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Reductive Dechlorination of Tri- and Tetrachloroethylenes Depends on Transition from Aerobic to Anaerobic Conditions

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Aerobic enrichment cultures from contaminated groundwaters dechlorinated trichloroethylene (TCE) (14.6 mg/liter; 111 μmol/liter) and tetrachloroethylene (PCE) (16.2 mg/liter; 98 μmol/liter) reductively within 4 days after the transition from aerobic to anaerobic conditions. The transformation products were equimolar amounts of *cis*-1,2-dichloroethylene and traces of 1,1-dichloroethylene. No other chlorinated product and no methane were detected. The change was accompanied by the release of sulfide, which caused a decrease in the redox potential from 0 to −150 mV. In sterile control experiments, sulfide led to the abiotic formation of traces of 1,1-dichloroethylene without *cis*-1,2-dichloroethylene production. The reductive dechlorination of PCE via TCE depended on these specific transition conditions after consumption of the electron acceptor oxygen or nitrate. Repeated feeding of TCE or PCE to cultures after the change to anaerobic conditions yielded no further dechlorination. Only aerobic subcultures with an air/liquid ratio of 1:4 maintained dechlorination activities; anaerobic subcultures showed no transformation. Bacteria from noncontaminated sites showed no reduction under the same conditions.

Chlorinated ethenes and ethanes are widely used as solvents, as degreasing agents, or in various applications of technical processes. In the industrialized nations, trichloroethylene (TCE) and tetrachloroethylene (perchloroethylene; PCE) are the most frequently found chlorinated groundwater contaminants (30). Since these compounds or their transformation products are suspected to be carcinogens (20), an extensive understanding of their fate in the environment is necessary to reduce risk for human health and to apply remediation techniques successfully.

Up to the early 1980s, PCE and TCE were considered to be persistent in aquatic environments and to be resistant to microbial degradation (20). However, recent studies showed that TCE is oxidized by methylotrophic bacteria (23, 27, 36), by propane (34)- and ammonia (1)-oxidizing bacteria, and by other heterotrophic enrichment cultures (12). *Pseudomonas* species degrade TCE completely to CO_2 when aromatic degradation pathways are preinduced by phenol or toluene (25, 33). However, for PCE no microbial attack under aerobic conditions was found.

Under strictly anaerobic conditions, both TCE and PCE are subject to reductive dechlorination. Anaerobic biotransformation was observed in methanogenic cultures and fixed-film reactors (4, 10, 11, 13, 32) and in sediment and aquifer microcosms (3, 29, 35) in which small amounts (≪5 mg/liter) of PCE and TCE were transformed. It is generally accepted that the anaerobic—widely understood as methanogenic—transformation of PCE proceeds by sequential reductive dechlorination (3) via TCE, dichloroethylenes, and vinyl chloride to CO_2 (4, 32) or ethylene (13). Vinyl chloride was found to accumulate transiently during this reaction sequence (32). Several authors suggested that primarily acetate-utilizing methanogenic bacteria are involved in the reductive dechlorination of chlorinated ethenes (4, 32). Two strains of *Methanosarcina* species and one 3-chlorobenzoate-dehalogenating bacterium able to dechlorinate PCE to TCE at concentrations of ≪1 mg/liter were actually found (9, 10, 11).

In contaminated aquifers, different patterns of dechlorination products were observed. Examinations of aquifers in Germany revealed high concentrations of *cis*-1,2-dichloroethylene (cDCE) in groundwaters which had been contaminated with PCE and TCE (5, 21, 26). In many cases, cDCE was the main component of the actual contamination, whereas only traces of vinyl chloride and methane were found. Bioremediation investigations of a contaminated site in Braunschweig, Germany, showed fast dechlorination of PCE and TCE to cDCE without subsequent dechlorination of cDCE under specific conditions. The accumulation of cDCE suggested that other than methanogenic conditions supported the reductive dechlorination of PCE and TCE to cDCE in contaminated aquifers. This study was carried out to define the conditions under which the accumulation of cDCE occurs.

MATERIALS AND METHODS

Chemicals. Unless otherwise indicated, all chemicals were purchased in analytical grade from Merck, Darmstadt, Germany. PCE in spectroscopic grade and TCE in analytical grade (>99% pure, as determined by gas chromatography) were obtained from Fluka AG, Neu-Ulm, Germany. 1,1-Dichloroethylene and cDCE were acquired in gas chromatography grades (97 and 99% pure, respectively) from Aldrich Chemicals, Steinheim, Germany. Vinyl chloride and technical gases were obtained from Linde AG, Braunschweig, Germany.

Groundwater sampling. Samples from the groundwater at the contaminated site in Braunschweig, Germany, were taken with a submersible pump. The groundwater was pumped into glass bottles without degassing.

Media and cultures. The basic medium (M; modified from Brunner et al. [6]) used in the experiments contained the following compounds per liter: $Na_2HPO_4 \cdot 2H_2O$, 2.4 g; KH_2PO_4, 1.5 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; 3 ml of vitamin solution (28); and 10 ml of trace element

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TABLE 1. Groundwater analyses for different wells of a contaminated site

Compound	Concn (mg/liter) in:			
	Uncontaminated well	Contaminated well		
	A	B	C[a]	D
CH_4	<0.05	0.3	1.3	0.5
Dichloromethane	<0.001	18.4	43.3	7.7
Trichloromethane	0.002	0.77	0.53	0.78
Carbon tetrachloride	<0.0001	<0.002	<0.002	<0.002
1,1-Dichloroethane	<0.001	0.96	1.58	1.16
1,1,1-Trichloroethane	0.001	0.33	0.21	0.36
1,2-Dichloroethane	<0.002	1.38	0.78	<0.04
Vinyl chloride	<0.004	0.79	1.67	0.71
1,1-Dichloroethylene	<0.0005	0.19	0.10	0.17
trans-1,2-Dichloroethylene	<0.002	<0.004	<0.004	<0.004
cDCE	<0.002	124.2	73.7	172.3
TCE	0.042	10.2	34.6	3.02
PCE	0.005	1.80	3.51	1.29
Benzene	<0.1	0.93	2.0	0.56
Toluene	<0.05	29.3	45.0	37.9
Xylene	<0.05	39.8	36.1	28.4
Chemical oxygen demand (mg of O_2/liter)	5	2,760	19,050	1,990
Total organic carbon	ND[b]	751	4,269	ND
Cl^-	15	269	372	177
SO_4^{2-}	330	260	100	ND
NO_3^-	34	0.17	0.5	ND
E_h (mV)	+457	−184	−342	−340

[a] Groundwater used for the experiments.
[b] ND, not done.

solution (6); the pH of the medium was 6.9. For the dechlorination experiments, a complex xenobiotic medium (XM) was prepared by mixing medium M and contaminated groundwater 1:1 (well C, Table 1). Autoclaving for 30 min sterilized the groundwater and eliminated the volatile contaminants. Medium XM was prepared in each culture vessel separately. After the components were mixed, the medium contained a small quantity of a yellow precipitate of iron phosphates.

For the dechlorination experiments with organic sulfur compounds, a synthetic xenobiotic medium (SM; based on the composition of the contaminated groundwater) was created by adding the following components per liter to medium M: yeast extract, 0.4 g; KNO_3, 0.05 g; $CaSO_4$, 0.05 g; $(NH_4)_2Fe(SO_4)_2$, 0.04 g; Na_2S, 0.05 g; 2-butanone, 100 mg; butyl acetate, 100 mg; 2-chlorobenzene, 2.5 mg; ethyl acetate, 200 mg; isopropanol, 100 mg; and toluene, 50 mg.

Additional media were used for separating different physiological groups of bacteria. Nutrient broth (NB) contained, per liter, $Na_2HPO_4 \cdot 2H_2O$, 0.24 g; KH_2PO_4, 0.15 g; peptone (caseine hydrolysate), 2.5 g; and meat extract, 1.5 g. Methanogenic (ME) and sulfidogenic (P) media were as previously described (no. 63 and 120, respectively [14]). Cultures with the latter two media were incubated at 37°C. All other experiments were carried out at 25°C. Anaerobic media were prepared under a nitrogen atmosphere.

Experiments with different types of electron acceptors were carried out with 200 ml of medium and 300 ml of oxygen or nitrogen in 500-ml bottles sealed with Teflon-lined screw caps. The bottles had lateral septum ports with Hungate butyl rubber septa (Bellco International, Feltham, United Kingdom). All other reductive dechlorination experiments were performed with 200 ml of medium and 50 ml of air in 250-ml serum bottles sealed with screw caps and Teflon-lined butyl rubber septa. Two microliters of cDCE (12.84 mg/liter; 132 μmol/liter), TCE (14.64 mg/liter; 111 μmol/liter), or PCE (16.23 mg/liter; 97.7 μmol/liter) was added to the media with a Hamilton syringe. Closed bottles with the media were stored for 36 h to adjust the equilibrium of the volatile compounds between the gas and liquid phases before inoculation. The partition ratios of the chlorinated compounds (concentration of gas/concentration of liquid) in 250- and 500-ml bottles were determined by comparing the added amounts to the measured concentrations in the liquid phase. Ninety five to ninety eight percent of the added compounds was recovered in completely filled bottles. The partition ratios of PCE were determined for the gas/liquid ratio at the beginning of incubation. Because of the change in the liquid volume from sampling, the partition ratios of cDCE were measured for the actual gas/liquid ratio at the end of incubation in each set of experiments. PCE was added to the media at a concentration of 16.23 mg/liter. In 500-ml bottles a partition ratio of 1.33 was measured; only 43% (6.98 mg/liter; 42.1 μmol/liter) of the added PCE was detected in the liquid phase. The partition ratio of cDCE was 0.31 (76% detected in the liquid phase). In 250-ml bottles the partition ratio of PCE was 0.26 (79% detected in the liquid phase). The partition ratio of cDCE was 0.30 (77% detected in the liquid phase). The concentrations measured in the liquid phase were corrected for the partition ratios to determine the molar ratio of the dechlorination reaction. The partition ratios of PCE found in this study were much lower than those found in previous studies (16), whereas those of cDCE were of the same order of magnitude. The differences in the partition ratios of PCE between our study and previous studies may have been caused by high concentrations of hydrophobic organic compounds in the contaminated groundwater in medium XM.

Cultures with different types of electron acceptors were inoculated with 5 ml of activated sludge. Subcultures were inoculated with 1 ml of the culture which exhibited complete dechlorination of PCE. The activated sludge was harvested from a bench-scale continuous-flow reactor with pure oxygen aeration (19). The reactor consisted of an aeration vessel and a sedimentation vessel with biomass backflow. The reactor was developed for the biodegradation of contaminants in the groundwater from well C (Table 1) and was inoculated only with bacteria from the groundwater.

The bacteria of the dechlorinating cultures were isolated on agar plates of medium XM or NB and soft agar tubes of anaerobic medium XM, NB, P, or ME. For examination of the dechlorination activity of anaerobic bacteria, subculturing was carried out with PCE and TCE in medium XM, NB, P, or ME. In addition to the cultivation method, the epifluorescence method was used to examine the appearance of methanogenic bacteria as described by Mink and Dugan (24). Only two types of anaerobic bacteria could be detected in the dechlorinating subcultures: a facultative, anaerobic, rod-shaped bacterium characterized by aerobic and anaerobic growth on different media and by sporeforming capabilities (*Bacillus* sp.) and a slightly vibrio-shaped, sulfidogenic, sporeforming bacterium characterized by growth with the formation of FeS in medium P (*Desulfotomaculum* sp.). Cultures for the kinetic experiments were inoculated with a single colony of an aerobic isolate from an agar plate and 0.5 ml of a mixed culture consisting of the *Bacillus* sp. and the *Desulfotomaculum* sp.

Each set of experiments was accompanied by controls

with sterilized inoculum. Further control experiments with cultures and sterilized media were conducted by decreasing the redox potential with 50 mg of Na_2S added to 200 ml of medium (to obtain the redox potential of −150 mV of seeded cultures after reductive dechlorination).

Analytical methods. The chlorinated hydrocarbons, their degradation products, and other volatile organic compounds were determined by head-space capillary gas chromatography. To measure high and low concentrations of the chlorinated compounds simultaneously, we used a special detecting unit on a Carlo Erba Mega series gas chromatograph (HRGC 5300; Carlo Erba Instruments, Hofheim, Germany) with a headspace autosampler (HS 250) and a Mega series computing integrator (type 4270). The detecting unit was built with a linearized ^{63}Ni electron capture detector and a serially connected flame ionization detector.

A fused silica capillary column (SE-54; 50-m length; 0.32-μm inner diameter; 0.3-μm film thickness; Perkin Elmer) was used with helium as the carrier gas (1.45 ml/min). Nitrogen was used as the make-up gas (40 ml/min). The temperature program was as follows: 18 min of isothermal conditions at 40°C, 4 min of heating at a rate of 25°C/min, and 6 min of isothermal conditions at 140°C. Samples were collected in 10-ml headspace vials (5-ml sample volume) sealed with Teflon-lined butyl rubber septa and aluminum crimp caps. Before analysis, the vials were placed in the headspace autosampler for 3 h at 65°C to adjust the equilibrium between the gas and liquid phases. Analytical standards were prepared by dissolving the volatile compounds in dimethyl formamide. Ten microliters of the diluted standard solutions was injected into 5 ml of medium M in the headspace vials. The calibration standard vials were immediately closed and treated like sample vials. Peak areas were calculated by the external standard method. Detection limits ranged from 0.02 μg/liter for PCE to 4 μg/liter for vinyl chloride. Data given are means of duplicate analyses.

Total organic carbon was measured with a total organic carbon monitor (type TCM 480; Carlo Erba Instruments). Chloride ions were determined with an ion-sensitive electrode (type 6.0502) and a reference electrode (type 6.0726) (both from Metrohm, Filderstadt, Germany). Redox potentials were measured electrometrically with a platinum electrode and a calomel reference electrode (Ingold, Steinbach, Germany). The data were corrected to normal potentials. The following parameters were determined with photometric test kits from Dr. Bruno Lange GmbH, Berlin, Germany: chemical oxygen demand with LCK 114 and LCK 314, sulfate ions with LCK 153, and nitrate ions with LCK 339. Total sulfide content was measured with a Spectroquant test kit (no. 14779.0001; Merck, Darmstadt, Germany). Bacteria were microscopically counted in a Thomae chamber. Because of their morphological differences, the numbers of cells of the different strains were estimated by microscopic observation.

RESULTS

PCE and TCE dechlorination in a contaminated aquifer. In this study, groundwater and bacteria from a contaminated site of a former solvent-recycling factor in Germany were used for the dechlorination experiments (well C, Table 1). The highest contamination was found in well C, with a chemical oxygen demand of 19,000 mg/liter (summarizing parameter of the organic content) (Table 1). The contamination consisted of aliphatic and aromatic solvents, alcohols, and volatile chlorinated compounds. More than 100 xenobiotic compounds were detected in the groundwater (19). No *trans*-1,2-dichloroethylene was detected. High concentrations of cDCE in contaminated wells B, C, and D correlated with low concentrations of TCE and PCE. The highest content of organic compounds was found together with the highest TCE, PCE, and vinyl chloride concentrations and the lowest cDCE concentrations. Vinyl chloride concentrations reached only 3% of cDCE concentrations. Vinyl chloride concentrations seemed to be independent of cDCE concentrations but showed a dependence on methane, which was found only in traces. The high chlorine contents of the contaminated wells indicated transformation of the chlorinated compounds. As shown by the redox potential and the nitrate concentration in uncontaminated well A, the aquifer was originally aerobic.

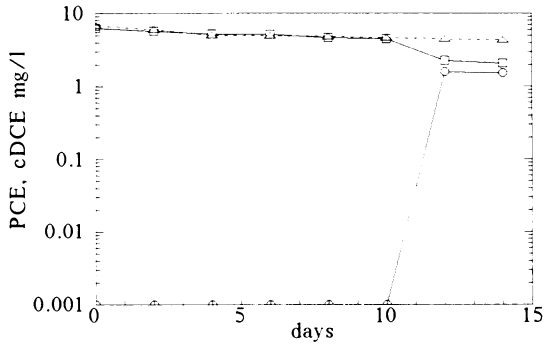

FIG. 1. Reductive dechlorination of PCE under aerobic conditions in medium XM inoculated with activated sludge. Symbols: △, PCE sterile control; □, PCE; ○, cDCE. (Error bars are smaller than the symbols.)

Dechlorination experiments with aerobic sludge. To prove the aerobic degradation of the groundwater contaminants, we conducted batch experiments with single compounds and activated sludge. These batch cultures showed reductive dechlorination of PCE and TCE when insufficient oxygen for complete degradation of the organic compounds was provided in the medium. This phenomenon, which is in contrast to previous reports (4, 13, 32), was examined more closely under various redox conditions. The following electron acceptors were supplied to medium XM inoculated with activated sludge: 1,500 ml of O_2 per liter, 1 g of NO_3^- per liter, and oxygen dissolved in the medium (the atmosphere in the vessel was replaced by N_2). Culturing under anaerobic conditions was carried out with different media (XM, NB, ME, and P). The results are presented in Fig. 1 to 3 (note the logarithmic scale of the ordinate).

Bacterial growth was observed in all media during the first 6 days of incubation. The initially aerobic culture started transformation of PCE to cDCE at day 10 (Fig. 1). The reaction was accompanied by a change in the color of the medium sediment from yellow to grey, indicating the onset of sulfidogenic conditions. The culture under denitrification conditions showed the same reaction after the consumption of nitrate (Fig. 2). Complete transformation of PCE to cDCE was found in the culture with aerobically prepared medium and a gas atmosphere replaced by nitrogen (Fig. 3). In this case, the dechlorination was accompanied by a change in the color of the medium to blackish at day 7. At day 8, traces of 1,1-dichloroethylene (5 μg/liter) appeared with the onset of sulfidogenic conditions. TCE concentrations increased during transformation, but after 10 days, TCE was no longer

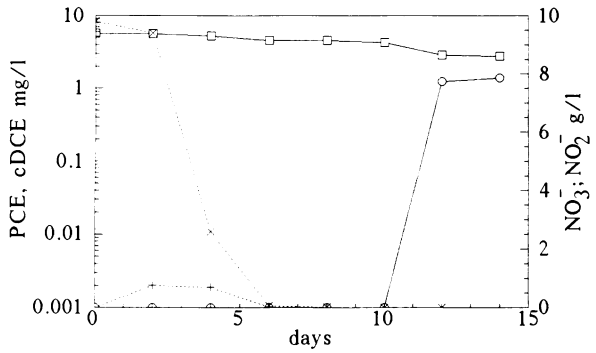

FIG. 2. Reductive dechlorination of PCE under denitrifying conditions in medium XM inoculated with activated sludge. Symbols: □, PCE; ○, cDCE; ×, NO_3^-; +, NO_2^-. (Error bars are smaller than the symbols.)

TABLE 2. Parameters of PCE-dechlorinating cultures under different physiological conditions[a]

Culture	Time (days)	pH	E_h (mV)	SO_4^{2-} (mg/liter)	S^- (mg/liter)
Control	0	6.9	110	275 ± 22	<0.1
	14	6.9	100	269 ± 28	<0.1
Aerobic	0	6.9	150	272 ± 24	<0.1
	14	7.8	−150	180 ± 8	16 ± 0.8
Denitrifying	0	6.9	110	271 ± 19	<0.1
	14	8.0	−170	166 ± 12	21 ± 1.3
Oxygen dissolved in the medium[b]	0	6.9	110	268 ± 22	<0.1
	14	7.6	−210	87 ± 6	25 ± 1.2

[a] Cultures in medium XM were inoculated with 5 ml of activated sludge from an aerobic continuous-flow reactor.
[b] The gas atmosphere was replaced by nitrogen.

detectable. The initial concentration of PCE in the medium was 6.98 mg/liter. Corrected for the partition ratio between the gas and liquid phases, this value represented 97.7 μmol/liter overall. At day 14, the cDCE concentration of 7.1 mg/liter corrected for the partition ratio represented 96 μmol/liter. This calculation indicates an equimolar transformation of PCE to cDCE. Repeated additions of PCE to cultures after the formation of cDCE yielded no additional dechlorination of PCE. No subsequent reduction of cDCE was observed after four more months of incubation (data not shown). In cultures that were anaerobic from the beginning of incubation, no transformation of PCE was observed. Methane or methanogenic bacteria were not detected in the cultures. In medium P (sulfidogenic), 4.5 μg of 1,1-dichloroethylene per liter was found after the growth of sulfidogenic bacteria.

Physicochemical data from the cultures are presented in Table 2. The redox potential of the aerobic and denitrifying cultures decreased from +110 (150) mV to −150 (170) mV. At this redox potential, small amounts of cDCE were produced. In the culture with oxygen dissolved only in the medium, the redox potential decreased from 110 mV to −210 mV. Therefore, redox potentials between −150 and −210 mV seemed to be necessary for complete dechlorination of PCE to cDCE. Sulfide concentrations increased with decreasing redox potentials. The fact that the sulfide concentrations were low compared with the amount of sulfate removed can be explained by incomplete reduction of sulfate to sulfur or thiosulfate.

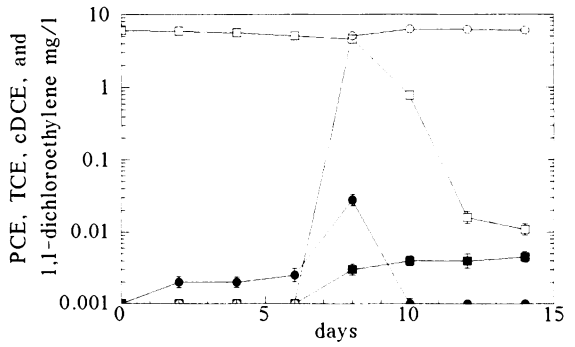

FIG. 3. Reductive dechlorination of PCE in the culture with oxygen dissolved only in the liquid phase (medium XM; the gas atmosphere was replaced by nitrogen) and inoculated with activated sludge. Symbols: □, PCE; ○, cDCE; ●, TCE; ■, 1,1-dichloroethylene. (Error bars are shown if they are larger than the symbols.)

The results showed that reductive dechlorination of PCE via TCE to cDCE could be achieved with the transition from aerobic to anaerobic conditions. In subcultures of the dechlorinating culture with oxygen dissolved only in the medium, the amounts of anaerobic bacteria increased in consecutive cultures and aerobic bacteria were lost. PCE transformation rates decreased from subculture to subculture. Stable dechlorination capabilities were maintained only in cultures with a higher initial oxygen content in the gas phase. Therefore, further experiments were carried out with an air/liquid ratio of 1:4. These cultures were defined as cultures with a limited oxygen supply.

Dechlorination experiments with sulfur compounds. Since sulfidogenic cultures showed no formation of cDCE, the influence of sulfide was examined. Experiments were conducted to simulate the change in the redox potential in seeded cultures. Na_2S (50 mg/liter) was added to sterile controls of medium XM, M, or NB with PCE and TCE. At 48 h after the addition of sulfide, only 1,1-dichloroethylene was found in the media. Approximately 0.08 to 0.09% of the PCE or TCE molecules were dechlorinated to 1,1-dichloroethylene in medium XM (7.5 μg/liter [77 nmol/liter] and 9.4 μg/liter [98 nmol/liter], respectively). The transformation did not depend on specific compounds in the groundwater. 1,1-Dichloroethylene was also found in medium M (3.7 and 8.9 μg/liter, respectively) and in medium NB (4.3 and 6.9 μg/liter, respectively). The amounts of 1,1-dichloroethylene released were in the same range as in cDCE-forming cultures or in cultures with sulfidogenic bacteria. This result demonstrates the abiotic involvement of sulfide in the formation of 1,1-dichloroethylene.

The experiments showed that anaerobic cultures were not able to dechlorinate PCE and TCE to cDCE. Sulfide was also not involved in the formation of cDCE. These facts led to the hypothesis that aerobic or aerotolerant bacteria were necessary for transformation. Sulfide could be involved in the reaction only by decreasing the redox potential to nonphysiological values for these bacteria. Therefore, the reaction could be initiated by the release of sulfide from proteins or by the degradation of other organic sulfur compounds. To test this hypothesis, we carried out experiments with medium SM and NB with TCE. Different SM cultures were supplemented with L-cysteine (150 mg/liter), glutathi-

one (150 mg/liter), dimethyl sulfide (50 mg/liter), and diethyl sulfide (75 mg/liter). After 28 days, no cDCE was found in controls or in the culture with cysteine. cDCE was found at 115 μg/liter with gluthathione, 490 μg/liter with dimethyl sulfide, and 6,860 μg/liter with diethyl sulfide. In addition, 6 μg of 1,1-dichloroethylene per liter was detected with diethyl sulfide. In medium NB, 7,850 μg of cDCE per liter and 6.2 μg of 1,1-dichloroethylene per liter were measured after 8 days. The data showed that sulfide release was necessary for reductive dechlorination in synthetic media.

Characterization of the dechlorinating mixed cultures. Experiments to isolate the dechlorinating bacteria were carried out with medium NB or SM supplemented with diethyl sulfide. Dechlorination activity was lost in consecutive subcultures, although growth was observed. Stable dechlorinating capabilities were maintained only in groundwater medium XM with a limited oxygen supply. However, in these subcultures the complexity of the sludge population was preserved. Therefore, single strains isolated from the dechlorinating mixed cultures were tested for dehalogenating activity. All aerobic and anaerobic strains alone were unable to dechlorinate PCE and TCE in different media. Only a gram-positive, sporeforming, slightly vibrio-shaped, sulfidogenic bacterium (*Desulfotomaculum* sp.) released traces of 1,1-dichloroethylene in medium P. So far, the formation of cDCE could not be related to single bacterial strains.

Several combinations of strains were tested for their dechlorinating activities in medium XM with a limited oxygen supply. Dechlorination of PCE was found only in cultures in which an aerobic bacterium was mixed with the facultative anaerobic *Bacillus* sp. and the anaerobic *Desulfotomaculum* sp. These cultures could be subcultivated and retain stable dechlorination capabilities. Of seven aerobic strains tested in cocultures, three strains, one gram-negative and two gram-positive, pleomorphic bacteria, supported dechlorination. All cultures showed sulfidogenic conditions after 15 days. These results showed that aerobic bacteria were necessary for reductive dechlorination of PCE to cDCE.

Kinetics of dechlorination. The kinetics of dechlorination of PCE to cDCE were studied further in time course experiments with medium XM. Data from the culture of an aerobic, gram-positive, pleomorphic strain with the *Bacillus* and *Desulfotomaculum* species are presented in Fig. 4 and 5 (note the logarithmic scale of the ordinate). Reductive dechlorination of PCE to cDCE began after 6 days and finished at day 10 (Fig. 4). The main transformation occurred when the total number of cells began to decrease (Fig. 5). During dechlorination of PCE, the concentration of TCE increased up to 168 μg/liter (day 8; Fig. 4). At the end of the reaction, TCE was no longer detectable. 1,1-Dichloroethylene (4.5 μg/liter; 47 nmol/l) appeared when sulfidogenic conditions were established (days 8 to 10). The concentration of vinyl chloride in medium XM did not increase during reductive dechlorination. The initial concentration of PCE in the liquid phase was 12.8 mg/liter. Corrected for the partition ratio between the gas and liquid phases, this value represented 97.7 μmol/liter. At the end of transformation, 7.3 mg of cDCE per liter was found in the medium. This value represented 97.5 μmol of cDCE per liter overall. The data demonstrated that PCE was dechlorinated to equimolar amounts of cDCE. In the dehalogenation reaction, 6.9 mg of chloride ions per liter should have been released; 5.8 mg/liter was actually found (Fig. 5). The release of 4.0 mg of chloride ions before dechlorination of PCE (days 4 to 6) was caused

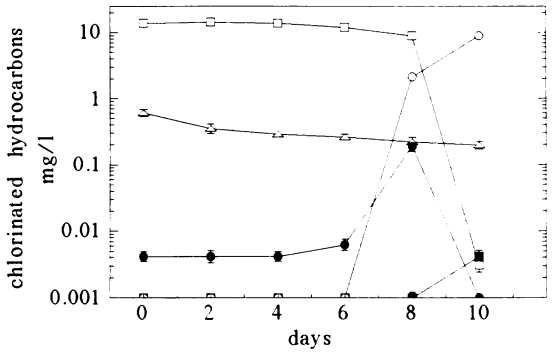

FIG. 4. Kinetics of reductive dechlorination (concentrations of chlorinated hydrocarbons) of PCE with a three-species mixed culture and an air/medium ratio of 1:4 (limited oxygen supply). Symbols: □, PCE; ○, cDCE; ●, TCE; ■, 1,1-dichloroethylene; △, vinyl chloride. (Error bars are shown if they are larger than the symbols.)

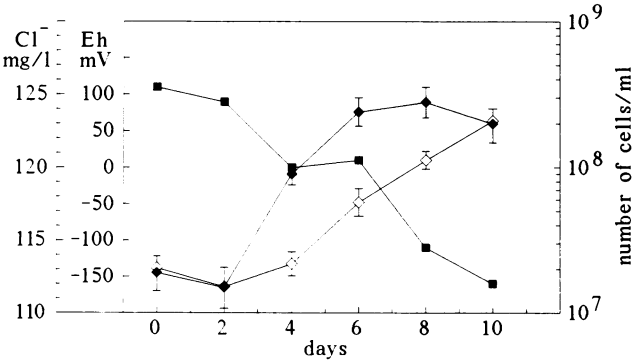

FIG. 5. Kinetics of reductive dechlorination (redox potential, number of cells, and chloride ions) of PCE with a three-species mixed culture and an air/medium ratio of 1:4 (limited oxygen supply). Symbols: ■, redox potential; ◆, number of cells; ◇, chloride ions.

by the degradation of unknown, nonvolatile, chlorinated compounds in medium XM (data not shown).

After 4 days of incubation, visible growth of the aerobic strain was observed (Fig. 5). The organism exhibited a growth cycle including myceliumlike clusters, fragmentation to rod-shaped clusters, and single coccoid cells. Microscopic observation showed that after 6 days, about 10% of the total bacterial cells were cells of the *Bacillus* sp. At this time, reductive dechlorination of PCE started and the first *Desulfotomaculum* cells could be detected. The redox potential (Fig. 5) decreased to 0 during growth of the aerobic strain (day 4) and was constant up to the beginning of growth of the sulfidogenic bacteria (day 6). After 8 days, all cells of the aerobic strain were coccoid. From the sporogenic *Bacillus* sp., mostly spores were released. The relative number of *Desulfotomaculum* cells reached 20%. The total number of cells began to decrease at this stage. At the beginning of growth of the sulfidogenic bacteria, a further decrease in the redox potential was observed. The main transformation of PCE, including the formation of 1,1-dichloroethylene, occurred between −100 and −150 mV.

Dechlorination experiments with bacteria from other sites. Experiments with samples from other sites were carried out to examine the distribution of PCE transformation capabilities in bacteria from different environments. Cultures were

inoculated with 5 g of garden soil (high humus content), 50 ml of water from an uncontaminated aquifer (waterworks in Peine, Germany), or 50 ml of water from an aquifer contaminated with PCE and TCE (Mannheim, Germany). Doubly concentrated medium XM was prepared to allow inoculation with 50 ml of groundwater. All cultures showed growth and sulfidogenic conditions and released 1,1-dichloroethylene after 30 days of incubation. In the culture inoculated with soil, 6 mg of CH_4 per liter appeared. TCE concentrations increased from 4 to 75 μg/liter. In all cases except one, no formation of cDCE or vinyl chloride was observed. Only in the culture inoculated with water from the contaminated aquifer was equimolar transformation of PCE to cDCE found.

DISCUSSION

In this study, the conditions of reductive dechlorination of PCE and TCE to cDCE were examined. Aerobic enrichment cultures from a contaminated aquifer incubated with a limited oxygen supply showed reductive dechlorination to equimolar amounts of cDCE after the transition from aerobic to anaerobic conditions. Dechlorination in batch cultures was restricted to the beginning of anaerobic conditions (2 to 4 days). No dechlorination was observed after this time. The number of cells decreased during transformation. Aerobic conditions in the first days of incubation were necessary to initiate dechlorination and to maintain the activity in subcultures. No dechlorination of PCE to cDCE was found in cultures which were anaerobic from the beginning of incubation. TCE was the intermediate product, indicating sequential dechlorination. The transformation reaction was highly stereoselective. cDCE but not *trans*-1,2-dichloroethylene was found.

Transformation required an additional decrease in the redox potential caused by sulfide. The decrease must be considered the driving force for the onset of reductive dechlorination, although the particular reaction mechanism is still unclear. Cultures without this release of sulfide or cultures of sulfidogenic bacteria showed no transformation. In dechlorinating cultures, sulfide could be derived from degradable organic sulfur compounds (proteins or diethyl sulfide) or from the successive growth of sulfidogenic bacteria. This result suggests that sulfide was the mediating factor (decrease in the redox potential) for other bacteria.

Sulfide itself caused abiotic dechlorination of PCE and TCE. However, the dechlorination product was different. Traces of 1,1-dichloroethylene were found after the addition of sulfide to sterile controls. The formation of 1,1-dichloroethylene was also observed in sulfidogenic cultures. Abiotic formation was not further examined in this study but may be a reaction of sulfur nucleophiles, as shown for other halogenated compounds (2). Biogenic formation of 1,1-dichloroethylene was proposed by several authors (3, 29, 32), but no proof of the reaction was published. The formation of 1,1-dichloroethylene can be considered a reaction mediated by the microbial release of sulfide. The occurrence of 1,1-dichloroethylene in groundwaters contaminated with TCE and PCE can be explained conclusively for the first time by this reaction.

Previous reports described sequential reductive dechlorination of PCE and TCE only under strictly anaerobic, particularly methanogenic, conditions (3, 4, 13, 29, 32, 35). Most of these data were obtained from long-term experiments conducted with large amounts of biomass and low concentrations of the chlorinated compounds. Chlorinated metabolites did not exceed 20% of the initial concentrations of PCE and TCE. Vinyl chloride accumulated in methanogenic batch cultures and continuous-flow reactors (13, 32). In some cases, cDCE occurred as a metabolite (3, 29, 32), but equimolar transformations to cDCE without the formation of vinyl chloride were not observed in methanogenic cultures.

In the dechlorinating cultures described in this study, no methanogenic bacteria could be detected. All anaerobic enrichment cultures and isolates showed no dechlorination activity. In sulfidogenic cultures, traces of 1,1-dichloroethylene were found. Reductive dechlorination of PCE and TCE to cDCE was achieved only in remixed cultures of different aerobic isolates in a coculture with a *Bacillus* sp. and a *Desulfotomaculum* sp. under conditions of limited oxygen supply. Other aerobic isolates also supported the growth of the *Bacillus* and *Desulfotomaculum* species, but these cultures showed no dechlorination. These findings and the amounts transformed showed that the conditions of reductive dechlorination found in this study were completely different from the conditions described in previous reports.

The dechlorination reaction could not be related to axenic cultures. However, it was shown in the cultures with three species that aerobic bacteria were necessary to initiate the reaction. Further studies must clarify which of the three species actually catalyzes the reductive dechlorination of PCE to cDCE. The transformation may be catalyzed by aerobic or facultative anaerobic bacteria (*Bacillus* sp.) if the redox potential drops to nonphysiological values. Another possibility is that these bacteria excrete an unknown factor that enables the sulfidogenic bacteria (*Desulfotomaculum* sp.) to catalyze the dechlorination under these specific conditions. The reaction may even be conducted by a nondetectable obligate anaerobic bacterium carried over from the enrichment cultures. The last two assumptions, however, are not very likely, because no traces of cDCE were found in anaerobic cultures. In addition, the reaction should not have been restricted to the transition conditions in the cultures with a limited oxygen supply. The results more likely suggest that aerobic or facultative anaerobic bacteria were involved in the dechlorination. This hypothesis is supported by the facts that these cultures could only be subcultivated and retain stable dechlorinating activities under conditions of limited oxygen supply. The anaerobic *Desulfotomaculum* sp. could only survive under these conditions because of its sporeforming capability. Transformation in the dechlorinating cultures occurred when the redox potential in the medium decreased to values between −50 and −150 mV and when carbon sources (electron donors) were present in excess. After consumption of the electron acceptor oxygen or nitrate by growth of the aerobic bacteria, the redox potential values reached only 0 mV. To reach the low redox potentials required for dechlorination, a further decrease in the redox potential caused by sulfide was necessary. However, dechlorination was also stimulated by the release of sulfide from the degradation of organic sulfur compounds without the growth of sulfidogenic bacteria. The number of cells in dechlorinating cultures was already decreasing during dechlorination. This result implies that the release of cell compounds from dying cells may be also involved in dechlorination.

In addition, several indications for the involvement of aerobic or facultative anaerobic bacteria were already published in previous studies. Aerobic biodegradation of TCE with the formation of $^{14}CO_2$ from radiolabeled compounds was reported in heterotrophic enrichment cultures. The

authors mentioned that some of their cultures generated cDCE and traces of 1,1-dichloroethylene (12). This result indicates reductive dechlorination in cultures which were initially aerobic. Transformation of PCE to equimolar amounts of cDCE without the formation of 1,1-dichloroethylene was demonstrated in aerobic groundwater solely contaminated with PCE. Dechlorination occurred in contaminated groundwater supplemented with ethanol, glycerol, acetate, or lactate in batch cultures with a limited gas space (18).

Thermodynamic considerations show that chlorinated compounds can serve as acceptors for electron-carrying coenzymes and parts of respiration chains (31). The redox potentials of PCE/TCE and TCE/cDCE range between those of O_2/H_2O and NO_3^-/NO_2^-. Several authors described the reduction of other chlorinated compounds by reduced cofactors, for instance, iron(II)porphyrines, which are essential parts of respiration chains (7, 22). Thus, aerobic or facultative anaerobic bacteria may also be involved in the reductive dechlorination of PCE and TCE to cDCE, whereas dechlorination to vinyl chloride is apparently conducted by strictly anaerobic or methanogenic bacteria. The first enzymes of aerobic bacteria able to dechlorinate reductively were found in cell extracts of an aerobic, polychlorophenol-degrading *Rhodococcus* sp. These enzymes had increased activities under anaerobic reaction conditions (17). Reductive dechlorination of carbon tetrachloride to chloroform by *Escherichia coli* under fumarate-respiring conditions was recently published. Carbon disulfide was found as a possible minor product of carbon tetrachloride, indicating that sulfur may play a role in the dechlorination of carbon tetrachloride. (8).

In comparison with previous reports, the differences in the new dechlorination conditions found in this study demonstrate that physiologically different bacteria compete in the reductive dechlorination of TCE and PCE. Different patterns of dechlorination products found in contaminated aquifers can be explained by different dechlorination conditions. The finding that reductive dechlorination to cDCE under the described conditions was only observed with bacteria from contaminated sites emphasizes the significance of this transformation in contaminated groundwaters.

The accumulation of vinyl chloride in methanogenic cultures explains the appearance of this component in methanogenic aquifers contaminated with TCE and PCE (5, 35). In these aquifers, high concentrations of vinyl chloride were found together with higher concentrations of methane, ethane, and ethene and traces of *trans*-1,2-dichloroethylene (5). Reductive dechlorination in methanogenic cultures depended on large amounts of degradable electron donors and on the formation of methane (11, 13).

In the aquifer described in this study, vinyl chloride concentrations depended on methane concentrations but were independent of cDCE concentrations. *trans*-1,2-Dichloroethylene was not observed. cDCE concentrations were about 100 times higher than those of vinyl chloride, indicating that there is an additional cDCE forming process, described in this study. This process can be initiated by variations of the height of groundwater layers during the year. In addition, high concentrations of sulfate may have inhibited the methanogenic dechlorination of cDCE to vinyl chloride. Sulfate inhibits methanogenesis and reductive dechlorination, as shown with chloroaromatic compounds (15). The findings of this study are in agreement with those of a study which reported the dechlorination of PCE and TCE to cDCE in microcosms of a contaminated aquifer after a few days of incubation. Further dechlorination to vinyl chloride and the formation of methane were observed only after a longer adaptation period of 2 months (26). The new type of dechlorination described in this study may be of technological importance for the treatment of contaminated groundwaters.

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