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Effect of Chlorinated Ethenes on S_{min} for a Methanotrophic Mixed Culture

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The presence of chlorinated aliphatic hydrocarbons (CAHs) was found to increase the minimum methane concentration for net growth (S_{min}) of a methanotrophic mixed culture expressing particulate methane monooxygenase (pMMO). Without CAHs, S_{min} was 5 μg/L methane. S_{min}, however, increased to 20−25 μg/L in the presence of 0.05 mg/L 1,1-dichloroethylene (1,1-DCE), 2 mg/L *trans*-1,2-dichloroethylene (t-DCE), or 4 mg/L trichloroethylene (TCE). A lower maximum methane oxidation rate was required to model growth rates at these low methane concentrations, a result that was attributed to reducing energy limitation. A derived equation for possible factors by which CAHs increased S_{min} included reducing energy limitation, competitive inhibition, and transformation product toxicity. However, a simplified model that incorporated these effects into a single parameter was adequate to model the overall effect on growth rate and S_{min}.

Introduction

Biological degradation of chlorinated aliphatic hydrocarbons (CAHs) is an increasingly promising treatment alternative, because contaminants are destroyed and rendered nonhazardous. With *in situ* bioremediation, contaminants are treated in place, and desorption from soil may be accelerated. Under aerobic conditions, trichloroethylene (TCE) and the dichloroethylene isomers are mostly recalcitrant to degradation. However, bacteria containing oxygenase enzymes can cometabolically oxidize these and other CAHs, but require oxygenase-inducing substrates such as methane, propane, phenol, toluene, or ammonia to be active against CAHs (*1*−*3*).

With methanotrophic bacteria, both methane and CAHs are oxidized by methane monooxygenase (MMO). As a result, competitive inhibition of methane utilization by CAHs (*4*) and inhibition of CAH transformation by methane (*3, 5, 6*) are observed when present together. The products of CAH cometabolism have been shown to be toxic to the organism doing the transformation (*6*−*9*), although some CAHs exhibit much greater transformation product toxicity (TPT) than others (*10, 11*). Oxidation of methane or CAH by MMO also requires reducing energy from the cofactor nicotinamide adenine dinucleotide (NADH) (*12*). Whereas methane is further oxidized to regenerate the NADH consumed, CAHs are not. Thus CAH cometabolism may deplete the intracellular pool of necessary reducing energy (*13*).

The following model for simultaneous growth substrate utilization and CAH transformation with competitive inhibition and TPT was described previously (*14*) and has been cited by others in a modified form (*13*):

$$\mu = \frac{1}{X_a}\frac{\partial X_a}{\partial t} = Yk_s\left(\frac{S}{S + K_s(1 + C/K_c)}\right) - \frac{k_c}{T_c}\left(\frac{C}{C + K_c(1 + S/K_s)}\right) - b \quad (1)$$

growth rate = yield from substrate utilization − TPT − decay

where μ is the net growth rate (d^{-1}), X_a is the active biomass concentration (μg/L), t is the time (d), Y is the true biomass yield (μg of biomass/μg of methane), k_s is the methane maximum oxidation rate (μg of methane/μg of biomass-d), S is the methane concentration (μg/L), K_s is the methane half-saturation concentration (μg/L), C is the CAH concentration (mg/L), K_c is the CAH half-saturation concentration (mg/L), T_c is the transformation capacity (μg of CAH/μg of biomass), k_c is the CAH maximum oxidation rate (μg of CAH/μg of biomass-d), and b is the decay rate (d^{-1}). As suggested by the model, the addition of CAHs results in significant reductions in the net growth rate (*15, 16*). Growth rate is increased by substrate utilization and decreased by TPT from CAH oxidation and from decay due to endogenous respiration and predation.

A biofilm model was previously developed that considered cometabolism of CAHs in the presence of a primary growth substrate (*14*). The model was used for hypothesis testing and to identify the processes of significance that were in need of further evaluation. The results indicated the importance of the minimum substrate concentration for net growth (S_{min}), a concentration below which a culture will not grow and an active biofilm will not develop. Here the rate of new cell growth just equals the total loss rate of organisms due to endogenous respiration and other destructive processes, i.e., the net growth rate is zero. An equation for S_{min} (μg/L of methane) in the presence of CAHs can be derived from eq 1 by setting μ equal to zero and solving for methane concentration (*14*):

$$S_{min} = K_s\frac{\frac{C}{K_c}\left(\frac{k_c}{T_c} + b\right) + b}{Yk_s - b} \quad (2)$$

In the absence of CAHs $C = 0$, and the typical equation for S_{min} is obtained (*17*):

$$S_{min_{C=0}} = \frac{K_s b}{Yk_s - b} \quad (3)$$

The effects of CAH presence, type, and concentration on S_{min} were examined to determine, if possible, the relative importance in each case of decreased methane oxidation rate due to competitive inhibition, an increased death rate due to TPT, or the diversion of reducing energy from growth to CAH oxidation, thus decreasing bacterial yield.

Materials and Methods

Chemicals. Both 99.0% methane (Liquid Carbonic Specialty Gas Co., San Carlos, CA) and 1.00% methane in air (Scott Specialty Gases, Inc., Plumsteadville, PA) were used. CAH-saturated aqueous solutions (*8*) were used for addition of CAHs and were prepared with TCE (99+% pure ACS reagent, Aldrich Chemical Co., Milwaukee, WI), *trans*-1,2-dichloroethylene (t-DCE) (98% purity, Aldrich), and 1,1-dichloro-

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ethylene (1,1-DCE) (neat standard for EPA methods, Sigma Chemical Co., St. Louis, MO). Further dilution in mineral medium was required for 1,1-DCE (*15*).

Bacterial Inoculum. A methanotrophic mixed culture (*8*) served as the bacterial inoculum. The dominant methanotroph isolated from this mixed culture showed 92% 16S rRNA homology to *Methylosinus trichosporium* OB3b (*18*). Inoculum consistency was ensured by freezing aliquots of cells from the growth reactor for use in separate experiments. An aliquot was thawed, diluted, and grown up on methane prior to use as an inoculum in each experiment as described elsewhere (*15*). The dominant methanotroph in this mixed culture expressed soluble MMO (sMMO) under the copper-limited conditions maintained in the growth reactor (*18*). However, the inoculum cultures used for the studies reported here were first grown with a high copper to biomass ratio (at all times greater than 78 μmol/g) that relieved the copper limitation and caused a reversion to pMMO expression, as confirmed by a lack of detectable naphthalene oxidation (*19*). This is a typical response of type II methanotrophs such as *M. trichosporium* OB3b (*20, 21*).

Mineral Medium and Sample Bottle Preparation. Deionized water was first air-stripped to remove dissolved methane. The complete mineral medium (*22*) consisted of inorganic nutrients containing 0.074 μM copper, as well as 1.0 mg/L sodium thiosulfate, and 1 mM sodium bicarbonate, the latter added after autoclaving and cooling.

Bottles (250 mL) were prepared as described elsewhere (*15*). Test tubes (15 mL, Kimax), inserted upside-down, were used for methane addition. Medium was added so that 16.0 $\pm$ 0.1 mL headspace would remain after subsequent liquid additions. Tight control over headspace volume ensured reproducible aqueous methane concentrations.

Methane was added with a Pressure-Lok valved gas-tight syringe (Precision Sampling Co., Baton Rouge, LA) and a long "J"-shaped needle. The needle reached to the bottom of the bottle so that methane could be dispensed up into the inverted medium-filled test tube. CAH stock solutions were added beneath the liquid surface just before tightly sealing the bottles with both a Teflon-lined silicone septum and a Teflon-lined rubber septum. Controls received 220 mg/L sodium azide or no inoculum. (After sealing, bottles were briefly inverted to release the methane bubble from the inverted tube.)

S_{min} Evaluation. The value of S_{min} was determined in two phases. In the "incubation" phase, bottles containing a small biomass inoculum were given a range of methane solution concentrations (determined as described later) bracketing the anticipated S_{min} value. The methane concentration resulting in transition between no methane utilization and full utilization over the incubation period served as a preliminary estimate of S_{min}. A more rigorous evaluation resulted from the "enumeration" phase, which involved the estimation of active biomass concentrations before and after incubation in order to identify changes over the incubation period and to estimate net growth rates (μ_{inc}, d^{-1}). S_{min} was estimated as the incubation methane concentration causing no change in active biomass during the 34-d incubation period, thus corresponding to a zero net growth rate. The effect of CAH type and concentration on S_{min} was tested by adding aqueous concentrations of 0.05 mg/L 1,1-DCE, 1.9 mg/L t-DCE, or 1, 2, or 4 mg/L TCE to different sets of bottles also containing methane.

Incubation Phase. Most sample bottles were inoculated with 0.5 mL of a 6% dilution of pregrown cells expressing pMMO, giving an initial active cell concentration of approximately 0.0013 μg/L as estimated during the enumeration phase. Bottles with 4 mg/L TCE instead received 0.5 mL of a 30% dilution. Methane was added to provide aqueous concentrations ranging from 0 to 20 μg/L for bottles with no CAH added and at higher concentrations for bottles containing CAHs in anticipation of higher S_{min} values. The bottles were then incubated in the dark at 20 °C with 150 rpm rotary shaking. After 34 d, bottles were sampled for methane and CAHs. Of the three CAHs, only t-DCE removal was significantly greater than losses in controls. Appropriate volumes of t-DCE stock solution were added to restore the solution concentration to 1.9 mg/L prior to the enumeration phase. As explained in the Results and Discussion, methane removal exceeding 20% was used as a positive indicator of net growth.

Enumeration Phase and S_{min}. The purpose of the enumeration phase was to determine whether the active population of methanotrophic bacteria had increased ($\mu_{inc} > 0$), decreased ($\mu_{inc} < 0$), or remained the same ($\mu_{inc} = 0$) over the 34 d of the incubation phase in each bottle set containing a given CAH concentration. The methane concentration for which $\mu_{inc} = 0$ was designated as S_{min}.

The active methane-oxidizing population at the start of the 34-d incubation period and at the end were enumerated in the same way. Here, a 250-mL bottle received either 0.5 mL of methane (460 μg/L solution concentration) or, in the case of bottles inoculated with 2−4 mg/L TCE, 1.0 mL of methane (920 μg/L solution concentration). Headspace methane was then monitored daily until removed below the detection limit (0.1 μg/L in solution). From these results, the time required for 90% methane removal ($t_{90\%}$) was determined. The $t_{90\%}$ values are functions of starting population sizes. The above higher methane concentration used with 2 and 4 mg/L TCE was in order to increase the growth rate so that 90% methane depletion here occurred within a reasonable time. By comparing $t_{90\%}$ values for bottles after 34 d of incubation with a comparable subset of bottles enumerated before incubation began, it could readily be determined whether $t_{90\%}$ had increased, decreased, or remained the same; that is, whether population size had decreased, increased, or remained the same. For example, for bottles incubated with 1 mg/L TCE, comparisons were made among $t_{90\%}$ values determined for some bottles at day 0 and for the rest at day 34, but in both cases 1 mg/L TCE was present during the enumeration procedure.

While changes in $t_{90\%}$ values over the 34-d incubation period could be used to estimate S_{min} values for methane, it was thought better to convert the $t_{90\%}$ values into growth rate values, which are of more fundamental significance. This can be done rather simply by the following procedure. Using the appropriate growth rates (μ_{enum}, d^{-1}) previously measured for this culture in the absence and presence of CAHs (*15*), the $t_{90\%}$ values measured after 34 d ($t_{90\%,34}$) can be used to calculate relative changes in biomass concentration (X_{34}/X_0) using the exponential growth equation:

$$\ln(X_{34}/X_0) = \mu_{enum}\Delta t \tag{4}$$

where

$$\Delta t = \overline{t_{90\%,0}} - t_{90\%,34} \tag{5}$$

and where $\overline{t_{90\%,0}}$ is the average time to 90% methane removal in bottles at incubation day 0 (d) and $t_{90\%,34} = t_{90\%}$ for a bottle at incubation day 34 (d). The additional Δt needed to remove the added methane by the initial culture is related to the ratio of X_{34} and X_0. Values for μ_{enum} with no CAH, 0.05 mg/L 1,1-DCE, 1.9 mg/L t-DCE, 1 mg/L TCE, 2 mg/L TCE, and 4 mg/L TCE were 1.50, 1.11, 0.94, 1.14, 1.15, and 0.92 d^{-1}, respectively (*15*). The latter two rates include the effect of the 2-fold higher methane concentration used during enumeration.

Next, growth rates over the 34-d incubation period (μ_{inc}) were calculated for each bottle from the relative biomass concentration change. As the above analysis was only applied to bottles in which less than 20% methane removal was observed during the 34-d incubation period, methane concentrations and thus growth rates can be assumed to be constant for this time period. The exponential growth

equation can be used to relate the relative biomass concentration changes to μ_{inc} values as follows:

$$\mu_{inc} = \frac{\ln (X_{34}/X_0)}{34\ \text{d}} \quad (6)$$

Combining eqs 5 and 6 then yields the following relationship for the incubation growth rate as a linear function of $t_{90\%}$ values:

$$\mu_{inc} = \frac{\mu_{enum}(\overline{t_{90\%,0}} - t_{90\%,34})}{34\ \text{d}} \quad (7)$$

As expected, $\mu_{inc} = 0$ corresponds to $t_{90\%,34} = \overline{t_{90\%,0}}$. The 95% confidence intervals for $\mu_{inc} = 0$ for each CAH concentration were calculated from the μ_{inc} values obtained from inserting each of the three to five replicate $t_{90\%,0}$ values into eq 7 in place of $t_{90\%,34}$.

In an attempt to estimate which of the several possible effects of CAHs on S_{min} were operative, values for the coefficients in eq 1 were estimated. Here, eq 1 was fitted to the growth rate versus incubation methane concentration data by varying Yk_s, K_s, or b for the data without CAHs and varying k_c/T_c and K_c for the data with CAHs. Unweighted nonlinear least squares (NLS) parameter estimation was performed on a Microsoft Excel 5.0 spreadsheet by the method described previously (*28*, *29*). This procedure also provided 95% confidence intervals and correlation coefficients for the parameter value best estimates. The S_{min} estimate was the methane concentration corresponding to zero net growth using the best fit growth rate curve and was calculated using eqs 2, 3, or 8 and the fitted parameter values. The 95% confidence intervals for the S_{min} estimates include the errors associated with the parameter values and with $\mu_{inc} = 0$.

Concentration Measurements. Methane and CAHs were sampled (0.1 mL) from bottle headspace and analyzed as reported elsewhere (*15*). Solution concentrations were calculated using dimensionless 20 °C Henry's constants of 28.5 for methane (*10*) and 0.299, 0.305, and 0.862 for TCE, t-DCE, and 1,1-DCE, respectively (*30*). One-third of the methane mass was present in solution after partitioning between headspace (16 mL) and liquid (230 mL), whereas CAHs partitioned primarily into the aqueous phase, with 94–98% of the total CAH mass in solution.

Results and Discussion

Incubation Phase. The fraction of methane remaining after the 34-d incubation period for bottles having no CAHs is shown in the inset to Figure 1. Methane was not significantly removed in bottles incubated at less than 7 μg/L methane, yet it was nearly completely removed above 7 μg/L as a result of the higher growth rates obtained at higher methane concentrations. When CAHs were added to the bottles, higher methane concentrations were required to obtain measurable methane removal (Figure 1). The transition between zero and 100% methane utilization also appeared to occur more gradually.

Using the estimated Monod kinetic parameter values for methane utilization by this culture (given later), the estimated inoculum concentration (0.0013 μg/L) would be expected to remove only 0.2% of the initial methane over the 34-d incubation period if no net growth had occurred. However, growth would occur if the initial methane concentration was greater than S_{min}, leading to removal of more than 0.2% of the initial methane. For the results shown in Figure 1, methane removal at low methane concentrations ranged from 0 to 15%, similar to that in controls. Thus, an upper estimate for S_{min} was obtained by assuming at least 20% methane removal

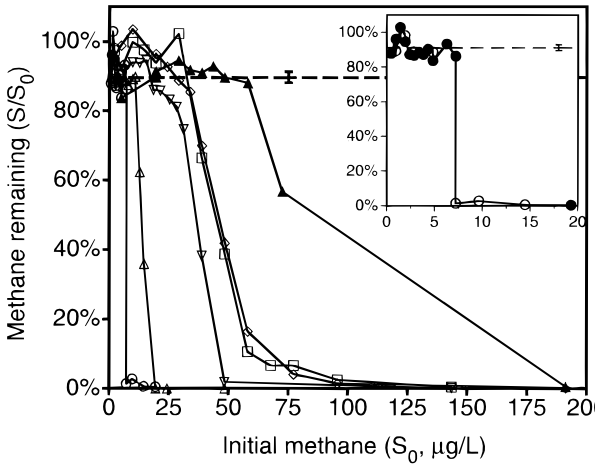

FIGURE 1. Methane remaining after 34 days as a function of initial methane concentration and CAH presence. (○) No CAH present, (△) 1 mg/L TCE, (▽) 2 mg/L TCE, (▲) 4 mg/L TCE, (□) 0.05 mg/L 1,1-DCE, (◇) 1.9 mg/L t-DCE, and (– –) average of controls with 95% confidence intervals. Inset shows expanded view of data with no CAH present including data (●) from this experiment and (○) a preliminary experiment conducted under identical conditions.

TABLE 1. Effect of CAHs on S_{min}

CAH type	CAH concn (mg/L)	S_{min} (μg/L) based on $S_{20\%}$[a]	S_{min} (μg/L) based on μ fit[b]	K_c (L/mg of CAH-d)	
none		7	4.9 ± 1.9[c]		
t-DCE	1.9	36	20.4 ± 10.6	0.140	0.019
1,1-DCE	0.051	35	25.0 ± 14.9[d]	6.00	0.89
TCE	1.0	11	7.4 ± 2.9	0.066	0.012[e]
TCE	2.0	29	10.5 ± 4.3		
TCE	4.0	62	20.0 ± 10.8		

[a] Incubation methane concentrations resulting in 20% methane removal over 34 days. [b] S_{min} values determined from fitting of eq 8 to growth rate data in Figures 3–6. [c] 95% confidence interval. [d] Value determined after excluding outlying data point. Inclusion of the outlying data point resulted in $S_{min} = 21.3 \pm 12.8$ μg/L. [e] Value determined for combined fit using 1, 2, and 4 mg/L TCE data.

($S_{20\%}$) was required to indicate net growth had occurred. The S_{min} values so obtained are listed in the third column in Table 1, and represent a preliminary, though not conclusive, indication that S_{min} increased in the presence of CAHs.

At the conclusion of the incubation period, possible TCE and 1,1-DCE removal was not enough to be detected relative to controls (Figure 2), although a separate study using a higher methane to CAH ratio showed that this culture does transform small quantities of TCE and 1,1-DCE (*10*). In contrast, transformation of t-DCE was significant. The mass of t-DCE removed depended on the mass of methane utilized, giving a very high transformation yield of up to 3.8 mg of t-DCE removed/mg of methane consumed.

Enumeration Phase and S_{min}. While the above approach supports the hypothesis that CAH presence increased S_{min}, a better means for estimating S_{min} was thought to result from growth rate estimates. By definition, incubation at a methane concentration equal to S_{min} should result in a zero net growth rate over the 34-d incubation time, i.e., no change in active biomass.

Traditional methods for quantifying active biomass changes were inadequate or inappropriate. The necessary small inoculum concentration used eliminated methods with higher quantification limits, such as those based on total and volatile suspended solids, absorbance, protein, and total organic carbon analyses. These techniques also failed to discriminate between active methanotrophic cells, inactive cells, dead cells,

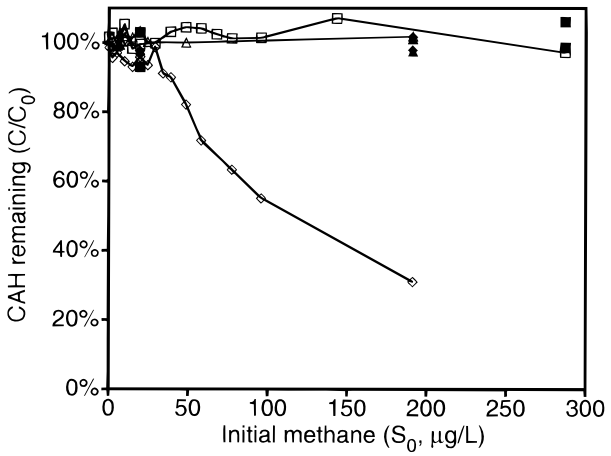

FIGURE 2. CAH remaining after 34 days as a function of initial methane concentration. Initial CAH concentrations: (△) 1 mg/L TCE, (□) 0.05 mg/L 1,1-DCE, and (◇) 1.9 mg/L t-DCE. Live samples indicated by open symbols; no cell and azide-killed controls indicated by filled symbols.

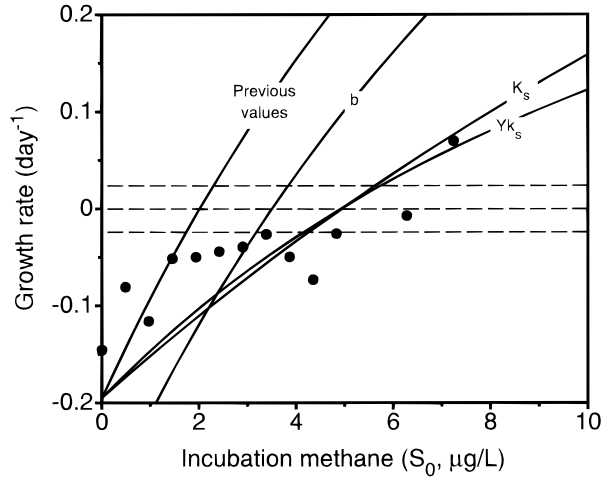

FIGURE 3. Measured growth rate versus incubation methane concentration in the absence of CAHs (●). (– –) Zero growth rate with 95% confidence intervals, and (—) eq 1 fits using previously determined parameter values except for changes in the parameter noted on each curve.

and other organisms possibly present in the mixed culture. Plate counting was rejected because of the imprecision as well as the questionable plating efficiency for methane-oxidizing bacteria (*23*), though this technique has been used by others for determination of S_{min} for other growth substrates (*24, 25*). Microscope-aided cell counting was rejected also because of the lack of sufficient precision, the tendency for the cells to aggregate, and the inability to discriminate between active methane-oxidizing and other cells. The activity-based measurement technique employed here overcame these difficulties (*26, 27*).

For bottles incubated with 0−10 μg/L methane in the absence of CAHs, growth rates as a function of methane concentration are shown in Figure 3. The horizontal lines indicate the 95% confidence interval for a zero growth rate. In general, growth rates were negative at incubation methane concentrations less than 6 μg/L and positive at methane concentrations greater than 6 μg/L. As expected, even greater positive growth rates were found for bottles with more than 20% methane utilization during the 34-d incubation period. However, these results are not shown because they are underestimates of true growth rates due to the fact that methane concentration declined during the incubation period.

In order to obtain the best estimate of S_{min} using all the available growth rate data, curves were fitted to the data. Equation 1 is a logical choice for an expression to describe the variation of μ as a function of methane concentration. The left-most curve in Figure 3 shows the poor fit of eq 1 using the previously determined parameter values (±95% confidence intervals): $Yk_s = 1.71 \pm 0.05$ d^{-1}, $K_s = 15.6 \pm 1.2$ μg/L, and $b = 0.20 \pm 0.05$ d^{-1} (*15*). The overprediction may be due to the fact that these parameter values were determined at methane concentrations much greater than S_{min}, ranging from 9 to 460 μg/L. To obtain a better fit to the data, each parameter value was allowed to vary while holding the others constant. The resulting best fits using NLS regression are shown in Figure 3. The least improvement was obtained by increasing b to 0.31± 0.08 d^{-1}. The best fit was obtained when K_s was approximately doubled to 38.3 ± 9.5 μg/L or when Yk_s was approximately halved to 0.81 ± 0.17 d^{-1}.

A question arises as to why the Monod model values obtained at higher methane concentrations need to be modified for growth rates near S_{min}. In response to starvation conditions, gene, enzyme, and organism-level adaptations are known to allow bacteria to survive under increasingly oligotrophic conditions (*31*). Bacteria may reduce the rate of endogenous respiration, i.e., lower b (*31*), or shift to lower capacity/higher affinity enzyme systems, i.e., lower k_s and lower K_s (*32*). Also, when the rate of methane oxidation is low, intracellular reducing energy may become depleted. As a result, the observed k_s is likely to decrease because MMO requires NADH as a cofactor for methane oxidation (*13, 33*). As the choice here was between a lower k_s or a higher b or K_s, only a reduction in Yk_s was considered to be consistent with the above arguments, and thus was the value changed in order to better fit the lower methane data, and was used in all subsequent fittings.

Using the reduced Yk_s value and the previously determined K_s and b, the eq 3 S_{min} value becomes 4.9 ± 1.9 μg/L methane. This S_{min} value, determined more precisely here from use of methane concentrations near S_{min}, is significantly greater than (with 95% confidence) the value predicted from the Monod kinetic parameters determined at higher methane concentrations (2.0 ± 0.5 μg/L methane).

One additional possibility is that the growth rate near S_{min} was limited by the rate of methane diffusion to the cell surface. Schmidt et al. (*34*) presented an equation to predict cell doubling time as limited by substrate diffusion and maintenance energy requirement. The rod-shaped cells in this culture had a length of 0.1−1 μm (*8*). At most [i.e., for a 1-μm diameter sphere with density of 305 mg dry wt/cm^3 (*34*)], a methane concentration of only 0.06 μg/L methane would be sufficient to supply the maintenance energy requirement (b/Y) of 0.30 mg/mg-d (*15*), using an aqueous diffusion coefficient of 1.64 cm^2/d for methane (*14*). Diffusional limitation to the cell surface was thus not a factor in the increased S_{min} value.

Effect of CAHs on S_{min}. CAHs were added to additional sets of bottles to test the effect on S_{min}. In the presence of 1.9 mg/L t-DCE, a higher methane concentration was required for a zero growth rate (Figure 4). Equation 1 was fitted to the data with t-DCE present using NLS regression in order to arrive at the best estimates of $k_c/T_c = 0.59 \pm 0.39$ d^{-1} and $K_c = 2.5 \pm 1.6$ mg/L for t-DCE. The resulting best fit curve is shown in Figure 4. The high uncertainty in the k_c/T_c and K_c estimates is due to a high correlation among parameter estimates (correlation coefficient = 0.94) (*28*) and leads to great uncertainty in the S_{min} value (20.2 ± 15.4 μg/L methane) when calculated from eq 2 using these parameter values. For bottles incubated with 0.05 mg/L 1,1-DCE (Figure 5) and 1, 2, and 4 mg/L TCE (Figure 6), similar results were obtained. The curves fit the data well, but the k_c/T_c and K_c values were strongly correlated and the S_{min} estimates had large confidence intervals.

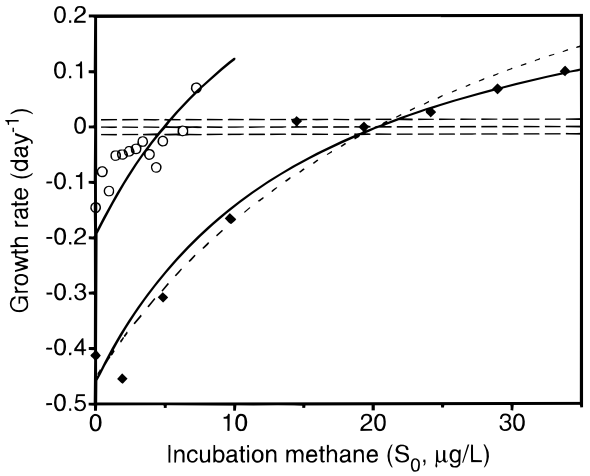

FIGURE 4. Measured growth rate versus incubation methane concentration in the presence of (○) 0 mg/L t-DCE and (♦) 1.9 mg/L t-DCE. (– –) Zero growth rate with 95% confidence intervals, (—) eq 8 fit, and (---) eq 1 fit.

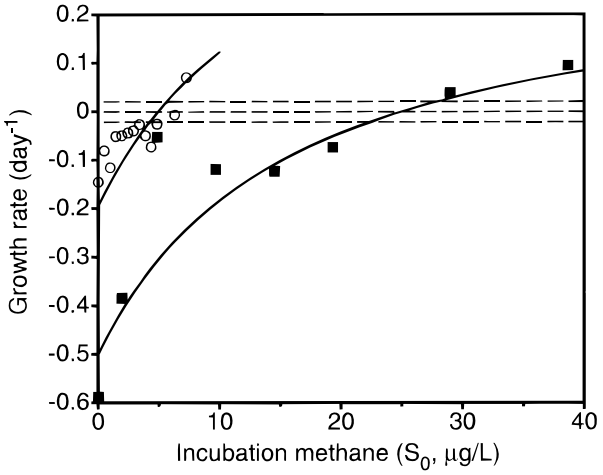

FIGURE 5. Measured growth rate versus incubation methane concentration in the presence of (○) 0 mg/L 1,1-DCE and (■) 0.05 mg/L 1,1-DCE. (– –) Zero growth rate with 95% confidence intervals, and (—) eq 8 fit. Fitted curve does not include outlying data point at 5 μg/L methane.

The high degree of correlation among parameter estimates indicates that a simpler model formulation using fewer parameters may be appropriate given the data available. The most straightforward simplification of eq 1 that retains a CAH concentration dependence is the following:

$$\mu = Yk_s\left(\frac{S}{S + K_s}\right) - k'_cC - b \qquad (8)$$

where $k'_c = k_c/T_cK_c$ (L/mg of CAH-d) and represents a growth rate reduction that is first order in CAH concentration. High values of k'_c are expected for compounds that have a large negative effect on growth rates and S_{min}. In the absence of CAHs, eqs 8 and 1 simplify to the same expression. The equation for S_{min} corresponding to eq 8 is identical in form to that without CAHs present (eq 3), except that a modified decay term (b', d^{-1}) results that includes both natural decay as well as CAH-related effects:

$$S_{min} = \frac{K_sb'}{Yk_s - b'} \qquad (9)$$

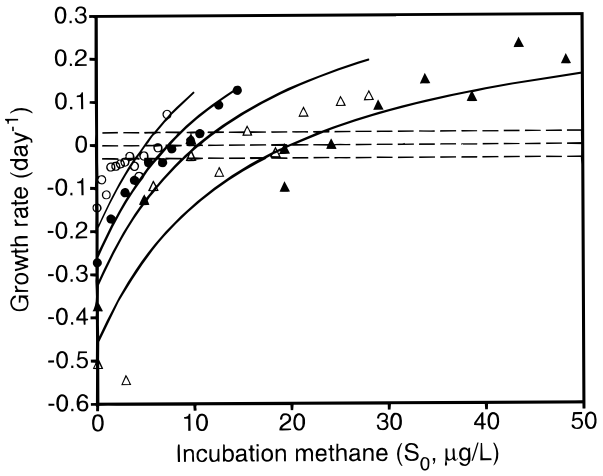

FIGURE 6. Measured growth rate versus incubation methane concentration in the presence of (○) 0 mg/L TCE, (●) 1 mg/L TCE, (△) 2 mg/L TCE, and (▲) 4 mg/L TCE. (– –) Zero growth rate with 95% confidence intervals, and (—) eq 8 fit.

where

$$b' = k'_cC + b \qquad (10)$$

By reducing the effect of CAHs on μ to a single parameter, the uncertainty in the estimation of k'_c and S_{min} is improved. The fit of eq 8 to the growth rate data was also slightly better than that for eq 1 (Figure 4). More importantly, the estimated parameter k'_c (0.140 ± 0.019 L/mg-d) has a much smaller 95% confidence interval (Table 1). The resulting S_{min} value using eq 9 is 20.4 ± 10.6 μg/L methane, which is significantly greater than the value determined without CAHs present (4.9 ± 1.9 μg/L).

For bottles incubated with 0.05 mg/L 1,1-DCE, the best fit k'_c was 6.00 ± 0.89 L/mg-d and S_{min} was 25.0 ± 14.9 μg/L, after excluding the outlying data point at 5 μg/L methane (Figure 5). When this datum was included, k'_c and S_{min} were 10 and 15% less, respectively, with wider confidence intervals. As with t-DCE, the fit with eq 8 was slightly better than the fit with eq 1.

In the presence of 1, 2, and 4 mg/L TCE, progressively higher methane concentrations were required for a zero growth rate (Figure 6). Equation 8 was fitted to the combined 1, 2, and 4 mg/L TCE data (Figure 6) in order to arrive at the best overall estimate of k'_c for TCE (Table 1). The predicted S_{min} values ranged from 7.4 μg/L methane in the presence of 1 mg/L TCE to 20 μg/L methane with 4 mg/L TCE (Table 1). The effect of 4 mg/L TCE on growth rates and S_{min} was roughly equivalent to that of 1.9 mg/L t-DCE or 0.05 mg/L 1,1-DCE.

According to eq 2, S_{min} should increase with an increase in CAH concentration or a decrease in TPT. It is also affected by competitive inhibition, increasing as the relative affinity for the CAH compared to methane increases (increased K_s/K_c ratio). Unfortunately, it was not possible to discriminate between the processes, because the eq 1 parameters k_c/T_c and K_c could not be estimated accurately with the available data. These parameters were successfully determined in a previous study of the effect of these chlorinated ethenes on growth rates at a relatively high methane concentration (460 μg/L) (*15*). These results indicated that competitive inhibition was the most important process affecting growth rate for t-DCE and TCE, but TPT was the most significant process for 1,1-DCE. Because TPT from 1,1-DCE cometabolism is typically much more severe than for the other chlorinated ethenes (*6*, *11*, *15*, *35*), it is no surprise that 1,1-DCE here also had the greatest effect on S_{min} even though only small amounts of 1,1-DCE were apparently transformed.

Equation 8 provides a simple model that adequately combined the various effects of CAHs on growth rate into a single term, k'_cC (d^{-1}). When divided by the cell yield, Y, this term is equivalent to the maintenance energy requirement for cometabolism as proposed by Criddle (*36*). Such an increase in maintenance energy was recently reported for a toluene-oxidizing pure culture degrading TCE in a chemostat in which the toluene supplied was just sufficient to satisfy the maintenance demand (*37*).

The k'_c values are independent of CAH concentration and represent a convenient means for comparing the overall effect of CAHs on growth rates at methane concentrations near S_{min}. The much higher k'_c value for 1,1-DCE indicates that growth rate and S_{min} are much more sensitive to this CAH than to TCE, which had a k'_c 90 times lower. The k'_c for TCE was also two times lower than for t-DCE. Reducing energy (NADH) limitation from t-DCE cometabolism may have been a significant factor here as up to 3.8 mg of t-DCE was oxidized per milligram of methane utilized. Based upon calculations made elsewhere (*10*), 38% of the methane removed must have been oxidized completely to carbon dioxide just to regenerate the NADH required for the t-DCE that was oxidized. This additional demand leaves less reducing energy available for growth and maintenance and thus probably contributed to the effect t-DCE presence had on S_{min}.

The one-parameter equation for growth rate (eq 8) would not necessarily be applicable at higher methane concentrations, but due to its simplicity, this should be evaluated. The S_{min} equation (eq 9), however, is only relevant at low methane concentrations. Further evaluation using other CAHs and other oxygenase-inducing enzyme systems is needed to evaluate the general applicability of the one-parameter model.

Implications. The S_{min} values estimated through use of a growth rate model were found to be 29−68% less than values based simply upon detectable methane removal ($S_{20\%}$) (Table 1). The $S_{20\%}$ approach has limitations since near S_{min} organisms may grow, but not enough to appreciably decrease the methane concentration. For this reason, the $S_{20\%}$ method overestimates S_{min}. The values based upon growth rate equal to zero are thus believed to be much better.

These results suggest potential limitations in the application of aerobic cometabolic bioremediation when certain CAHs are present. The severe TPT associated with 1,1-DCE transformation may make it difficult to establish and sustain an active cometabolic population in areas contaminated with this compound. For example, the addition of 800 μg/L 1,1-DCE to methane-stimulated soil columns decreased the rates of methane and oxygen utilization and reduced vinyl chloride removal from 90% to 20% (*11*). Likewise, *in situ* phenol-stimulated TCE transformation deteriorated significantly when 65 μg/L 1,1-DCE was present (*38*). Further, residual phenol concentrations increased at intermediate monitoring wells. This latter observation is of concern, as aromatic growth substrates (phenol and toluene) are themselves regulated chemicals. Theoretically, in such a system, growth substrates are removed to a residual or threshold concentration equal to S_{min} (*39, 40*). Based on the findings presented here, this residual will increase in relation to residual CAH concentration. However, this study suggests that by increasing the growth substrate dosage, CAH removal can be improved. Such a strategy may then actually reduce the residual of both chemicals. Also, in the presence of multiple microbial strains, downgradient residual concentrations may be determined more by organisms incapable of transforming CAHs, thus potentially resulting in a lower S_{min} for phenol than would otherwise be expected in the presence of CAHs. Thus, potential methods appear to be available to circumvent some of the problems associated with CAH effects on increasing S_{min}.

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