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EFFECT OF TEMPERATURE, pH AND ELECTRON DONOR ON THE MICROBIAL REDUCTIVE DECHLORINATION OF CHLOROALKENES

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ABSTRACT

In order to elucidate the effect of environmental conditions such as pH, temperature, and electron donor on the microbial reductive dechlorination of tetrachloroethylene and its transformation products, batch, acetate-fed methanogenic cultures were developed from a field contaminated soil and incubated within a pH range of 4 to 9.5 and a temperature range of 5 to 45°C. Optimum conditions for reductive dechlorination were achieved at pH and temperature values of around 7 and 35°C, respectively. Methane production was more affected by pH and temperature than was the rate of dechlorination. Increased levels of electron donor (acetate) resulted in faster rates of both methanogenesis and reductive dechlorination. The effect of pH and temperature on the dechlorination rate and methane production were simulated by using previously established relationships pertaining to the activation and deactivation of enzymatic processes.

INTRODUCTION

The contamination of soil and groundwater by volatile organic compounds (VOCs), especially halogenated alkenes -- such as tetrachloroethylene (PCE), trichloroethylene (TCE), cis- and trans-dichloroethylene (cDCE and tDCE), and vinyl chloride (VC) -- is an ever-growing environmental concern since some of these contaminants have been found to be either carcinogenic or potentially carcinogenic thereby posing a hazard to human health. Although compounds with low chlorine content (e.g., TCE, DCE and VC) are degradable under aerobic conditions, completely chlorinated compounds (e.g., PCE) are persistent in aerobic natural environments. Transformation of chloroalkenes under anaerobic conditions via biological reductive dechlorination has been well documented (Bagley and Gossett, 1990; Barrio-Lage *et al.*, 1986; Bouwer and McCarty, 1983; de Bruin *et al.*, 1992; DiStefano *et al.*, 1991; DiStefano *et al.*, 1992; Freedman and Gossett, 1989; Holliger *et al.*, 1993; Parsons *et al.*, 1984; Pavlostathis and Zhuang, 1991a; Pavlostathis and Zhuang, 1993; Vogel and McCarty, 1985; Zhuang and Pavlostathis, 1994).

The impact of either low or high pH values on reductive dechlorination has not been studied. However, since reductive dechlorination is mediated by anaerobic bacteria which prefer either neutral or slightly alkaline environments, inhibition of bacterial activities (e.g., methanogenesis) at pH values outside the optimum range may also affect the rate and extent of the dechlorination process. Temperature is also an important environmental constraint, especially in the in-situ bioremediation of contaminated sites. Its effect on the primary metabolism of the requisite microorganisms will inevitably affect the rate and extent of dechlorination. Although reduced methanogenic activity is expected to lead to low rates of microbial reductive dechlorination, the relationship between these two processes and the extent of the effect that low temperatures may have on dechlorination have not been fully documented.

The objective of this paper is to report on the effect of environmental conditions such as pH and temperature and nutritional conditions (e.g., electron donor level) on the reductive dechlorination of PCE and its dechlorination products under methanogenic conditions.

MATERIALS AND METHODS

Culture

An acetate-fed, methanogenic culture was developed using a PCE- and TCE-contaminated soil as the inoculum and maintained as a semicontinuously-fed batch reactor with a hydraulic retention time of 50 d (Pavlostathis and Zhuang, 1993). The final concentration of the basal media was as follows (in g/L): NH_4Cl, 0.5; K_2HPO_4, 0.87; KH_2PO_4, 0.54; $MgSO_4 \cdot 7H_2O$, 0.1; $MgCO_3$, 0.1; and $CaCl_2 \cdot 2H_2O$, 0.06. To the basal medium, 1 mL vitamin solution (Wolin *et al.*, 1963) and 1 mL trace metal solution (Mah and Smith, 1981) were added per liter of medium. Before adding the vitamin solution, the basal medium was autoclaved at 121°C for 30 min and then purged with ultra high purity nitrogen (>99.998%) for 1 h to remove any oxygen. Reducing agents were not added to this medium and the pH was adjusted to 7.0 with either 1 N NaOH or 2 N HCl. Acetate was used as the electron donor and the liquid-phase PCE concentration was ca. 1 mg/L immediately after feeding this reactor. The culture, which dechlorinated PCE to VC, served as the inoculum for all methanogenic cultures used to test the various factors affecting the dechlorination process, unless otherwise stated. Before use, the culture inoculum was purged with nitrogen gas to remove all VOCs. Batch cultures were developed in serum bottles and all incubations were carried out in the dark at 35°C, except when a temperature range was used to assess the effect of temperature on the reductive dechlorination of PCE.

Temperature Assay

Two series of cultures were developed and incubated at 5, 15, 25, 35 and 45°C. Half of the cultures were developed using the methanogenic culture as inoculum and the other half were developed with a PCE- and TCE-contaminated soil. The soil inoculum was prepared as a soil slurry (5% w/v) using the basal medium and was kept anaerobic. The soil slurry was purged with nitrogen gas to remove all VOCs. Duplicate cultures were developed in 280-mL serum bottles (200 mL liquid volume) and incubated without shaking. Acetate (750 mg/L) and yeast extract (250 mg/L) were added to the basal medium. The initial PCE concentration was 1.6 mg/L. The initial pH and oxidation-reduction potential (ORP) values varied from 7.04 to 7.11 and from -14 to -32 mV, respectively.

pH Assay

A series of batch, methanogenic cultures were developed with initial concentrations of 1.7 g/L acetate, 625 mg/L yeast extract, 0.9 mg/L PCE at five pH values: 4, 6, 7, 8, and 9.5. All cultures were prepared in 165-mL serum bottles (120 mL liquid volume) in duplicate. At the beginning of the incubation the following ORP values were recorded for the pH 4, 6, 7, 8 and 9.5 cultures, respectively: +83, +5, -49, -104 and -166 mV. The increase in ORP values with decreasing pH is expected since an inverse relationship exists between pH and ORP. In general, a pH decrease of one unit causes the potential to become more positive by ca. 58 mV (Jacob, 1970).

Electron Donor Assay

Another series of cultures were developed in 165-mL (100 mL liquid volume) serum bottles in order to quantify the effect of the electron donor on the reductive dechlorination of TCE. The PCE-dechlorinating, methanogenic culture was used as inoculum after three washings with basal medium, centrifugation (1500 rpm for 20 min) and purging with nitrogen gas to remove all VOCs. Four duplicate cultures were developed with the same methanogenic inoculum level (74 mg/L protein measured as Bovine Serum Albumin, BSA) and an initial TCE concentration of 2.4 mg/L. These cultures were then amended with 0, 150, 300 and 1500 mg/L acetate. Yeast extract (50 mg/L) was also added.

Analyses

Chloroalkenes were identified and quantified by gas chromatography (GC) either by direct injection of gas and/or aqueous samples or by purge-and-trap as previously described (Pavlostathis and Jaglal, 1991). Methane was quantified by GC (thermal conductivity detector). Acetate and other volatile fatty acids were quantified by high performance liquid chromatography (HPLC). Protein measurements were performed using a protein-dye binding assay (Bradford, 1976).

RESULTS AND DISCUSSION

Effect of Temperature

Incubation of these cultures lasted between 25 and 78 days, depending on the inoculum source and incubation temperature (Table 1). The pH in all cultures increased and ranged from 7.09 to 7.35. At the end of the incubation, the following ORP values were recorded for the culture-inoculated microcosms incubated at 5, 15, 25, 35 and 45°C, respectively: -90, -127, -182, -203 and -158 mV. For the same series of incubation temperatures, the soil-inoculated microcosms achieved the following ORP values: -76, -118, -145, -172 and -164 mV. PCE was sequentially dechlorinated to TCE and then to cDCE. It is noteworthy that lag periods for active PCE dechlorination were not observed in either the culture- or soil-inoculated microcosms (Fig. 1). The rate and extent of dechlorination increased with increasing temperatures of up to 35°C and then declined as the temperature rose to 45°C. The distribution of VOCs at the end of incubation is shown in Table 1. By assuming the sequential reductive dechlorination to be a series of first-order reactions, the rate constants (k_1, k_2 for PCE to TCE, and TCE to cDCE, respectively) were estimated using regression analysis (Zhuang, 1994) and results are shown in Table 2. The soil-inoculated cultures

incubated at 15, 25 and 35°C also dechlorinated cDCE to VC and the respective rates (k_3 for cDCE to VC) increased with increasing temperature, as follows: 7.0×10^{-4}, 7.4×10^{-3} and 1×10^{-2} h^{-1}, respectively.

Table 1. Distribution and mass balance of VOCs near completion of PCE dechlorination as a function of incubation temperature

Inoculum	Temp. (°C)	Incubation Time (d)	VOC distribution[a] (%)				Mass Balance[b] (%)
			PCE	TCE	cDCE	VC	
Enriched Culture	5	78	4.2	TR[c]	95.8	ND[d]	92.1
	15	78	1.4	ND	98.6	ND	91.5
	25	25	TR	TR	100	ND	92.2
	35	28	TR	TR	100	ND	87.1
	45	41	TR	TR	100	ND	94.0
Contaminated Soil	5	78	33.9	14.4	51.7	ND	88.3
	15	78	2.8	2.8	40.3	54.1	96.9
	25	34	1.0	2.7	6.6	89.7	86.5
	35	25	0.5	1.5	2.8	95.2	99.0
	45	78	13.1	4.6	82.3	ND	88.9

[a] Based on moles of VOCs accounted for at the end of incubation; [b] Based on initially added moles of PCE; [c] TR, traces; [d] ND, not detected

Table 2. Protein concentration and dechlorination rate constants as a function of incubation temperature

Inoculum	Temperature (°C)	k_1 (h^{-1})	k_2 (h^{-1})	Protein[a] (mg BSA/L)
Enriched Culture	5	1.8×10^{-3}	1.1×10^{-2}	0.8
	15	2.2×10^{-2}	4.8×10^{-2}	1.8
	25	2.7×10^{-2}	2.2×10^{-1}	5.5
	35	3.1×10^{-2}	2.5×10^{-1}	7.1
	45	2.2×10^{-2}	1.9×10^{-1}	2.3
Contaminated Soil	5	6.4×10^{-4}	1.2×10^{-3}	0.5
	15	2.1×10^{-3}	7.0×10^{-3}	0.6
	25	6.4×10^{-3}	1.1×10^{-2}	4.1
	35	8.6×10^{-3}	4.7×10^{-2}	4.8
	45	1.2×10^{-3}	8.9×10^{-3}	1.1

[a] Protein concentration after 22 d of incubation.

The PCE dechlorination rate followed very closely that of methane production (Fig. 2). The optimum temperature for both processes was 35°C. Temperature ranges for maximum dechlorination rates have been reported as follows: 25 to 35°C for PCE (Holliger *et al.*, 1993); 30 to 35°C for 2,4-dichlorophenol (Kohring *et al.*, 1989). In addition to the direct effect of temperature on the dechlorination rates, temperature also affects the acclimation period and leads to a selection of distinct dechlorinating populations (Mohn and Tiedje, 1992). The precipitous decline of the dechlorination rates below 20°C has far-reaching repercussions on the application of reductive dechlorination for in situ remediation of subsurface, natural environments where relatively low temperatures typically prevail (Pavlostathis and Zhuang, 1991b).

The effect of temperature on the rate of dechlorination and methane production was simulated by using an Arrhenius relationship which accounts for both enzyme activation and deactivation (Bailey and Ollis, 1986):

$$R_d = \beta_d A \tag{1}$$

$$R_m = \beta_m A \tag{2}$$

$$A = \frac{T\left[\exp\frac{-E_a}{RT}\right]}{1 + \left[\exp\frac{\Delta S_d}{R}\right]\left[\exp\frac{-\Delta H_d}{RT}\right]} \tag{3}$$

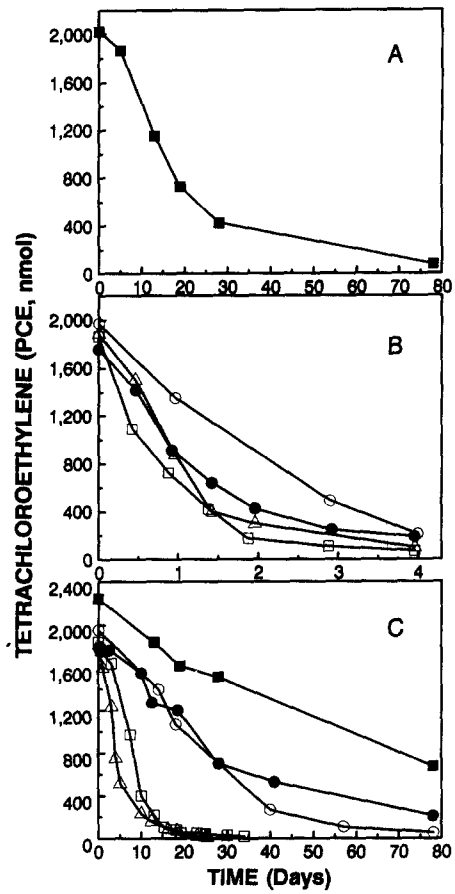

Figure 1. Effect of temperature on the dechlorination of PCE (A and B, culture-inoculated and C, soil-inoculated microcosms) [Temperature, °C: 5 (■), 15 (○), 25 (□), 35 (△) and 45 (●)].

where: R_d, R_m = dechlorination and methane production rate, respectively (nmol/h); β_d, β_m = dechlorination and methane production proportionality factor, respectively (nmol/h-K); T = absolute temperature (K); E_a = activation energy (kcal/mol); R = gas constant (= 1.9872 x 10^{-3} kcal/mol-K); ΔS_d = entropy change of enzyme deactivation (kcal/mol-K); and ΔH_d = enthalpy change of enzyme deactivation (kcal/mol). By using the mean methane production rate data for the first 20 d of incubation, the parameters in equations 1 through 3 were estimated and are listed in Table 3. For temperatures equal or below the optimum temperature, the temperature coefficient (Q_{10}), defined as the ratio of the rates for a temperature difference of 10°C is as follows: $Q_{10} = \exp[10\, E_a/R\, T_1\, T_2]$. In a previous study, anaerobic cultures were developed with a subsurface soil and incubated within a temperature range from 5 to 20°C (Pavlostathis and Zhuang, 1991b). The effect of temperature on the metabolic rates (i.e., nitrate-reduction,

sulfate-reduction and methanogenesis) was quantified. Methanogenesis was most sensitive to temperature, followed by sulfate-reduction. At 10°C or below, the methane production rate was insignificant. The Q_{10} values for the methanogenic cultures varied between 4.4 and 5.0. In the present study, the Q_{10} values for methanogenesis and microbial reductive dechlorination varied between 4.2 and 4.7 and between 2.2 and 3.1, respectively. Therefore, the reductive dechlorination process appeared to be less sensitive to temperature variation than was methanogenesis.

By using the Monod equation and ignoring the bacterial decay, the biokinetic coefficients for acetate utilization were estimated for both the culture-inoculated and soil-inoculated microcosms at temperatures 15, 25, 35 and 45°C. At 5°C, acetate utilization in both microcosm series was negligible. A least-squares technique was used for the estimation of the biokinetic coefficients and Newton's method was then used to estimate substrate concentrations as a function of incubation time (Zhuang, 1994). The estimated values of the biokinetic coefficients ranged as follows (for the culture- and soil-inoculated microcosms, respectively): μ_{max} = 0.001-0.01 and 0.001-0.02 h^{-1}; Y = 0.02-0.07 and 0.03-0.05 mg/mg; and apparent K_s = 520-970 and 700-1190 mg/L. It is noteworthy that these K_s values are much higher than previously reported values. K_s values for acetate utilization by mixed, methanogenic cultures as low as 15 mg/L have been reported (Pavlostathis and Giraldo-Gomez, 1991). Mass transfer limitations may have led to the high apparent K_s values, especially for the soil-inoculated cultures.

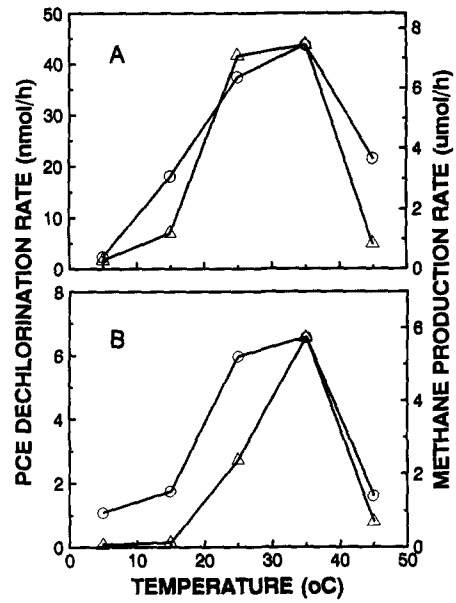

Figure 2. PCE dechlorination (O) and methane production (Δ) rates as a function of incubation temperature (A, B: culture- and soil-inoculated microcosms, respectively).

Table 3. Parameter values for the simulation of the effect of temperature on the rate of methane production and dechlorination (see equations 1 through 3)

Process	Inoculum Source	E_a (kcal/mol)	ΔH_d (kcal/mol)	ΔS_d (kcal/mol-K)	β (mol/h-K)
Methane	Enriched Culture	26.17	68.56	0.225	3.88×10^{11}
Production	Contaminated Soil	28.22	72.19	0.234	3.78×10^{12}
Reductive	Enriched Culture	20.61	33.95	0.113	2.59×10^{5}
Dechlorination	Contaminated Soil	14.15	36.68	0.120	5.91×10^{-1}

Effect of pH

Incubation of this series of cultures lasted for 157 h and insignificant pH deviations (i.e., less than 0.1 pH units) were observed from the pH values at the beginning of the incubation. At the end of incubation the following ORP values were recorded for the pH 4, 6, 7, 8 and 9.5 cultures, respectively: +77, -24, -162, -138 and -170 mV. The pattern of PCE dechlorination at the five pH values tested is shown in Figure 3. The pH 7 culture achieved both the highest PCE dechlorination rate and extent as well as methane production (Table 4). The PCE dechlorination rate correlates fairly well with the methane production rate ($r^2 = 0.84$). However, the methane production rate declined more than the dechlorination rate at pH values below and above 7 (Fig. 3B). Maximal PCE dechlorination by an enriched, anaerobic culture was also observed between pH 6.8 and 7.6 (Holliger *et al.*, 1993). These results agree with the widely accepted optimum pH range (from 6.5 to 7.5) for anaerobic bacteria (Lowe *et al.*, 1993). The VOC distribution was also affected by the culture's pH. As shown in Table 5, although pH 7 and 8 cultures achieved a comparable extent of PCE dechlorination, the predominant dechlorination products were VC and cDCE, respectively. Production of VC was not observed in the pH 8 culture. Significant accumulation of cDCE was also observed in the pH 6 culture. The pH 4 and 9 cultures followed very similar patterns of PCE dechlorination: VC was not observed and the limited PCE dechlorination resulted in predominantly cDCE production. The dechlorination of PCE to TCE and that of cDCE to VC were affected by pH values outside the neutral range. In contrast, dechlorination of TCE to cDCE was not inhibited. When the PCE dechlorination rates were normalized to the biomass concentration (i.e., protein concentration) of each culture, these rates became comparable regardless of the culture's pH (Table 4).

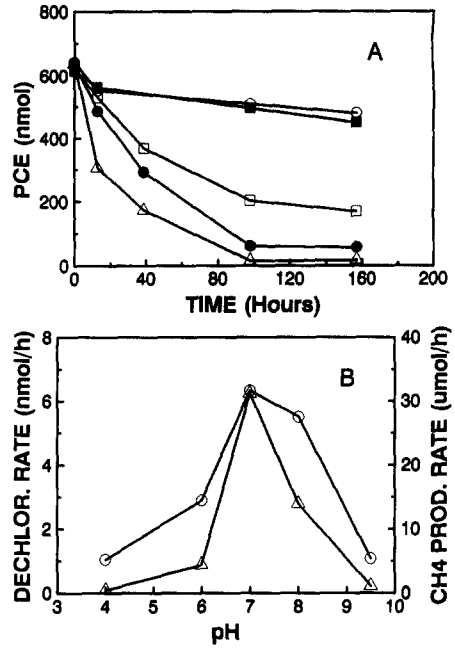

Figure 3. Effect of pH. A: PCE dechlorination pattern [pH values: 4 (○), 6 (□), 7 (△), 8 (●) and 9.5 (■)]. B: PCE dechlorination (○) and methane production (△) rates.

The effect of pH on the rate of methane production and PCE dechlorination was simulated by using an enzyme deactivation model as follows (Bailey and Ollis, 1986):

$$R_d = k_d B \tag{4}$$

$$R_m = k_m B \tag{5}$$

$$B = \frac{1}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}} \tag{6}$$

where: R_d, R_m = dechlorination and methane production rate, respectively (nmol/h); k_d, k_m = dechlorination and methanogenesis constant, respectively (nmol/h); $[H^+]$ = proton concentration (mol/L); K_1 and K_2 equilibrium constants for inactivation of requisite enzyme by protonation and deprotonation, respectively (mol/L). By using the mean rate of methane production and PCE depletion, the kinetic parameters in equations 4 through 6 were estimated by regression analysis and the results are shown in Table 6.

Table 4. Results of PCE dechlorination at a range of pH values

pH	Rate Constant[a]		Extent of Dechlorination (%)	Methane Production (%)[c]	Protein[d] (mg BSA/L)
	Actual (h^{-1})	Normalized (L/mg-h)[b]			
4	1.6×10^{-3}	2.3×10^{-3}	25.4	1.3	0.7
6	8.5×10^{-3}	3.1×10^{-3}	72.9	7.3	2.7
7	3.6×10^{-2}	2.0×10^{-3}	97.2	100	18.2
8	1.7×10^{-2}	1.4×10^{-3}	90.9	43.5	11.9
9.5	1.9×10^{-3}	1.0×10^{-3}	27.3	3.3	1.9

[a] First-order rate constant for PCE to TCE dechlorination; [b] PCE dechlorination rate constant normalized to the biomass present (mg BSA/L); [c] Based on the methane production of pH 7 culture; [d] Protein concentration at the end of 157 h of incubation.

Table 5. VOC distribution in methanogenic cultures incubated at a range of pH values[a]

pH	VOC distribution[b] (%)				Mass Balance[c] (%)
	PCE	TCE	cDCE	VC	
4	79.5	7.7	12.8	ND[d]	93.7
6	28.3	13.7	58.0	ND	96.1
7	2.9	4.0	7.0	86.1	95.2
8	9.4	5.4	85.2	ND	97.1
9.5	76.2	9.8	14.0	ND	95.5

[a] After 157 h of incubation at 35°C; [b] Based on moles of VOCs accounted for at the end of incubation; [c] Based on initially added moles of PCE; [d] ND, not detected

Table 6. Estimated kinetic parameters for methane production and PCE dechlorination as affected by pH (see equations 4 through 6)

Process	k (nmol/h)	K_1 (mol/L)	K_2 (mol/L)
Methane Production	113	4.0×10^{-8}	6.27×10^{-8}
Dechlorination	7.8	5.92×10^{-7}	4.24×10^{-9}

Effect of Acetate Concentration

The dechlorination pattern of TCE within 28 d of incubation as a function of the initial acetate concentration is presented in Figure 4A. Within this incubation period, TCE was primarily dechlorinated to cDCE. The extent of dechlorination was proportional to the initial acetate concentration. The culture which did not receive any acetate exhibited limited TCE dechlorination for the first 3 h of incubation with dechlorination ceasing thereafter. Cultures amended with 150, 300 and 1500 mg/L acetate achieved the following initial TCE dechlorination rates: 1.4×10^{-2}, 1.7×10^{-2} and 2.4×10^{-2} mg/L-h, respectively (Fig. 4B) The dechlorination rate increased drastically as the acetate concentration increased within a relatively low acetate concentration range, but as the acetate concentration was increased further, the effect of acetate on the dechlorination rate became less significant. These results confirm previous observations where the dechlorination rate was not affected by increasing electron donor concentrations at relatively high levels (Zhuang, 1994). Methanogenesis was also affected by initial acetate concentrations. Although the inoculum had been washed to remove any residual energy source before inoculation, some carbon source was still available in the culture not amended with acetate and yeast extract, initially resulted in very low, but quantifiable, methane production. In contrast to the dechlorination process, methane production was more affected by acetate levels (Fig. 4B).

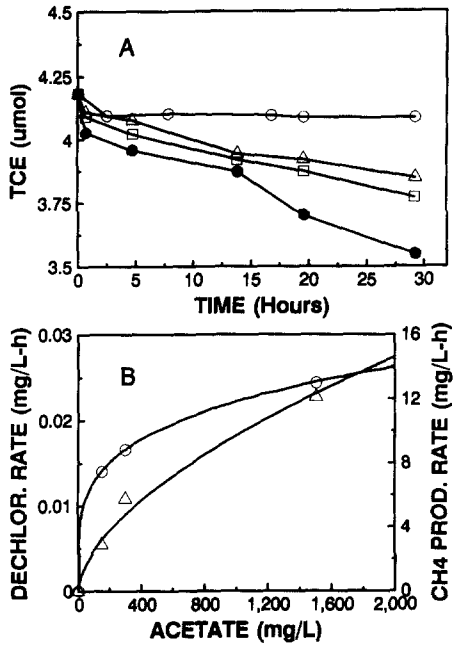

Figure 4. Effect of electron donor (acetate). A: TCE dechlorination pattern [Initial acetate concentration (mg/L): 0 (○), 150 (△), 300 (□) and 1500 (●)]. B: TCE dechlorination (○) and methane production (△) rates.

Electron Balance

Based on the electroequivalents of acetate used and the extent of reductive dechlorination of chloroalkenes (Pavlostathis and Zhuang, 1993), electron balance calculations showed that the distribution of electrons between methanogenesis and TCE dechlorination favored the former process. At a constant TCE concentration, increased initial acetate concentrations did not have a significant effect on the electron distribution. It is noteworthy that an extremely small fraction (0.01 to 0.05%) of the total reducing equivalents consumed by these cultures was actually utilized for the dechlorination process. In a previous study of TCE dechlorination at 35°C by mixed, sulfate-reducing cultures using acetate and lactate as electron donors, the fractions of the total reducing equivalents used for sulfate reduction, methane production and dechlorination were 98.66, 1.29 and 0.05%, respectively (Pavlostathis and Zhuang, 1991a). Mixed, sulfate-reducing cultures grown on lactate, dechlorinated PCE to TCE with the following electron distribution: 99.12, 0.84 and 0.04% for sulfate reduction, methane production and dechlorination, respectively (Bagley and Gossett, 1990). In contrast to these studies where a very low fraction of electrons was directed toward reductive dechlorination,

a high electron flow (31%) toward PCE dechlorination (and 69% toward acetogenesis) was achieved by an enriched anaerobic culture under inhibition of methanogenesis brought about by high PCE levels and/or its dechlorination products (DiStefano *et al.*, 1991). An enriched, anaerobic culture dechlorinated PCE by using H_2 or formate as electron donors and all derived electrons were completely recovered in dechlorination products and biomass (Holliger et al., 1993). Therefore, although there is in most cases a disproportionate distribution of electrons between the primary metabolism (e.g., methanogenesis) and dechlorination, under certain conditions a high electron flow toward dechlorination can be achieved.

CONCLUSIONS

To elucidate the applicability of the microbial reductive dechlorination process for the remediation of soil and groundwater contaminated with chloroalkenes, parameters such as pH, temperature, and electron donor concentration were examined for their effect on the dechlorination process mediated by a mixed methanogenic culture. The optimum conditions for reductive dechlorination were achieved at pH and temperature levels of around neutrality and 35°C, respectively. While very low levels of TCE (2.1 to 8.4 mg/L) adversely affected the methane production rate they did not significantly affect the rate of TCE dechlorination. The availability of the electron donor had the greatest impact on the dechlorination process. However, only 0.01 to 0.05% of the total electroequivalents used were channeled towards reductive dechlorination. Therefore, microbial reductive dechlorination requires not only a relatively high biomass concentration, but more importantly an ample supply of electron donor(s) to provide a steady flow of reducing power to the requisite microorganisms. Conditions, yet to be determined, favoring the use of electron donors predominantly for dechlorination would be highly desirable for the development of efficient and cost-effective bioremediation technologies.

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