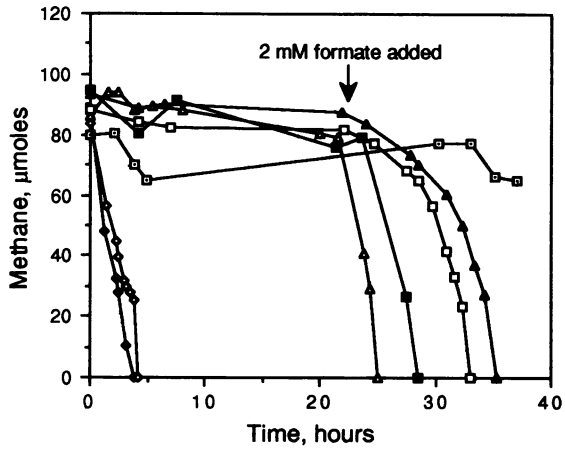

FIG. 5. Inhibition of methane oxidation by CO. Significant oxidation of methane did not occur until 2 mM formate was added to the bottles. Total micromoles of methane in the bottles are plotted against time in hours. The aqueous methane concentration at 90 µmol was 23 µM. The cell density was 0.27 g of cells (dry weight) liter^{-1}. Symbols: ◆, no CO, replicate 1; ◇, no CO, replicate 2; △, 10 µM CO; ■, 18 µM CO; □, 25 µM CO; ▲, 29 µM CO; ⊡, control (sterile mineral medium blank).

subcultures were incubated with methane. There was no difference in the methane utilization rates between bottles A and B, which had been incubated with a concentration of CO greater than that used in the above experiments (66 µM CO), and bottles C and D, which had not been exposed to CO (Fig. 7). There was also no difference in number of viable cells, with (1.44 ± 0.13) × 10^8 CFU for bottles A and B and (1.39 ± 0.08) × 10^8 CFU for bottles C and D.

DISCUSSION

Our data indicate that CO is a potent inhibitor of TCE oxidation and methane oxidation in *Methylomonas* sp. strain MM2. One mode of inhibition appears to be consumption of reducing power. The oxidation of CO requires electrons. The addition of formate as an exogenous source of electrons doubled the CO oxidation rate (Fig. 2). CO concentrations that merely reduced the TCE oxidation rate when formate was present (Fig. 3) caused complete inhibition of TCE oxidation when no formate was provided. In the concurrent oxidation of CO and methane, the oxidation of both substrates was inhibited by the lack of reducing power until formate was added (Fig. 5 and 6). This correlates with the findings of researchers who observed complete inhibition of methane oxidation in whole cells and partial or no inhibition in cell extracts (4, 9–11, 43, 44). In all of the experiments with whole cells, no exogenous source of reducing power had been added (9–11, 43), whereas a source of reductant was added when cell extracts were used (4, 10, 11, 43, 44). It is possible that the complete inhibition of methane oxidation observed in whole cells in these studies resulted from lack of reducing power, in that CO oxidation depleted the cells of available reductant and was preventing the regeneration of reductant by blocking methane oxidation through competitive inhibition. In one study with whole cells of *M. capsulatus* (Bath) (45), CO was not oxidized when no exogenous supply of electrons was provided but was oxidized when formaldehyde was supplied as a source of reductant. CO toxicity was not the cause of inhibition in the studies with whole cells, because methanol utilization was not inhibited (4, 9–11, 43, 44). (CO inhibits growth in the majority of

TABLE 2. Calculation of K_i for methane inhibition of TCE oxidation

Methane concn (µM) (I)	TCE oxidation rate (k' [liter mg^{-1} day^{-1}])[a]	Inhibition coefficient[b] (K_i [µM])
0 (replicate 1)	1.24	
0 (replicate 2)	1.24	
111	0.60	102
225	0.44	123
479	0.26	130
998	0.12	107

[a] Pseudo-first-order rate coefficient $k' = k/(K_s[1 + I/K_i])$.
[b] The mean inhibition coefficient was 116 ± 13 µM.

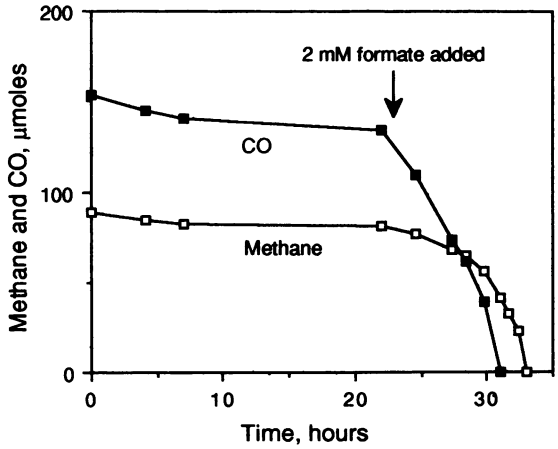

FIG. 6. Simultaneous oxidation of CO and methane for 25 µM CO data from Fig. 5. CO disappeared before methane did. A similar behavior was observed for all CO concentrations.

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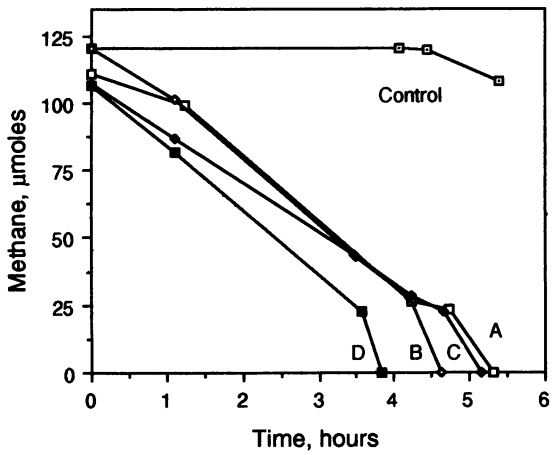

FIG. 7. Evaluation of CO toxicity: effect of incubation for 17 h with CO on subsequent methane utilization. Replicate subcultures in bottles A and B received 66 µM CO, whereas bottles C and D received no CO. Total micromoles of methane in the bottles are plotted against time in hours. The aqueous methane concentration at 110 µmol was 28 µM. The cell density was 0.18 g of cells (dry weight) liter^{-1}. The control was a sterile mineral medium blank.

aerobic microorganisms by binding to the terminal oxidase [8]). CO at 66 µM, a concentration greater than those used in our inhibition studies, was not toxic to *Methylomonas* sp. strain MM2.

CO also competitively inhibited TCE oxidation, as did methane. The K_i for CO, 4.2 µM, was much less than that observed for methane, 116 µM. The K_i for methane is greater than most K_s values reported for methane oxidation by whole cells (16, 27, 30, 36, 42, 48, 52) but is comparable to the K_s reported for *M. trichosporium* OB3b by Oldenhuis et al. (92 µM) (34) and is less than the K_s reported for crude extracts of *M. capsulatus* (Bath) (160 µM) (6). The K_i for CO is comparable to the K_s values for CO reported for *M. methanica* (3 µM) (11) and ammonia oxidizers (0.1 to 14 µM) (28, 49) and to the K_i for CO inhibition of ammonia oxidation by *Nitrosomonas europaea* (3 µM) (46). In *M. trichosporium* OB3b, the K_s values for several chlorinated aliphatic compounds were less than the K_s for methane, in particular for dichloromethane and 1,1-dichloroethylene, which had K_s values of 4 and 5 µM, respectively (34).

During TCE oxidation by *Methylomonas* sp. strain MM2, approximately 9 mol% of the TCE was transformed to CO. This is consistent with our previous work with ^{14}C-labeled TCE, in which approximately 75% of the radiolabel remained in aqueous intermediates and the remaining 25% was partitioned into carbon dioxide and cells (21, 22). The labeled carbon dioxide was most likely produced by the oxidation of CO and formate, and at least a portion of the radiolabel in the cells could have come from incorporation of labeled carbon from the carbon dioxide. A study with ^{14}C-labeled carbon dioxide showed that *Methylomonas* sp. strain MM2 incorporates carbon from carbon dioxide (unpublished data), as do many methanotrophs, including several other strains of *Methylomonas* sp. (39). Fox et al. (13) reported that 88 mol% of TCE was transformed to CO and formate in their work with the purified MMO from *M. trichosporium* OB3b. The relative ratios of compounds produced from the breakdown of the reactive intermediate TCE epoxide was shown to be dependent on pH and other factors (24, 33). Therefore, it can be expected that variable and potentially large amounts of CO will be produced, depending upon the surrounding environment and the concentration of TCE.

Given the marked inhibitory effect of CO on TCE oxidation, there is a potential for the inhibition of TCE oxidation by the CO produced in the TCE transformation process. The extent of inhibition would depend on a number of factors: the relative affinities of the enzyme for CO and TCE, the availability of reductant, the amount of CO produced, and the removal of CO by other organisms in the consortium. The reported K_s values for TCE for mixed and pure methanotrophic cultures range from 4 to 145 µM (1, 3, 22, 34). In *Methylomonas* sp. strain MM2, competitive inhibition of TCE oxidation by the CO produced is not likely to be significant, since the K_i for CO (4.2 µM) is comparable to the K_s for TCE (3.9 µM) (22), and only 9 mol% of the TCE was transformed to CO. If the methanotrophs in a process application do have a low K_i for CO similar to that of *Methylomonas* sp. strain MM2, and a high K_s for TCE similar to that reported for *M. trichosporium* OB3b (145 µM) (34), then the potential for competitive inhibition would be greatly pronounced, particularly if the amount of CO produced was as great as that observed in the work with the purified MMO from *M. trichosporium* OB3b (13). Together with TCE oxidation, CO oxidation may drain the cell of endogenous reducing power. If methane is not concurrently available or if CO concentrations are so high that methane oxidation is blocked, an exogenous source of reducing power will be required. Formate enhances TCE oxidation in the absence of methane (1, 23, 35) and was used during the in situ biodegradation study conducted at the Moffett Naval Air Station in California (40). However, formate addition on a large scale is expensive, and continued provision of formate could result in the enrichment of nonmethanotrophic biomass capable of growth on formate but incapable of transforming chlorinated aliphatic compounds.

Inhibition by CO in mixed cultures may not be as pronounced as that observed for pure cultures. Other bacteria capable of oxidizing or utilizing CO could be present among the diverse microorganisms in the subsurface environment or in a waste treatment bioreactor, thus mitigating the inhibitory effect of CO on the methanotrophic population. Bacteria that oxidize and utilize CO are "ubiquitously distributed in nature" (32). Bacteria that metabolize CO have been isolated from a wide variety of sources, including soils, waters, wastewaters, sewage sludges, and oligotrophic environments (7, 29, 32, 53).

The effect of CO as well as other transformation intermediates should be considered in the modeling of TCE oxidation, and it may be necessary to include a term that describes both the competitive inhibition by CO and the requirement for reducing power. Since CO oxidation has the same requirement for reductant that the TCE oxidation does but, unlike TCE oxidation, does not produce toxic products that inactivate the cell (1, 23, 34, 50), CO may be an ideal cometabolite with which to work in evaluating the effects of reductant availability on cometabolism. In process applications, the production of sufficient CO from TCE oxidation to have a significant effect on the methanotrophs would depend upon the operational parameters and the nature of the bacterial population. TCE oxidation may not be the only source of CO in a process application. Toxic concentrations of CO can contaminate air supplied by air compressors (15). Since CO is not only inhibitory to methane and TCE oxidation but also is toxic to most aerobic microorganisms

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(8), the source and purity of the air used in aerobic treatment processes should be evaluated.

ACKNOWLEDGMENTS

This work has been funded by EPA grant EPA CR 812220, awarded to Paul V. Roberts (D. Grbić-Galić was a Co-Principal Investigator on that project); by EPA grant EPA R815738-01-OCS awarded to D. Grbić-Galić; and by a grant from the Switzer Foundation awarded to S. M. Henry.

We thank Craig Criddle for thoughtful discussions and guidance with modeling and TCE analysis and Peter Adriaens and Ned Black for reviewing the manuscript.

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