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Interaction between methanogenic and sulfate-reducing microorganisms during dechlorination of a high concentration of tetrachloroethylene

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A methanogenic and sulfate-reducing consortium, which was enriched on medium containing tetrachloroethylene (PCE), had the ability to dechlorinate high concentrations of PCE. Dehalogenation was due to the direct activity of methanogens. However, interactions between methanogenic and sulfate-reducing bacteria involved modification of the dechlorination process according to culture conditions. In the absence of sulfate, the relative percentage of electrons used in PCE dehalogenation increased after an addition of lactate in batch conditions. The sulfate reducers would produce further reductant from lactate catabolism. This reductant might be used by methanogenic bacteria in PCE dechlorination. A mutualistic interaction was observed in the absence of sulfate. However in the presence of sulfate, methanogenesis and dechlorination decreased because of interspecific competition, probably between the H_2-oxydizing methanogenic and sulfate-reducing bacteria in batch conditions. In the semicontinuous fixed-bed reactor, the presence of sulfate did not affect dechlorination and methanogenesis. The sulfate-reducing bacteria may not be competitors of H_2-consuming methanogens in the reactor because of the existence of microbial biofilm. The presence of the fixed film may be an advantage for bioremediation and industrial treatment of effluent charged in sulfate and PCE. This is the first report on the microbial ecology of a methanogenic and sulfate-reducing PCE-enrichment consortium.

Key Words——dechlorination; methanogenesis; microbial interactions; reductants; sulfate reduction; tetrachloroethylene

Tetrachloroethylene (PCE) is a chlorinated aliphatic compound essentially used 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. Biotransformation of PCE in ethane or ethylene has been studied by many researchers at low concentrations under strict anaerobic conditions (De Bruin et al., 1992; Freedman and Gossett, 1989).

The dechlorination process was observed in methanogenic, acetogenic, or sulfate-reducing habitats (Holliger and Schraa, 1994; Mohn and Tiedje, 1992). In anaerobic PCE-enrichment culture, methanogenesis and acetogenesis disappeared with high concentrations of PCE (Maymo-Gatell et al., 1995). Ecological understanding of such communities will probably be important in using dehalogenation in bioremediation processes (Mohn and Tiedje, 1992).

A methanogenic and sulfate-reducing consortium degrading PCE was obtained in our laboratory from anaerobic-digested sludge from a wastewater treatment plant (Bourg-en-Bresse, France). Initially, high concentrations of PCE were dechlorinated in trichloroethylene (TCE) by methanogenic fermentation in batch conditions. The sulfate-reducing bacteria were not involved in PCE dehalogenation (Cabirol et al., 1996). This methanogenic and sulfate-reducing consortium was then cultivated continually in the presence of high concentrations of PCE. The methanogenic bacteria always had a deciding role in the dechlorination process after this enrichment period. The pollutant was completely degraded to carbon biomass and CO_2. PCE dehalogenation has been studied in a semicontinuous fixed-bed reactor, revealing an attractive method for removing high concentrations of PCE in bioremediation processes (Cabirol et al., 1998).

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However, the presence of sulfate reducers in the microbial consortium may involve interactions with methanogens, which could decrease the capability of dechlorination and then the fixed-bed reactor performance. Indeed in the anaerobic treatment of wastewater containing sulfate, the sulfate reduction interferes with the methanogenesis: both mutualistic and competitive interactions between sulfate reducers and methanogens have been observed (Oude Elferink et al., 1994).

In the present work, we investigate the distribution of the reductants used in dechlorination of PCE at high concentration in relation with the presence or absence of sulfate and with the culture conditions. The knowledge of the relationships between methanogenic and sulfate-reducing microorganisms is necessary to consider an effective process of bioremediation.

Materials and Methods

Microorganisms and culture conditions. A methanogenic and sulfate-reducing mixed culture was obtained from anaerobic digested sludge from the Bourg-en-Bresse wastewater treatment unit. After several subcultures, the enrichment was conducted by using mineral salt medium, which contained 300 mM methanol and different concentrations of PCE (Cabirol et al., 1996, 1998). A PCE-acclimated consortium was maintained in a 1-L semicontinuous anaerobic reactor operated at 37°C.

Dechlorination experiments. All experiments were conducted in batch conditions with 20 ml-serum vials. Each microreactor was amended with 35 μM PCE (final concentration), 300 mM methanol, and 5 ml sterile mineral salt medium and inoculated with 5 ml of actively growing mixed culture. Four series of vials were conducted by supplementing the culture with 45 mM lactate ($C_3H_5O_3Na$) ("*Lac* series"); 45 mM lactate and 10 mM 2-bromoethane sulfonic acid (*BESA*), an inhibitor of methanogenesis ("*BESA* series"); 45 mM lactate and 6 mM SO_4^{2-} (0.85 g · L^{-1} Na_2SO_4) ("SO_4^{2-} series"); 45 mM lactate, 6 mM SO_4^{2-}, and 50 mg/L gentamycin (activity 297 μg) ("*Genta* series"). This antibiotic was an inhibitor of sulfate-reducing bacteria as previously demonstrated (Cabirol et al., 1996). At daily incubation periods, triplicates of active and control vials were used for analysis as previously described (Cabirol et al., 1996).

Procedure for bacterial counts. Enumerations of sulfate-reducing (SRB) and methanogenic bacteria (MB) were carried out separately by the most probable number (MPN) technique in anaerobic conditions. Enumeration was conducted initially and, after 3 days of incubation during dechlorination experiments. The medium for MPN determination of MB was the same mineral salt medium used for culture. After 1 month of incubation, methane formation was determined by gas chromatography. MPN determination of SRB was conducted with specific medium of these bacteria as previously described (Cabirol et al., 1996).

Fixed-bed reactor. Two cylindrical fixed-bed reactors were inoculated with an active culture (10 ml) and fed semicontinuously with mineral salt medium, which contained methanol (10 g · L^{-1}) and PCE loading of 310 μg (corresponding to the concentration of 40 μM) as previously described (Cabirol et al., 1998). During 15 days, the fixed-bed reactor was fed with mineral salt medium +4 g · L^{-1} lactate +0.6 g · L^{-1} SO_4^{2-} (0.85 g · L^{-1} Na_2SO_4) + PCE loading of 310 μg. The hydraulic retention time was 3 days. The sampling ports were situated at the middle of each column. Every week, 5 samples were taken from different points of the reactor, with a gas- and solvent-tight syringe, as a previously described procedure (Cabirol et al., 1998).

Analytical methods. The amounts of chlorinated compounds (PCE, TCE) were determined by the direct injection of samples into a Perkin Elmer model Sigma 2000 gas chromatograph using a previously described procedure (Cabirol et al., 1996). The amounts of CH_4 and CO_2 were determined by a Girdel 330 gas chromatographic analysis by using a previously described procedure (Cabirol et al., 1996). Sulfate was analyzed turbidimetrically as $BaSO_4$ according to the nephelometric method (Rodier, 1984). Biomass production was determined by the Lowry method for protein quantification.

Relative percentages of electrons used in sulfate reduction and dechlorination. The relative percentages of electrons used in methanogenesis, dechlorination, and sulfate reduction can be calculated according to the following equation proposed by Isa et al. (1986a) and adapted for dechlorination. The same amounts of substrate electrons (COD) are consumed for an equivalent amount of methane produced, sulfate and PCE reduced. For example, 4 mol of H_2 are utilized for 1 mol of methane produced or sulfate reduced (Ueki et al., 1989). De Bruin et al. (1992) observed PCE reduction with 4 mol of H_2.

So the relative percentages of electrons

$$\text{used in dechlorination} = \frac{\text{PCE-reduced}}{(\text{CH}_4\text{-produced} + \text{SO}_4^{2-}\text{-reduced} + \text{PCE-reduced})} \times 100,$$

$$\text{used in sulfate reduction} = \frac{\text{SO}_4^{2-}\text{-reduced}}{(\text{CH}_4\text{-produced} + \text{SO}_4^{2-}\text{-reduced} + \text{PCE-reduced})} \times 100.$$

By the relative percentage, we compared the amounts of electrons used by dechlorination in *Lac*, *BESA*, SO_4^{2-}, *Genta*, and standard series described as above. Experimental data were statistically studied to assess the significant differences between the series. So a *t*-test (p=0.05 or confidence intervals= 95%) was used to compare the relative percentage of electrons used in dechlorination in *Lac*, *BESA*, SO_4^{2-}, *Genta*, and standard series.

Chemicals. The following compounds were obtained in neat liquid form: PCE (CPG, 99%; Prolabo, Vaulx en Velin, France). CH_4 and CO_2 were obtained as mixed gases in reference bottles (Air Liquide, Lyon, France). PCE was added to cultures from solution in methanol (RP Normapur, 99.8%; Prolabo).

Results

PCE dechlorination in the absence of sulfate

In the absence of sulfate, the degradation of PCE was studied after the addition of lactate (a preferential carbon source of sulfate-reducing bacteria) in the mineral salt medium of microreactor. The presence of lactate significantly increased the relative percentage of electrons used in PCE dechlorination after 5 and 7 days of incubation (Fig. 1). A *t*-test confirmed these significant differences (p=0.05). After 9 days, concentrations of PCE were 10 and 5 μM in the standard and *Lac* series, respectively, for an initial concentration of 35 μM. PCE dehalogenation was greater in the presence than in the absence of lactate in batch conditions.

After 9 days of incubation, the relative percentages of electrons used in dechlorination were the same for the standard and *Lac* series (Fig. 1). However, lactate in the *Lac* series was completely degraded after 9 days.

The methane production did not change between the standard and *Lac* series (Fig. 2). Methanol was detected at a concentration of 148 mM after 9 days in both series.

PCE dechlorination was inhibited in the mixed culture supplemented with 10 mM BESA. The concentration of PCE did not decrease significantly in the *BESA* series (Fig. 3).

PCE dechlorination in the presence of sulfate

The presence of sulfate involved the activation of sulfate reduction. The relative percentage of electrons used in sulfate reduction was about 15% after 5 days of incubation, and 5.5 and 5.8 mM of SO_4^{2-} were reduced in the medium after 5 and 9 days, respectively. However, the presence of sulfate significantly decreased the relative percentage of electrons used in PCE dechlorination after 5 days of incubation (Fig. 1),

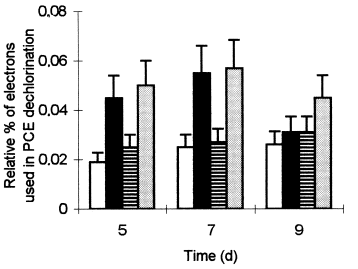

Fig. 1. Relative percentage of electrons used in complete dechlorination of 35 μM PCE by the acclimated microbial consortium.

□ standard series, ■ *Lac* series, ≣ SO_4^{2-} series, ▩ *Genta* series.

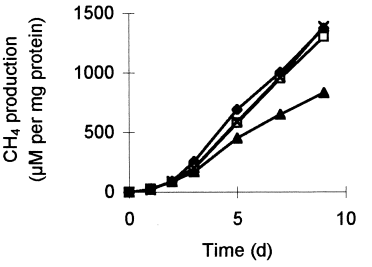

Fig. 2. Methane production by the different types of mixed culture in batch conditions.

The initial concentration of PCE was 35 μM. ◆ standard mixed culture, □ *Lac* mixed culture, ▲ SO_4^{2-} mixed culture, × *Genta* mixed culture.

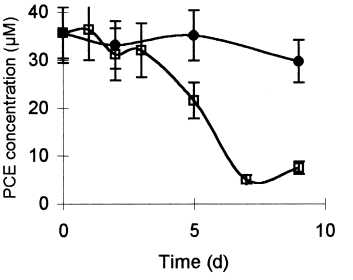

Fig. 3. PCE dechlorination by two types of mixed culture in batch conditions.

The initial concentration of PCE was 35 μM. □ *Lac* mixed culture, ● *BESA* mixed culture.

as confirmed by *t*-test (p=0.05).

But after 9 days of incubation, the relative percentages of electrons used in dehalogenation were identical in the *Lac* and SO_4^{2-} series (Fig. 1), especially since the culture medium contained no more lactate. Indeed, lactate was completely consumed by the microbial consortium after 9 days of incubation in both series.

The concentration of methanol after 9 days was then found to be 140 mM. The methane production decreased in the presence of sulfate (Fig. 2). The methanogenesis in the SO_4^{2-} series was inhibited at a

Table 1. Enumeration of SRB and MB bacteria in microbial consortium with 95% confidence limits.

Time (day)	MB[a] (*Lac* series)	MB[a] (SO_4^{2-} series)	SRB[a] (*Lac* series)	SRB[a] (SO_4^{2-} series)
0	5.6<6.4<7.1	5.6<6.4<7.1	6.2<6.9<7.7	6.2<6.9<7.7
3	7.2<7.9<8.7	7.2<7.9<8.7	6.2<6.9<7.7	7.6<8.4<9.1

[a] log bacteria number/ml.

Table 2. Influence of sulfate on PCE degradation in the semicontinuous fixed-bed reactor.

Medium	Loading of PCE (µg/j) in the influent	Amount of PCE (µg/j) in the effluent	PCE removal (%)
Absence of sulfate	309	17	93.4
Presence of sulfate	295	19	92.8

rate of 30% after 7 days.

An enumeration of sulfate-reducing bacteria revealed an increase of these types of bacteria in the SO_4^{2-} series (Table 1). However, the number of methanogenic archaea was stable in the *Lac* and SO_4^{2-} series.

Sulfate reduction was inhibited in the mixed culture supplemented with 50 mg/L gentamycin. PCE dechlorination and methanogenesis increased significantly in comparison with the SO_4^{2-} series, as confirmed by *t*-test ($p=0.05$ and $p=0.01$, respectively) (Figs. 1,2).

Fixed-bed reactor

The fixed-bed reactor performance in the presence of sulfate did not change with regard to this performance in the absence of sulfate (Table 2). PCE removal was about 93% in these two conditions. In the leaching biomass, the logarithms of methanogens number per ml were respectively 7.3 and 7.9 in the absence and presence of sulfate. The logarithms of sulfate-reducing bacteria number per ml were respectively 7.1 and 8.4 in the absence and presence of sulfate. The methane production did not vary in different conditions.

Discussion

The distribution of the reductants used in dechlorination of PCE at high concentrations was investigated in relation with the presence or absence of sulfate. The acclimated mixed culture was efficient to dechlorinate high concentrations of PCE by the activity of methanogens (Cabirol et al., 1996, 1998). This deciding role of methanogenic archaea in PCE dechlorination was confirmed in this study after inhibition of methanogenesis and dehalogenation by BESA (Fig. 3). However, the presence of sulfate reducers in the microbial consortium may involve interactions with methanogens, which could decrease the capability of dechlorination.

In the absence of sulfate, the relative percentage of electrons used in PCE dechlorination increased after the addition of lactate in batch conditions (*Lac* series in Fig. 1). Lactate cannot be metabolized by the methanogens; but the sulfate-reducing bacteria may consume it. They may produce further reductants used by the methanogenic archaea for PCE dechlorination. A syntrophy in the absence of sulfate could exist between the sulfate-reducing and methanogenic microorganisms. Mc Inerney and Bryant (1981) observed that in coculture, lactate is degraded into acetate and hydrogen by *Desulfovibrio* sp. and then into CO_2 and CH_4 by *Methanosarcina barkeri*. When lactate was completely consumed after 9 days of incubation, the relative percentage of electrons used in PCE dehalogenation was the same in the standard and the *Lac* series (Fig. 1). The increase of dechlorination that we observed after 5 and 7 days of incubation may then be in relation with the production of further reductants from lactate catabolism. The Flows of these extra electrons were deviated cometabolically to PCE dechlorination by methanogens to the detriment of methanogenesis. Further experiments would be necessary to investigate lactate catabolism and extra production of electrons by using tritium-enriched lactate.

In the absence of sulfate, the enumeration of sulfate-reducing bacteria did not change after 3 days of incubation (Table 1). Growth was lower in the absence than in the presence of sulfate. Lactate is a preferential carbon source, but sulfate is necessary to obtain an optimal growth of sulfate-reducing bacteria. Bryant et al. (1977) showed also that growth of desulfovibrios on ethanol or lactate was faster when sulfate was added as an electron acceptor.

In the presence of sulfate, the addition of lactate did not again induce an increase in the percentage of electrons used in PCE dechlorination (SO_4^{2-} series in Fig. 1). The sulfate reduction was active, demonstrating that sulfate-reducing bacteria had then an optimal energetic metabolism (15% electrons used in sulfate reduction). This suggests that these bacteria, rich in reduced transition-metal cofactors, did not dechlorinate PCE cometabolically in the considered consortium. However, the dehalogenation could coincide with an active sulfate reduction by cometabolism (Sonier et al., 1994). This might be due to the diversity of studied microbial consortia.

In batch conditions, the production of methane decreased slightly but significantly in the presence of sulfate, correlatively with the dechlorination diminution (Figs. 1,2). Sulfate reduction may involve the deviation

of electron flow at the expense of methanogenesis and dehalogenation. When sulfate reduction is inhibited by gentamycin, there was no decrease of methanogenesis and PCE dechlorination. An interspecific competition for the reductants may exist between the sulfate-reducing and methanogenic bacteria. The sulfate-reducing bacteria may be only competitors of the strict hydrogen-consuming methanogens for this substrate. The other methanogenic bacteria consumed methanol that was in excess. Kinetic investigations with pure culture revealed that sulfate reducers have higher affinities for H_2 than methanogens (Harada et al., 1994). The availability of electron acceptors may affect the flow of electrons required for PCE dechlorination and for methanogenesis via interspecific competition for electron donors. Electron acceptors are believed to be a limiting resource for anaerobic communities (Mohn and Tiedje, 1992).

This interspecific competition was observed only in batch microreactors. Indeed in the semicontinuous fixed-bed reactor, methanogenesis and dechlorination were not changed in the presence of sulfate (Table 2). The daily feeding could decrease the lack of electron donors. Furthermore, the presence of the fixed-film bacteria, which was reported by Cabirol et al. (1998), may involve mass transfer limitation of sulfate into the biofilm itself. The concentration of sulfate in the biofilm would be lower than the one in the liquid medium. Overmeire et al. (1994) reported mass transfer limitations in granules (R>1.5 mm) of UASB reactor system. The methanogenic archaea could also colonize and adhere much more efficiently to the glass support than the sulfate-reducing bacteria. The biofilm observation confirms that methanogens were dominant in the fixed film (Cabirol et al., 1998). This improved colonization and the adhesion of methanogens have been reported also in the literature (Isa et al., 1986b; Mc Cartney and Oleszkiewicz, 1993). With the use of our system, further experiments would be necessary to confirm the differences in microbial colonization by using small subunit rRNA-directed oligonucleotide hybridization probes specific for various populations of sulfate reducers and methanogen.

The sulfate-reducing bacteria would be poor competitors of H_2-oxydizing methanogenic archaea in the biofilm. The latter may have a protector effect for methanogen activity because there is probably a mass transfer limitation of sulfate and an improved colonization of methanogens. This protector effect of the fixed film may be an advantage for the bioremediation and industrial treatment of effluent charged in sulfate and PCE.

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