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Role of Methanogenic and Sulfate-Reducing Bacteria in the Reductive Dechlorination of Tetrachloroethylene in Mixed Culture

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Tetrachloroethylene (perchloroethylene, PCE) is widely used in many industries and particularly as a degreasing and dry-cleaning solvent. It is commonly found as a groundwater contaminant and because of its carcinogenic properties is considered a pollutant. which must be eliminated by proper treatment (Fawell and Hunt, 1988).

Several reports have demonstrated biotransformation of tetrachloroethylene at low concentrations under strict anaerobic conditions by sequential reductive dechlorination (Fathepure and Boyd, 1988a; Freedman and Gosset, 1989). During this biodegradation, trichloroethylene (TCE), 1,1-dichloroethylene, vinylidene chloride (DCE), and vinyl chloride (VC) are the intermediate products ; ethene or ethane are the end products (De Bruin et al, 1992; Di Stefano et al, 1992).

Tetrachloroethylene dechlorination was achieved by using methanogenic-enriched cultures (Freedman and Gossett, 1989) and in methanogen pure cultures (Fathepure and Boyd, 1988b). Some authors have also pointed out the ability of sulfate-reducing bacteria to degrade chlorinated compounds (ie, 3-chlorobenzoate, carbon tetrachloride, 1,2-dichloroethane, tetrachloroethylene) (Bagley and Gossett, 1990: Egli et al, 1987; Mohn and Tiedje, 1991).

The mixed culture used in this work was isolated from the sludge of an urban waste water treatment plant. It contains methanogenic, sulfate-reducing, and acetogenic bacteria which have an interdependant metabolism. Our research deals with the role of the mixed culture in PCE dechlorination at high concentration, from an ecological point of view. In this study, we investigated the respective role of sulfate-reducing and methanogenic bacteria in tetrachloroethylene dechlorination.

MATERIALS AND METHODS

A methanogenic and sulfate-reducing mixed culture was isolated from anaerobic digested sludge from the Bourg-en-Bresse (France) waste water treatment unit. After several subcultures, the enrichment was conducted using basal medium modified from Zehnder et al (1980) : KH_2PO_4 0.3 g, K_2HPO_4 0.3 g, NH_4Cl 0.3 g, NaCl 0.3 g, $CaCl_2$-$2H_2O$ 0.11 g, $MgCl_2$- $6H_2O$ 0.10 g,

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Na_2SeO_8 0.03 g, $NaHCO_3$ 3.2 g, CH_3OH 2.6 g, Yeast extract 1 g, Mineral solution 10 mL ($FeSO_4$-$7H_2O$ 0.3 g, H_3BO_3 0.05 g, $ZnCl_2$ 0.05 g, $CuCl_2$ 0.03 g, $MnCl_2$ 0.5 g, Na_2MoO_4-$2H_2O$ 0.05 g. $A1K(SO_4)_2$ 0.05 g, $CoCl_2$-$6H_2O$ 0.05 g. $NiCl_2$ 0.05 g. distilled deionized water qsp 1 L), Vitamin solution 10 mL, L-cystein 1.2 g, resazurin, distilled deionized water qsp 1L ; pH 7.5. Laboratory reactor was a 1-liter semicontinuous anaerobic digester operated at 37°C.

All experiments were conducted in batch conditions with 20 ml-serum vials. Each serum vial was amended with 50 µM tetrachloroethylene and 5 mL sterile basal medium. and inoculated with 5 mL of an actively growing mixed culture. The vials were sealed with PTFE-lined rubber septa and aluminium crimp caps. Incubation was conducted in the dark at 37°C. Each series of experiments with inoculated vials was accompanied by triplicate sterile basal medium controls (10 mL of sterile medium plus the chlorinated compound). At different incubation periods, triplicates of active and control vials were sacrificed and stored at -20°C for analysis: chlorinated compounds, biogas production. methanol degradation and biomass production. Biomass production was determined by the Lowry method for protein quantification.

Both gentamicin and 2-bromoethane sulfonic acid (BESA) inhibitors were separately applied to the mixed culture to investigate the roles of sulfate-reducing bacteria (SRB) and methanogens in PCE dechlorination. A series of experiments, prepared as above (with inoculated vials), was supplemented with 10 mM BESA. BESA-induced inhibition is specific to methanogens because of the BESA structural similarity to coenzyme M; this latter is unique to methanogens (Daniels et al, 1984). Another series of experiments was supplemented with 50 mg L^{-1} gentamicin. A preliminary study was conducted to determine the best inhibitor and its active concentration to inhibit sulfate-reducing bacteria without affecting methanogens, under our experimental conditions. Tanimoto et al (1989) screened growth inhibitors of sulfate-reducing bacteria which did not affect methanogenesis. Some antibiotics were found to be effective and specific to SRB. In our study, bacitracin and gentamicin were screened at several concentrations. Gentamicin at a concentration of 50 mg L^{-1} was the more suitable inhibitor (data not shown). This antibiotic binds to eubacterial ribosomes at multiple sites and specifically inhibits the translocation process (Gale et al, 1981).

For each measurement of the three series, enumerations of SRB were carried out by the most probable-number technique (three tubes) in anaerobic conditions. The anaerobic liquid medium contained the following substrates : Yeast extract 1 g. Sodium lactate 4 g, Ammonium chloride 0.5 g, K_2HPO_4 1 g, $MgSO_4$ 0.2 g, $CaCl_2$ 0.1 g, $FeSO_4$ 0.1 g, Na_2SO_4 0.5 g, distilled deionized water qsp 1 L plus iron thread (pH 7.2 - 7.4) (Gerhardt et al, 1994). A tube was positive only when sulfide production could be shown by a black precipitate.

The amounts of PCE, TCE, 1.1 DCE, 1.2 DCE, and VC were determined by direct injection of headspace samples into a Perkin Elmer model Sigma 2000 gas chromatograph equipped with a HS 100 autosampler, an electron capture detector and a flame ionization detector. Gas chromatographic separation was achieved with a fused-silica capillary column, CP Sil 8B methylsilicone (50 m X 0.23 mm i.d.. 1.2 µm film thickness). The time for thermostated vials in the headspace analyzer was 60 min at a temperature of 60°C (state of equilibration). The column gradient temperature was : 60°C (10 min) ; 5°C/min to 120°C ; 120°C (0 min); run-time 22 min; detectors temperature 300°C; carrier flow (He) 2 mL/min.

The amounts of CH_4, C_2H_2, and CO_2 were determined by a Girdel 330 gas chromatographic analysis of a 1 mL-headspace sample, injected with a gas-tight syringe. into a stainless-steel

column (2 m X 2 mm id.) packed with Porapak Q operated at 65°C, connected with a methanation oven (Ni catalytic) and a flame ionization detector. The temperature of the injector was 100°C. the temperature of the methanation oven 400°C, and the temperature of the detector 150°C; carrier pressure (H_2) 1 bar. The amount of methanol was determined by the internal standard method (ie mixed culture supernatant and ethanol [internal standard] vol/vol). A 1 µL-mixture was injected into a Girdel 300 gas chromatograph equipped with a flame ionization detector connected with a stainless-steel column (3 m X 2 mm id.) packed with Porapak Q 80/100 mesh operated at 170°C. The temperature of the injector was 200°C the temperature of the detector 210°C. Carrier gas was N_2.

Experimental data were statistically studied in order to assess the significant differences between the three series. PCE dechlorination followed an exponential decrease. The incubation time is linearly related to the logarithm (1n) of the PCE concentration, Then. the parameters of linear regression were calculated for the three series : correlation coefficient. regression coefficient and its 95 % confidence intervals (Table 1). A Student's t-test was used to compare the methane production of the three series.

The following compounds were obtained in neat liquid form : ICE (CPG, 99%, Prolabo, Vaulx en Velin, France), TCE (CPG, 99%, Prolabo, Vaulx en Velin, France). 1,1-DCE (99 %, Aldrich, Saint Quentin Fallavier, France), vinylidene chloride (98 %, mixture of isomers, Aldrich, Saint Quentin Fallavier, France), VC (ampoule 200 µg · mL^{-1} methanol, Supelco, Saint Germain en Laye, France). CH_4 C_2H_2, and CO_2 were obtained as mixed gases in reference bottles (Air Liquide, Lyon, France). PCE was added to cultures from a 5 mM solution in methanol (BP Normapur, 99.8 %, Prolabo, Vaulx en Velin, France). 2-Bromoethane sulfonic acid (BESA, Sodium salt) and Gentamicin Sulfate were obtained from Sigma Chemical (Saint Quentin Fallavier, France).

RESULTS AND DISCUSSION

The PCE completely disappeared within 37 days in a mixed culture without inhibitors (Fig. 1). Correlatively the formation of TCE was observed. During the first nine days of incubation, the PCE concentration decreased at a rate of 3.3 µM . day^{-1}. The TCE concentration increased at the same rate. During the 37 days of incubation, the dechlorination rate was 5.2 nmol of degraded PCE · mg $protein^{-1}$. day^{-1}. Low levels of DCE and VC were also detected. The whole mass balance of chlorine (0.43 µmol Cl) was conserved during the experiment

Our results suggest that this mixed culture has the potential to dechlorinate a high concentration of PCE via reductive dechlorination, as has been previously observed at low concentrations of PCE in other anaerobic environments (De Bruin et al. 1992; Fathepure and Boyd, 1988a; Freedman and Gossett, 1989). PCE reductive dechlorination led essentially to TCE. In other studies conducted by Freedman and Gossett (1989) and De Bruin et al (1992), PCE was completely degraded into ethene or ethane. But the concentrations of PCE were about ten times less than the concentration used in our study. High concentrations of PCE could inhibit methanogenesis (Di Stefano et al, 1991). Then, PCE toxicity could reduce the capacity of methanogens consortium to degrade TCE. In addition. the culture conditions (batch culture) could carry limiting factors : De Bruin et al (1992) observed a much slower dechlorination of PCE in batch cultures than in continuous flow, fixed-bed columns, Further studies could be carried out to investigate the ability of this mixed culture to dechlorinate TCE.

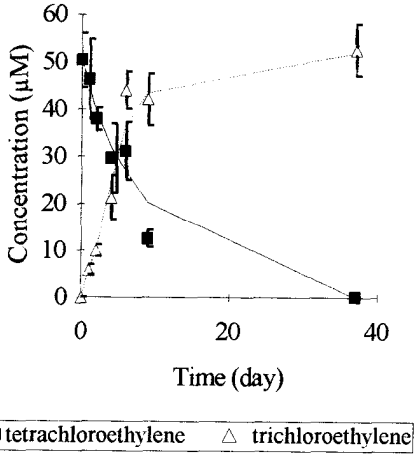

Figure 1. PCE dechlorination by the mixed culture without inhibitor (Mean ±SD).

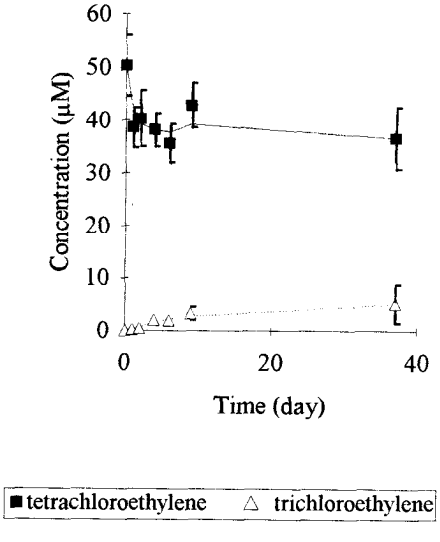

Figure 2. PCE dechlorination by the mixed culture supplemented with 10 mM BESA (Mean ±SD).

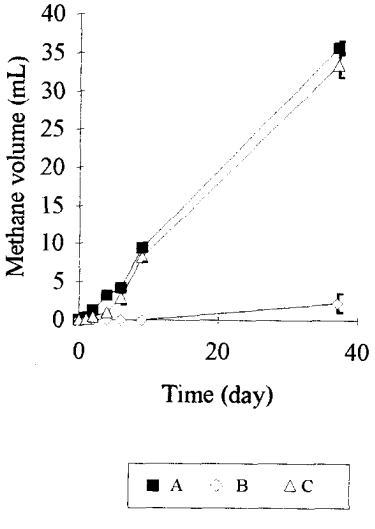

Figure 3. Methane production in mixed cultures A= Without inhibitor. B= +BESA, C= +Gentamicin (Mean ±SD).

In the mixed culture supplemented with 10 mM BESA, the concentration of PCE appeared to slightly decrease during the first four days and the dechlorination process stopped soon afterwards (Fig. 2).

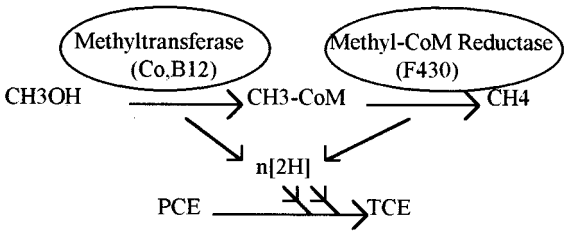

Figure 4. Hypothetical scheme for enzymatic complex involved in PCE dechlorination

BESA induced almost complete inhibition of methanogens, shown by the lack of methane production (Fig. 3). On the other hand. sulfate-reducing bacteria were not inhibited by BESA (Table 2) : their number was higher than the number in the standard mixed culture.

BESA inhibited methanogens because of its structural similarity to coenzyme M. Near total inhibition of methanogens led to the absence of methane production. The reductive dechlorination of PCE was nearly completely inhibited in this series of experiments as confirmed by a regression coefficient near zero (Table 1), demonstrating that methanogens had a deciding role in the dechlorination process. Fathepure and Boyd (1988a;1988b) proposed a hypothetical scheme : electrons transferred during methanogenesis are diverted to PCE by an electron transfer agent which is specific of methanogens and involved in methane formation. Holliger et al (1992a) observed that corrinoids or factor F_{430} were responsible for reductive dechlorination of 1,2-dichloroethane by *Methanosarcina barkeri*. Coenzyme M and factor F_{430} were indispensable for the methylcoenzyme M reductase activity. Also Holliger et al (1992b) demonstrated that the methylcoenzyme M reductase of *Methanobacterium thermoautotrophicum* DH catalyses the reductive dechlorination of 1.2-dichloroethane into ethene and chloroethane.

Table 1. Linear regression parameters used for modelling PCE dechlorination : $1n[PCE] = (a \times time) + b$

Series	Regression coefficient a	Correlation coefficient r	Lower 95% confidence intervals	Upper 95% confidence intervals
Without inhibitor	- 0.1859	- 0.9899	- 0.1977	- 0.1741
With BESA	- 0.0052	- 0.3096*	- 0.0125	- 0.0020
With gentamicin	- 0.1057	- 0.9228	- 0.1252	- 0 0861

* this correlation coefficient is low because the regression coefficient is near zero.

Table 2. Enumeration of sulfate-reducing bacteria in mixed cultures with the 95% confidence limits (Wardlaw, 1985) *: Lower 95% confidence limit < Mean < Upper 95% confidence limit*

Time (day)	Without inhibitor*	With BESA*	With gentamicin*
1	6.03 < **6.8** < 7.5	6.4 < **7.2** <7.9	0
37	3.5 < **4.3** < 5	6.4 < **7.2** <7.9	0

* Log bacteria number · mL^{-1}

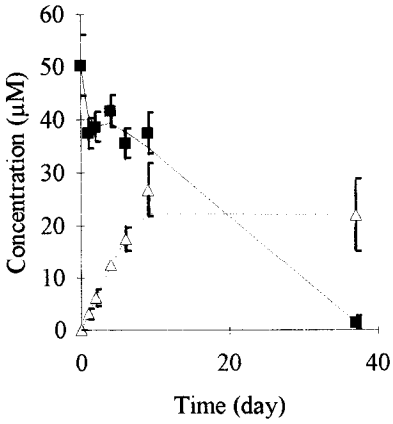

Figure 5. PCE dechlorination by the mixed culture supplemented with 50 mg · L^{-1} gentamicin (Mean ±SD).

Other enzymatic complexes might also play a part in dechlorination, for instance the methyl transferase complex which transferes the methyl-group from methanol to coenzyme M, via corrinoid (Holliger et al, 1992b). The low rate of dechlorination observed might be due to this methyl transferase complex. But the methyl coenzyme M reductase complex is responsible for the largest production of ATP. Since the methyl coenzyme M reductase complex is inhibited by BESA, the bacterial cells can not gain enough energy to support growth. The inhibition of BESA is then effective (Fig. 4).

Sulfate-reducing bacteria were not inhibited by BESA. The absence of methanogens permitted a better growth for sulfate-reducing bacteria, showing a competition between the two bacterial groups. Methanogens were dominant compared to sulfate-reducing bacteria in the standard mixed culture. The adapted culture medium was favorable to methanogens because of its lack of sulfates,which are the last acceptor of electrons for sulfate-reducing bacteria.

PCE was completely degraded into TCE in the mixed culture with 50 mg · L^{-1} gentamicin (Fig. 5). However, during the first nine days, the dechlorination rate of PCE (1.7 µM · day^{-1}) was lower than that calculated in the standard mixed culture (3.3 µM · day^{-1}). The observed decrease is noted too in Table 1 : the regression coefficient was lower than the standard regression coefficient. For all that, PCE dechlorination was effective : during the 37 days of incubation, the dechlorination rate of PCE was 5.08 nmol of degraded PCE mg $protein^{-1}$ · day^{-1}, which is similar to the standard rate.

Inhibition of all sulfate-reducing bacteria was noted (Table 2) when the activity of methanogenic bacteria was partially and temporarily inhibited (Fig. 3). After a lag time of nine days, the production of methane was slightly lower with gentamicin than without any inhibitor. Also, the t-

test gave a hightly significant difference in methane production between this mixed culture and the standard mixed culture during the first nine days (P = 0.01). The inhibiton of methanogens was about 50 % after six incubation days and was reduced to 13 % after nine incubation days.

The inhibition of sulfate-reducing bacteria, induced by gentamicin. did not stop the reductive dechlorination of PCE. So these bacteria did not have a major role in this biotransformation under our experimental conditions. However, at the beginning of the experiment, the reductive dechlorination rate was lower than the standard reductive dechlorination rate. In the same way. methane production was lower than the standard methane production during the first nine days. This lag time of methanogenesis seems to result from the momentary inhibition of methanogens by gentamicin. At a high concentration, this antibiotic could be toxic for methanogens (ie, 100 $mg \cdot L^{-1}$) (Tanimoto et al. 1989). This temporary inhibition would decrease the reductive dechlorination rate of PCE.

Only the methanogenic bacteria dechlorinate PCE under our experimental conditions, where the concentration of PCE is ten times higher than the most widely used one. The growth of sulfate-reducing bacteria increased when methanogens were inhibited by BESA. But since other studies have shown a positive role of sulfate-reducing and acetogenic bacteria in the dechlorination of PCE (Bagley and Gossett. 1990: Stromeyer et al. 1992). it is essential to have a better understanding of the relationships between sulfate-reducing and methanogenic bacteria during the dechlorination of PCE. We are currently investigating the competitive distribution of the reducing powers during dechlorination in our consortium. The knowledge of these intergroup relationships is necessary to optimize the reductive dechlorination of PCE, a carcinogenic product.

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