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Toxicity of 1,1,1-Trichloroethane and Trichloroethene on a Mixed Culture of Methane-Oxidizing Bacteria

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The influence of trichloroethene (TCE; 0 to 65 mg/liter) and 1,1,1-trichloroethane (1,1,1-TCA; 0 to 103 mg/liter) on methane consumption of a mixed culture of methane-oxidizing bacteria was studied in laboratory batch experiments. Increasing concentrations of TCE or 1,1,1-TCA resulted in decreasing methane consumption. Methane consumption was totally inhibited at a concentration of 13 mg of TCE per liter, while methane consumption was still observed at the upper studied concentration of 103 mg of 1,1,1-TCA per liter. The inhibition of methane consumption by TCE depended on the initial concentration of methane. A model accounting for competitive inhibition between methane and TCE or 1,1,1-TCA was used to simulate methane consumption at various concentrations of TCE or 1,1,1-TCA. The simulations indicated that competitive inhibition may be the mechanism causing the inhibitory effect of TCE on methane consumption, while this does not seem to be the case for 1,1,1-TCA.

Chlorinated aliphatics are among the most widespread contaminants in soil and groundwater due to their common use in industry for degreasing and dry cleaning and as solvents (19, 23). One possible method to remediate soils and groundwaters contaminated with chlorinated aliphatics is biological treatment utilizing the ability of methanotrophic bacteria to degrade chlorinated aliphatics during methane and oxygen consumption. However, the applicability of this technique may be hampered by the toxicity to the methanotrophic bacteria of the chlorinated aliphatics when present at high concentrations. In Denmark, trichloroethene (TCE) and 1,1,1-trichloroethane (1,1,1-TCA) have been observed at high concentrations (TCE at 9 mg/liter and 1,1,1-TCA at 13 mg/liter) in groundwater near two contaminated sites (K. Christiansen and S. Vedby, First Int. Meet. NATO/CCMS Pilot Study on Remedial Action Technologies for Contaminated Land and Groundwater, 1987). Because of the expected relatively low cost of biological treatment of contaminated soil and groundwater compared with that of other available techniques, it is important to determine the toxicity of TCE and 1,1,1-TCA to methane-oxidizing bacteria to evaluate the highest contaminant concentrations that can be treated efficiently by this method.

Aerobic biodegradation of chlorinated aliphatics by methane-oxidizing bacteria obtained from soil or groundwater has been shown in several studies (8, 13, 14, 17, 27) but primarily at quite low concentrations of chlorinated aliphatics (<500 μg/liter). Only a few studies (7, 16) have dealt with the effect of chlorinated aliphatics present at high concentrations (>1 mg/liter) on methane-oxidizing bacteria. These studies showed inconsistent results, and, furthermore, the fundamental mechanisms have not been examined.

The purpose of this study was to examine the effect of high concentrations of TCE and 1,1,1-TCA on a mixed culture of methane-oxidizing bacteria. A mechanistic methane consumption model incorporating competitive and noncompetitive inhibition of methane oxidation by TCE or 1,1,1-TCA was used for interpretation of the results.

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MATERIALS AND METHODS

Description of batch experiments. The batch experiments were carried out in 117-ml glass bottles equipped with Miniert valves (DYNATECH Precision Sampling Corp., Baton Rouge, La.), which enable frequent air sampling from the bottles. Each bottle contained inoculum and mineral medium to a total of 10 or 20 ml, methane at different initial concentrations at 0.3, 1.8 and 10.8% (vol/vol) of the headspace, and TCE or 1,1,1-TCA at different initial concentrations. Two stock solutions of TCE and 1,1,1-TCA were prepared by dissolving a known volume of TCE or 1,1,1-TCA in a nutrient solution. Different volumes of stock solution were added to the batches to achieve the desired concentrations of TCE or 1,1,1-TCA. Nutrient solution was added to each bottle to give the same final volume. Methane was added with a syringe. The headspace was atmospheric air. The bottles were incubated in the dark at 10°C, which is the groundwater temperature in Denmark. All experiments were carried out in duplicate or triplicate. The control bottles contained 0.3 g of sodium azide (NaN_3) per liter to kill the mixed culture. The concentrations of methane, TCE, and 1,1,1-TCA were monitored by taking air samples with a gas-tight syringe from each bottle at different times. The measured methane concentrations were normalized with the initial concentration before the remaining portion of methane relative to the control experiment was calculated. Protein concentrations were measured in water samples taken from bottles sacrificed before sampling. The batches for protein measurements were prepared in the same way as the other batches, but were capped with Teflon-coated rubber septa and sealed with aluminium caps instead of Miniert valves.

Microorganisms. A mixed culture of bacteria grown on methane as the sole organic carbon source constituted the microbial consortium. The culture originated from the upper aerobic sediment from Lake Lyngby, Denmark. The sediment was mixed in a nutrient solution to extract the bacteria from the sediment. After the sediment had settled, a water sample was transferred to a new nutrient solution and methane was added. Weekly, the culture was transferred to a new nutrient solution and methane was added. The nutrient solution contained the following macro- and micronutri-

ents per liter of distilled water: 5.8 mg of $Na_2HPO_4 \cdot 2H_2O$, 3.8 mg of NH_4Cl, 147 mg of $CaCl_2$, 122 mg of $NaNO_3$, 100 mg of $NaHCO_3$, 9.4 mg of KCl, 0.4 mg of $FeCl_3 \cdot 6H_2O$, 50 mg of $MgSO_4 \cdot 7H_2O$, 10 μg of $MnCl_2 \cdot 4H_2O$, 12 μg of $ZnSO_4 \cdot 7H_2O$, 6 μg of $CuSO_4 \cdot 5H_2O$, 6 μg of $CoCl_2 \cdot 6H_2O$, 5.5 μg of $Na_2MoO_4 \cdot 2H_2O$, and 2 μg of $Na_2B_4O_7 \cdot 10H_2O$. The pH of the solution was 7.

Chemicals. Methane, >99.95% pure, was purchased from A/S Dansk Ilt & Brintfabrik, Denmark. Sodium azide, >99%, and TCE, >99.5%, were purchased from Merck, Darmstadt, Federal Republic of Germany; 1,1,1-TCA, >99.0%, and bromotrichloromethane, >98%, were from Fluka AG, Buchs, Switzerland. Coomassie brilliant blue G-250 and bovine albumin for protein standards were purchased from Sigma Chemical Co., St. Louis, Mo. The nutrients were of reagent grade quality.

Analytical procedures. Methane and chlorinated aliphatics were analyzed on a DANI 8520 gas chromatograph equipped with a flame ionization detector (for methane) and an electron capture detector (for chlorinated aliphatics) and a 30-m wide coated open tubular fused silica column (0.53 mm inside diameter) coated with CP-SIL5 cross-bounded. The gas chromatograph was operated isothermally at a column temperature of 50°C and a detector temperature of 275°C. The carrier gas was nitrogen. Data acquisition and integration were performed on a MAXIMA chromatography work station. Air samples for methane were injected with a gas-tight syringe, and the concentrations were determined by comparison with a standard curve. The concentrations of 1,1,1-TCA and TCE were measured in air samples. Samples with low concentrations of TCE and 1,1,1-TCA were injected directly, and the concentrations were determined by comparison with a standard curve made in pentane. Samples with high concentrations of TCE and 1,1,1-TCA were dissolved in pentane containing bromotrichloromethane as internal standard, before a sample of pentane was injected. The concentrations were determined by comparison with a standard curve made in pentane containing internal standard.

Protein measurements were performed on liquid samples to which 3 M trichloroacetic acid solution was added to kill the bacteria before storage at 4°C. The samples were centrifuged for 20 min at 4,000 rpm at a Heraeus Christ Labofuge 111. The supernatant was discarded carefully, and 0.66 N sodium hydroxide solution was added to the pellet to lyse the bacteria. After incubation for 2 days at 35°C and staining with Coomassie brilliant blue G-250, the protein content was measured spectrophotometrically at 595 nm with a Beckman DB GT grating spectrophotometer. The preparation of Coomassie brilliant blue G-250 and the protein standards are described by Bradford (5).

Mathematical model for inhibition of methane degradation with TCE or 1,1,1-TCA. The degradation rate of methane without any inhibitor present can be described by the following Monod batch equation (4) adapted for two-phase (air and liquid) systems:

$$-dS/dt = V_L/(V_L + F\,V_A) \times X\,k\,S/(S + K_s) \quad (1)$$

where S is the methane concentration (milligrams per liter); X is the cell concentration (milligrams of cells per liter); k is the maximum utilization rate (grams of methane per gram of cells per day); K_s is the methane saturation constant (milligrams per liter); V_L and V_A are volume of liquid and air phases, respectively (liters); and F is the partition coefficient between air and liquid for methane (dimensionless).

The growth rate of bacteria can be described by the following equation accounting for bacterial decay (4):

$$dX/dt = X\,Y\,k\,S/(S + K_s) - X\,b \quad (2)$$

where Y is the yield coefficient (grams of cells per gram of methane) and b is the decay constant (day^{-1}).

Equations 1 and 2 may be modified also to account for competitive inhibition (or other inhibition models).

Competitive inhibition occurs when two substrates are converted by the same enzyme, resulting in competition between the two substrates. Mathematically, the saturation constant for methane (K_s) becomes a function of the concentration of TCE or 1,1,1-TCA and can be expressed as (2):

$$K_s(1 + C/K_i) \quad (3)$$

where C is the concentration of the secondary substrate (milligrams per liter) and K_i is the inhibition constant (milligrams per liter).

The assumptions of the model are that (i) the growth of bacteria is limited only by methane and not by oxygen or nutrients, (ii) the secondary substrate itself inhibits the methane consumption rate and not some intermediary degradation products of either methane or the secondary substrate, and (iii) the system is in equilibrium.

(i) The initial ratios of oxygen to methane in the experiments were 70, 11, and 1.7, which were sufficient to avoid oxygen depletion, because 1 mol of methane requires <1.7 mol of oxygen during growth of methane-oxidizing bacteria according to the utilization constant (see Table 1). The concentration of oxygen influences the degradation rate of methane and the growth rate of bacteria and can be incorporated in equations 1 and 2 by addition of a further Monod term (4). The saturation constant for oxygen is on the order of 0.01 to 0.1 mg/liter (18, 26), which means that the oxygen-based Monod term is close to 1 and can be neglected when oxygen concentrations exceed 1 mg/liter. Enhanced concentrations of nitrate (732 mg of $NaNO_3$ per liter) and phosphate (174 mg of $Na_2HPO_4 \cdot 2H_2O$ per liter) did not result in a faster degradation of methane (initial concentration of methane, 0.45 mg/liter), indicating that the degradation was not limited by nitrogen or phosphate.

(ii) The pathway for methane degradation is well known, and the rate-limiting step in the oxidation of methane to carbon dioxide is the initial oxidation of methane to methanol (1), so accumulation of intermediary products is unlikely to occur. A proposed pathway for TCE degradation indicates an epoxidation of TCE to trichloroepoxide, which is further abiotically transformed to either two-carbon compounds, which are oxidized by heterotrophic bacteria, or formate and carbon monoxide, which are oxidized by methanotrophic bacteria (12, 17). It is unlikely that any of these compounds directly influences methane monooxygenase. No pathway for 1,1,1-TCA degradation has been demonstrated.

(iii) The period of the experiments was longer than 10 days, supposedly sufficient to approach equilibrium.

Equations 2 and 3 were solved by a simple numerical method, and a program for simulating the nonlinear equations was used to estimate the appropriate parameters. The best fit was found by combining visual comparisons between fitted and measured curves and calculated standard deviations (σ) of the residuals between measured and fitted data and calculated coefficient of determinations (r^2) (15). The coefficient of determination represents the proportion of the

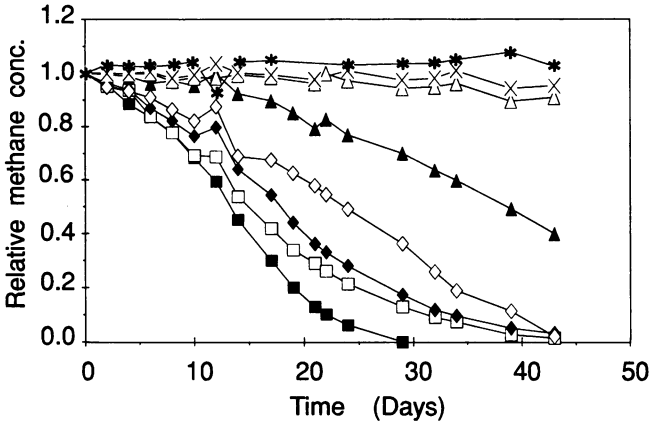

FIG. 1. Biodegradation of methane by a mixed culture of methanotrophs in the presence of the following concentrations of TCE: 0 (■), 0.3 (□), 1.7 (◆), 3.1 (◇), 6.5 (▲), 13 (△), 23 (×), and 65 (*) mg/liter. The initial concentration of methane was 0.45 mg/liter. The volume ratio of air to liquid was 107:10.

total variation of the observations that can be explained by the model.

RESULTS

The methane-oxidizing culture obtained from a lake sediment tended to grow in suspended colonies. The culture had the capability to degrade TCE and 1,1,1-TCA when grown on methane (data not shown). The culture was also able to grow on other primary substrates such as methanol and propane.

Toxicity of TCE. Figure 1 shows the consumption of methane at various concentrations of TCE in the range of 0 to 65 mg/liter in liquid. The initial concentration of methane was 1.8% (vol/vol) in the headspace, which equals 0.45 mg/liter in liquid. The growth of biomass measured as protein concentrations at TCE concentrations of 0, 6.5, 23, and 65 mg/liter is shown in Fig. 2. The initial concentration of protein was 2.1 mg/liter, which equals 8.4×10^6 bacteria per ml, using conversion factors of 0.5 mg of protein per mg (dry weight) (21) and 2×10^{12} bacteria per g (dry weight) (3).

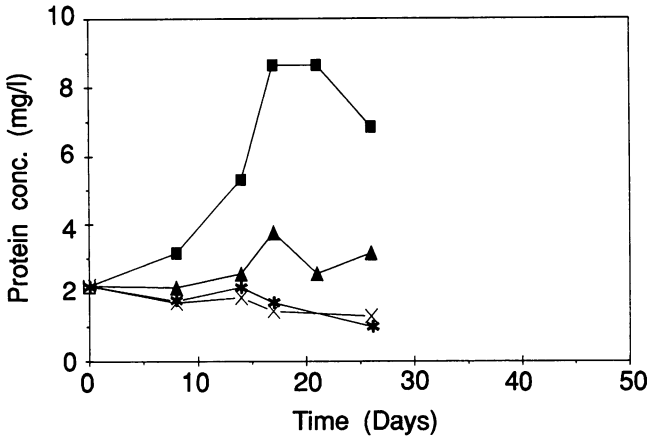

FIG. 2. Growth of a mixed culture of methanotrophs measured as protein in batch experiments in the presence of the following concentrations of TCE: 0 (■), 6.5 (▲), 23 (×), and 65 (*) mg/liter. The corresponding degradation of methane is shown in Fig. 1.

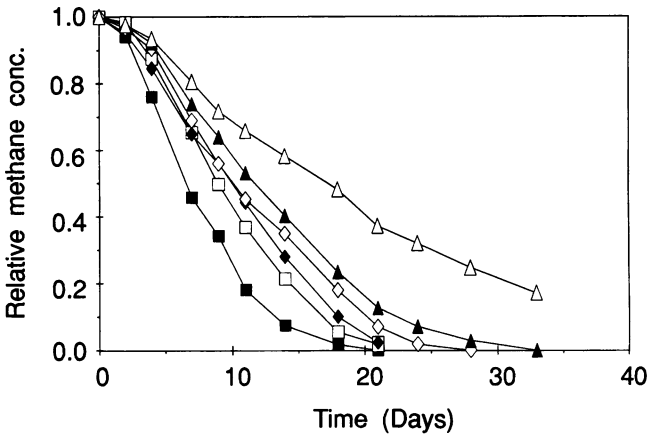

FIG. 3. Biodegradation of methane by a mixed culture of methanotrophs in the presence of the following concentrations of 1,1,1-TCA: 0 (■), 1(□), 8.6 (◆), 24 (◇), 48 (▲), and 103 (△) mg/liter. The initial concentration of methane was 0.45 mg/liter. The volume ratio of air to liquid was 97:20.

The percent TCE degradation was calculated as $[(C_{control} - C_{degradation})/C_{control}] \times 100$, where C is the concentration of TCE. The observed TCE degradation was 13 to 22% in the experiments with TCE concentrations of 0.3 to 6.5 mg/liter. No TCE degradation was observed at the other concentrations, which corresponds to the observed lack of methane consumption. The fastest degradation of methane and fastest growth of biomass were observed in the experiment without any TCE. The presence of increasing TCE concentrations decreased the methane consumption rate. Methane degradation was totally inhibited for 43 days at a TCE concentration of 13 mg/liter.

Toxicity of 1,1,1-TCA. Figure 3 shows the consumption of methane at various concentrations of 1,1,1-TCA in the range of 0 to 103 mg/liter. The initial concentration of methane was 1.8% (vol/vol) in the headspace, and the initial protein concentration was 4.4 mg/liter (= 18×10^6 bacteria per ml). For TCE, the methane consumption rate decreased with increasing concentrations of 1,1,1-TCA. No degradation of 1,1,1-TCA was observed in this experiment, which corresponds to previously reported experiments in which the degradation rate for 1,1,1-TCA was found to be lower than that for TCE (13). The time period necessary to achieve total degradation of methane was longer in the experiment with TCE than in that with 1,1,1-TCA because of a smaller volume ratio of liquid to air. A total inhibition of the methane-oxidizing bacteria was not observed at concentrations of 1,1,1-TCA as high as 103 mg/liter, which was quite different from the observation with TCE (total inhibition at 13 mg/liter).

Competition between TCE and methane. The toxicity experiments with TCE and 1,1,1-TCA were conducted with a fixed initial concentration of methane (0.45 mg/liter). A supplementary experiment was carried out to examine the effect of different initial concentrations of methane on the inhibition of methane degradation by TCE. The initial concentrations of TCE were 0.05, 0.3, and 1.8 mg/liter. Figure 4 presents the results from experiments with initial concentrations of methane of 0.08, 0.45, and 2.7 mg/liter. The methane degradation was inhibited by TCE at the highest concentration of TCE (1.8 mg/liter) only when the initial concentration of methane was 0.08 or 0.45 mg/liter (Fig. 4A and B). At the

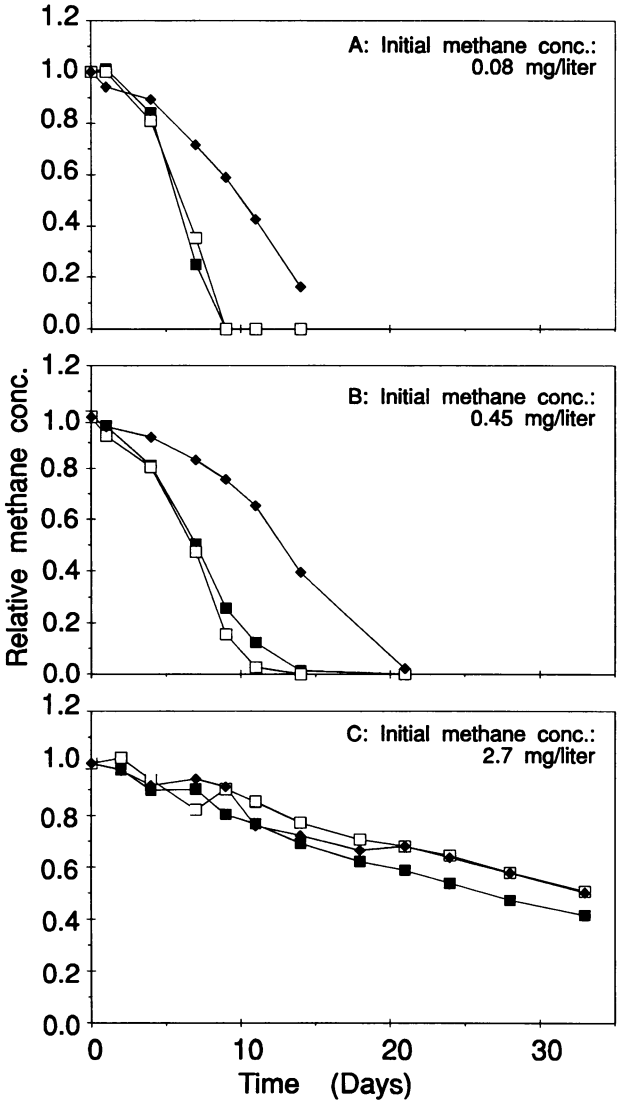

FIG. 4. Biodegradation of methane by a mixed culture of methanotrophs in the presence of the following concentrations of TCE: 0.05 (■), 0.3 (□), and 1.8 (◆) mg/liter. The volume ratio of air to liquid was 107:10.

highest initial concentration of methane (2.7 mg/liter), no inhibition of methane degradation was observed (Fig. 4C), indicating that a high initial concentration of methane prevents inhibition of methane-oxidizing bacteria by TCE.

Modeling the inhibition of methane degradation by TCE or 1,1,1-TCA. The basic parameters (k, K_S, Y, and b) were estimated by using the methane consumption curves from both toxicity experiments without any TCE or 1,1,1-TCA (Fig. 1 and 3) and the protein concentration curve for TCE (Fig. 2). The initial concentrations of methane and biomass and the estimated parameters are shown in Table 1 together with some values from the literature. To obtain the best fit for both methane consumption curves, slightly different constants were used for the two simulations. The four parameters are confounded, which makes it impossible to estimate each parameter separately. The coefficient of determination (r^2) was better than 0.99 for the methane consumption curves and 0.63 for the protein concentration curve. The low r^2 obtained for the simulation of the protein curve was due to relatively few observations and to relatively high uncertainty of the protein analysis. Figures 5 and 6 show the best simulations of the influence of TCE and 1,1,1-TCA on methane consumption, assuming a model accounting for competitive inhibition. The concentrations of TCE or 1,1,1-TCA were assumed to be constant during the experiments, which was the case for 1,1,1-TCA, while up to 22% of TCE was degraded during the experiment. The initial concentrations of TCE were used as the constant concentrations during the simulations. The inhibition constants (K_i) for TCE and 1,1,1-TCA are the only parameters expressing the effect of varying TCE and 1,1,1-TCA concentrations. The calculated r^2 and σ are given in the legends to Fig. 5 and 6.

DISCUSSION

The results of the toxicity experiments revealed a decreasing methane consumption for increasing concentrations of either TCE or 1,1,1-TCA (Fig. 1 and 3), which can be explained by a competition between methane and the secondary substrate for the same enzyme, methane monooxygenase. This theory also explains the observed lack of inhibition (Fig. 4C) at higher initial concentrations of methane.

The higher inhibitory effect observed for TCE than for 1,1,1-TCA is probably due to a higher affinity of TCE for methane monooxygenase, which also is reflected by the higher degradation rate of TCE versus 1,1,1-TCA (13). In

TABLE 1. Estimated and fixed parameters used to model the degradation of methane in the presence of various concentrations of TCE or 1,1,1-TCA and values from the literature

TCE or 1,1,1-TCA	Initial concn (mg/liter)[a] of:		Utilization constant, Y (g of protein/g of methane)[b]	Maximum utilization rate, k (g of methane/g of protein per day)[b]	Saturation constant, K_s (mg/liter)[b]	Decay constant, b per day[b]	Inhibition constant, K_i (mg/liter)[c]
	Methane	Protein					
TCE	0.45	2.15	0.20	1.72	0.20	0.12	12
1,1,1-TCA	0.45	4.40	0.20	1.50	0.22	0.12	1.9
Literature values[d]			0.26–0.55 (references 10, 11, 20, 24, and 26)	3.6–10 (references 11 and 20)	0.24–0.42 (references 6, 9, and 25)	0.33–0.4 (reference 11)	

[a] Measured values which were constant during the simulations.

[b] Y, k, K_s, and b were estimated on the basis of methane consumption curves without any TCE or 1,1,1-TCA and the corresponding protein curve. These values were constant during the simulations of competitive inhibition.

[c] The inhibition constants were estimated on the basis of simulation of methane consumption curves in the presence of different concentrations of TCE or 1,1,1-TCA.

[d] Literature values are based on biomass measured as milligrams (dry weight) per liter and converted to milligrams of protein per liter, using a conversion factor of 0.5 mg of protein/mg (dry weight) (21). Most of the literature values were measured at temperatures higher than 30°C.

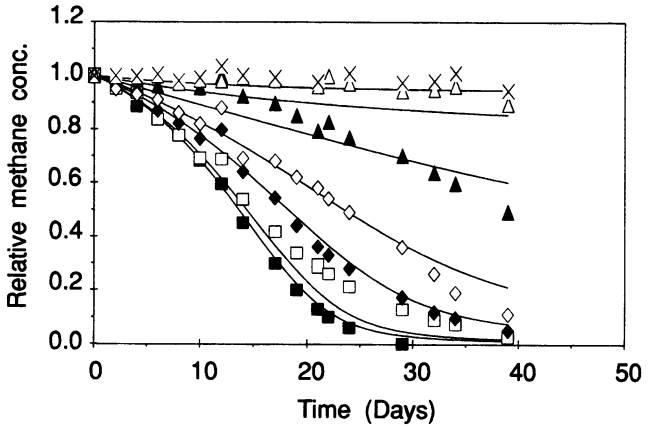

FIG. 5. Simulations by the competitive inhibition model of the biodegradation of methane (Fig. 1) in the presence of the following concentrations of TCE: 0 (■), 0.3 (□), 1.7 (◆), 3.1 (◇), 6.5 (▲), 13 (△), and 23 (×) mg/liter. The parameters used in the model are shown in Table 1. The coefficient of determination (r^2) and the standard deviation (σ), respectively, at each concentration were calculated to be: at 0 mg/liter, 0.9967 and 0.020; 0.3 mg/liter, 0.9646 and 0.061; 1.7 mg/liter, 0.9916 and 0.029; 3.1 mg/liter, 0.9699 and 0.048; 6.5 mg/liter, 0.8336 and 0.065; 13 mg/liter, not determined and 0.067; 23 mg/liter, not determined and 0.037.

contrast to the results reported here, Janssen et al. (16) did not observe any significant difference between the concentrations of TCE and 1,1,1-TCA causing toxic effects on the bacteria, probably due to differences between the methane-oxidizing cultures.

The methane consumption curves for different concentrations of TCE were fairly well simulated by the model accounting for competitive inhibition (Fig. 5). The inhibition constant was estimated to be 12 mg/liter for TCE. The same model failed to simulate methane consumption at different concentrations of 1,1,1-TCA (Fig. 6), indicating that competitive inhibition is not the dominating mechanism describing the inhibition of methane consumption by 1,1,1-TCA. Models for noncompetitive inhibition (2) and a combination of noncompetitive and competitive inhibition (2) were also tested, but these models failed as well.

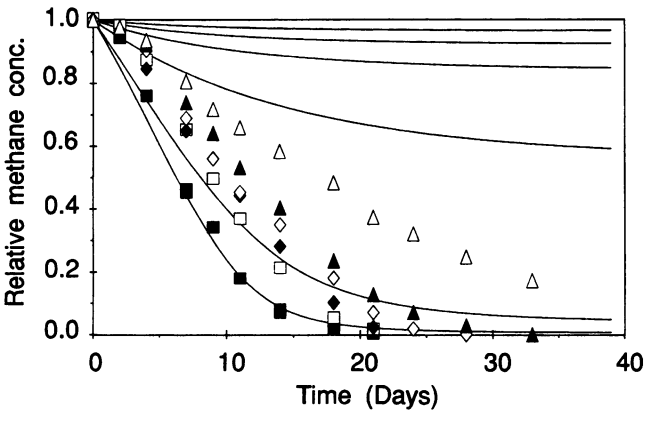

FIG. 6. Simulations by the competitive inhibition model of the biodegradation of methane (Fig. 3) in the presence of the following concentrations of 1,1,1-TCA: 0 (■), 1 (□), 8.6 (◆), 24 (◇), 48 (▲), and 103 (△) mg/liter. The parameters used in the model are shown in Table 1. The coefficient of determination (r^2) and the standard deviation (σ), respectively, at each concentration were calculated to be: at 0 mg/liter, 0.9908 and 0.049; 1 mg/liter, 0.9779 and 0.090; 8.6 mg/liter, 0.5614 and 0.407; 24 mg/liter, not determined and 0.600; 48 mg/liter, not determined and 0.645; 103 mg/liter, not determined and 0.502.

The results revealed that the time necessary to remediate contaminated soils or groundwaters depends on the concentrations of TCE and 1,1,1-TCA, because of the influence on the activity of methanotrophic bacteria. However, in some cases biological treatment may be impossible because of toxic effects of TCE or 1,1,1-TCA.

For TCE, total inhibition was observed at 13 mg/liter, which corresponds to a concentration in soil of 2.6 mg/kg assuming a distribution coefficient of 0.2 liter/kg, a representative value for sandy soils with a low content of organic carbon. This inhibitory soil concentration is not unrealistically high and may be exceeded near to the source of contamination. At lower TCE concentrations the methane consumption rate will be partially inhibited by TCE, resulting in a prolonged period to remediate contaminated soil or groundwater. The methane consumption rate needed for technical design may be estimated from the model accounting for competitive inhibition. The inhibition effect of TCE depends on the concentration of methane, but detrimental levels of TCE may occur that cannot be avoided by increasing the methane concentration.

For 1,1,1-TCA, no complete inhibition was observed in the concentration range tested in this study (<103 mg/liter), but a partial inhibition was observed. A groundwater concentration of 103 mg of 1,1,1-TCA per liter corresponds to a soil concentration on the order of 20 mg/kg, which normally would not occur at contaminated sites, indicating that toxic or inhibitory effects of TCE are more serious than those of 1,1,1-TCA.

ACKNOWLEDGMENT

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