Methanogenic and perchloroethylene-dechlorinating activity of anaerobic granular sludge

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Abstract

The biodegradation and toxicity of tetrachloroethylene (C_2Cl_4) and trichloroethylene (C_2HCl_3) were studied with different anaerobic enrichment cultures using the following electron donors: acetate, propionate, butyrate, methanol, formate and hydrogen. All of them sustained dechlorination except propionate, for which C_2Cl_4 biodegradation rates were not significant. The best results were obtained with butyrate. Hydrogen appeared to be a relevant electron donor for dechlorination with the present cultures. In the presence of specific inhibitors such as bromoethanesulphonate or molybdate, a slight inhibition of dechlorination was observed. According to dechlorination kinetics, Monod-type behaviour was observed up to 120 μM C_2Cl_4 or 200 μM C_2HCl_3 with K s values around 7 μM for both compounds. Dechlorination was partially inhibited at higher concentrations. In contrast, methanogens, or at least methane production, were more sensitive to the presence of chlorinated ethylenes and inhibition of methanogenesis was observed to different extents over all the C_2Cl_4/C_2HCl_3 concentration range tested, even at the lowest concentrations.

Introduction

Tetrachloroethylene or perchloroethylene (C_2Cl_4) is widely used as a solvent and is listed as a priority pollutant in many industrialized countries. Owing to its presumed carcinogenic and mutagenic properties, investigations have been undertaken to check itsbiodegradability and removal from groundwater or contaminated environments in general.

So far, it has not been possible to obtain evidence of any C2Cl4 biodegradation under aerobic conditions, although many reports have demonstrated the biodegradability of less-chlorinated ethylenes in the presence of oxygen by aerobic microorganisms (Alvarez-Cohen and McCarty 1991; Dobbins et al. 1995; Fogel et al. 1986; Nelson et al. 1987; Oldenhuis et al. 1991; Wilson and Wilson 1985). C2Cl4 is biodegradable by mixed cultures under anaerobic conditions (Bhatnagar et al. 1992; DiStefano et al. 1992; Smatlak et al. 1996; Tandol et al. 1994; Vogel and McCarty 1985; Wu et al. 1993). A few pure strains or highly enriched cultures able to degrade chlorinated ethylenes have also already been obtained, among which are Methanosarcina sp. (Fathepure and Boyd 1988) and other new genera or species (Fathepure et al. 1987; Holliger et al. 1993). A primary carbon and energy source is required to enable reductive dechlorination to take place. With mixed cultures or consortia, the choice of the primary carbon source plays a crucial role in the search of optimal dechlorination rates and pattern. The most suitable primary carbon source or electron donor will probably also depend on the origin of the inoculum. Acetate (Vogel and McCarty 1985), methanol (Fathepure and Boyd 1988; Freedman and Gossett 1989), lactate (Bagley and Gossett 1990), hydrogen (DiStefano et al. 1992; Smatlak et al. 1996) or mixtures thereof (Bhatnagar et al. 1992;Wuet al. 1993a, c) have been shown to support reductive dechlorination and to be suitable sources of electron donors.

In the present study, two cultures were selected from among several others for their ability to remove C_2Cl_4 anaerobically. Enrichments were undertaken in the presence of different electron donors including acetate, propionate, butyrate, methanol, formate and hydrogen, the best results being obtained with butyrate according to biodegradation rates. The influence of specificc inhibitors on C_2Cl_4 removal was checked to evaluate the roleof specific microbial groups. Biodegradation kinetics and toxicity of C_2Cl_4 and C_2HCl_3 were determined both for dechlorinators and for methanogens.

Materials and methods

Inocula and media

Culture A originating from pentachlorophenol-degrading methanogenic granules (Kennes et al. 1996; Wu et al. 1993b) was enriched with a medium containing C2Cl4 and a mixture of acetate, propionate, butyrate and methanol. Culture B was a mixture of different sludges from industrial wastewater treatment plants and a culture from a fixed-film reactor degrading a complex mixture of chlorinated compounds (Modesto Filho et al. 1991). The culture medium (50 ml) (Kenealy and Zeikus 1981) used in batch assays was introduced into 158-ml serum vials with Teflon-coated septa and aluminium caps. It contained a vitamin solution (Wolin et al. 1963) and Na2S 9H2O as reducing agent. The medium was buffered with a K_2HPO_4/KH_2PO_4 mixture and the pH was adjusted to 7.0± 7.2. Chloroethylenes were added after the vials had been autoclaved for 20 min at 120 °C. Initial chloroethylene concentrations were determined by GC analysis by sampling the vials 15 min after adding the chlorinated compounds in order to allow

equilibrium to be reached between the di sample phases. For each analysed during the course of the experiments, a vial was sacrificed, after methane measurement, for determination of chloroethylene concentrations and primary substrate consumption. All experiments were undertaken in duplicate.

Chemicals

Chlorinated compounds were obtained from Janssen Chimica (Beerse, Belgium), Fluka (Buchs, Switzerland) or Aldrich Chemical Company (Milwaukee, USA). Bromoethanesulphonate (BES) was purchased from Sigma (St. Louis, USA).

Analytical methods

Methane and fatty acids were determined by gas chromatography as described elsewhere (Kennes et al. 1996; Soto et al. 1997). Formate was analysed on a model 600 HPLC from Waters with a BioRad 87H column after acidification of the sample. Chlorinatedcompounds were determined by GC on a Hewlett-Packard, model 5890A gas chromatograph. The samples were either extracted with iso-octane or heated in order to transfer all the contaminants to the gas phase for analysis of headspace samples (Wu et al. 1993c). Other parameters, such as volatile suspended solids, were determined according to Standard Methods (APHA-AWWA-WPCF 1985).

Results

Influence of the source of electron donor on removal of chlorinated ethylenes and toxicity of chlorinated ethylenes According to data reported in the literature, the rate and extent of C_2Cl_4 biodegradation observed with anaerobic cultures are often di erent from one study to another. In the present case, the behaviour of two different cultures was checked. These cultures were selected from others after having shown their ability to dechlorinate C_2Cl_4 anaerobically. In order to establish the role of several electron donors in C_2Cl_4 dechlorination, the following substrates were tested with both cultures: acetate, propionate, butyrate, methanol, formate and a H_2/CO_2 (80:20) mixture. Two control bottles were used: (i) a non-inoculated medium, (ii) a bottle autoclaved with medium and cells in exponential growth phase prior to C_2Cl_4 addition. The synthetic medium described above for biodegradation experiments was initially prepared with tap water as well as with distilled water, leading to very similar removal rates. All further experiments were then undertaken with distilled water to ensure fully reproducible working conditions.

In order to adapt the cells to C_2Cl_4 and to reach a high biomass concentration in the bottles, the cultures were first grown on a volatile fatty acid (acetate, propionate, butyrate) plus methanol (1 mM each) mixture, in the presence of 5 lM C_2Cl_4. After complete exhaustion of the substrates, the bottles were ushed with nitrogen or H_2/CO_2 and the desired electron donors and C_2Cl_4 were supplied to the medium to check biodegradation of the chlorinated ethylenes.

The experiments were undertaken with the following initial C_2Cl_4 concentrations (lM): 7, 40, 80, 120 and 160. These concentrations represent approximate values and varied slightly from one vial to another but always with less than 5% deviation according to GC analysis. Both cultures were able to degrade C_2Cl_4 with each of the electron donors tested, except propionate. The rate and extent of C_2Cl_4 removal were, however, different, as appears in Table 1. Several successive C_2Cl_4 spiking steps were applied for each of the different primary carbon sources. C_2Cl_4 dechlorination rates increased after

each step. The maximal specific C_2Cl_4 dechlorination rates reported in Table 1 were obtained from the fourth spiking step, since the removal rates were very similar to those obtained on the third spiking. No C_2Cl_4 dechlorination was observed in non-inoculated bottles.

To check the influence of a heat treatment on C_2Cl_4 dechlorination in the presence of a H_2/CO_2 mixture, bottles inoculated with the enrichment cultures were autoclaved for 20 min at 120 °C. From the experimental results it could be concluded that the dechlorination capacity was lost after such treatment since basically no C_2Cl_4 biodegradation was found after 1 week. The removal rates were of the same order of magnitude with both cultures. C_2Cl_4 removal rates and complete dechlorination were, however, faster in the presence of butyrate. With the latter substrate, when yeast extract was added (0.1%), C_2Cl_4 dechlorination rates increased by a factor of approximately 1.5. The trend shown in Table 1 for C_2Cl_4 was, as a general rule, also observed for the less-chlorinated compounds, which were removed more slowly in the presence of acetate. Figure 1 shows a typical biodegradation pattern for culture B grown on butyrate. As appears in that figure, the less-chlorinated compounds were removed more slowly. Both the cis and trans $C_2H_2Cl_2$ isomers were formed during C_2Cl_4 biodegradation studies, at similar rates and concentrations. Peaks corresponding to trace levels of 1,1-$C_2Cl_2H_2$ were also detected by GC analysis.

Table 1 Maximal C2Cl4 dechlorination rates obtained with two di□erent anaerobic cultures using di□erent primary carbon sources. ± minor or slow removal, VSS volatile suspended solids, ND not done.

Electron donor	Maximal specific C_2Cl_4 dechlorination rates ($\mu mol\ h^{-1}\ mgVSS^{-1}$)	
	Culture A	Culture B
Acetate	52.7	92.2
Propionate	–	–
Butyrate	311.3	262.0
Methanol	185	242.0
Formate	196.7	ND
H_2/CO_2	202	229.9

In batch experiments undertaken with butyrate as primary carbon source, zero-order kinetics appeared best to represent the C2Cl4 biodegradation pattern for the 40±120 lM C_2Cl_4 concentration range. However, from the lower biodegradation rate observed at 7 lM it could be concluded that Monod kinetics does apply, with a Monod constant for C_2Cl_4 slightly below 7 lM (Fig. 2). Only at higher initial C_2Cl_4 concentrations (160 lM) was inhibition of C_2Cl_4 removal observed. However, inhibition of methane production was observed at all C_2Cl_4 concentrations tested (from 7 lM to 160 lM). Typical methane production experiments are shown in Fig. 3a for the different C_2Cl_4 concentrations.

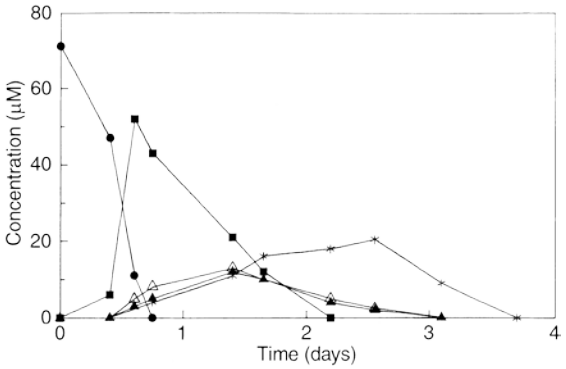

Fig. 1 Typical C_2Cl_4 dechlorination profile obtained with culture B and butyrate as source of electron donor. ● C_2Cl_4, ■ C_2HCl_3, Δ cis- $C_2H_2Cl_2$, ▲ trans- $C_2H_2Cl_2$, * vinylchloride

Toxicity and biodegradation rates were also determined with trichloroethylene and butyrate, for initial C_2HCl_3 concentrations of 9, 20, 40, 120, 200 and 300 lM. Here again, a zero-order biodegradation kinetics pattern was observed in the 40±200 lM C2HCl3 concentration range and inhibition was observed at 300 lM. At the lowest concentrations tested, Monod kinetics was observed with a Monod constant around 7 lM. As was observed with C_2Cl_4, C_2HCl_3 is also inhibitory to methane production and inhibition was already appreciable at 40 lM C_2HCl_3 (Fig. 3b). On the other hand, C_2HCl_3 removal rates remained basically the same in the concentration range 9±200 lM C_2HCl_3 and equal to 180 lmol h^{-1} mg volatile suspended $solids^{-1}$.

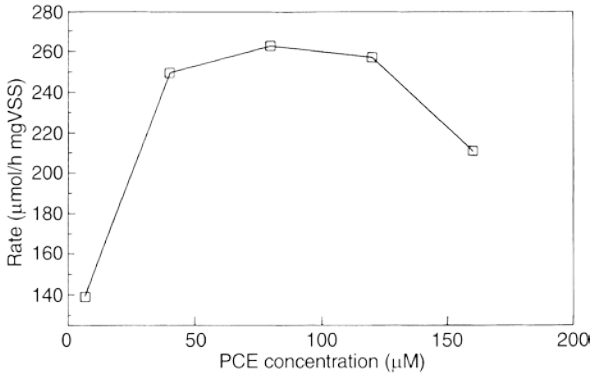

Fig. 2 Specific C_2Cl_4 removal rates versus initial C_2Cl_4 concentrations with butyrate as source of electron donor

Effect of specific inhibitors

Butyrate enrichments of culture B were grown batchwise in the presence of C_2Cl_4 and the following specific inhibitors: BES (5 mM), Na_2MoO_4 (5 mM) and hydrogen. In the case of bottles used to study inhibition of sulphate-reducing bacteria, sulphate was added to the medium in the form of Na_2SO_4 (3 mM). Bottles with sulphate were also used to demonstrate that the presence of sulphate did not significantly inhibit or enhance C2Cl4 biodegradation.

BES and molybdate, which specifically inhibited methanogenesis or sulphate reduction, did slightly inhibit C_2Cl_4 biodegradation. In the presence of BES or molybdate a 16% and an 8% inhibition were respectively observed according to C_2Cl_4 biodegradation rates.

Later on the C_2Cl_4 enrichment culture was grown in the presence of both butyrate and hydrogen (1.5 atm overpressure). In such experiments dechlorination started without any lag phase and complete C_2Cl_4 disappearence was observed while the butyrate concentration remained basically unchanged during C_2Cl_4 removal.

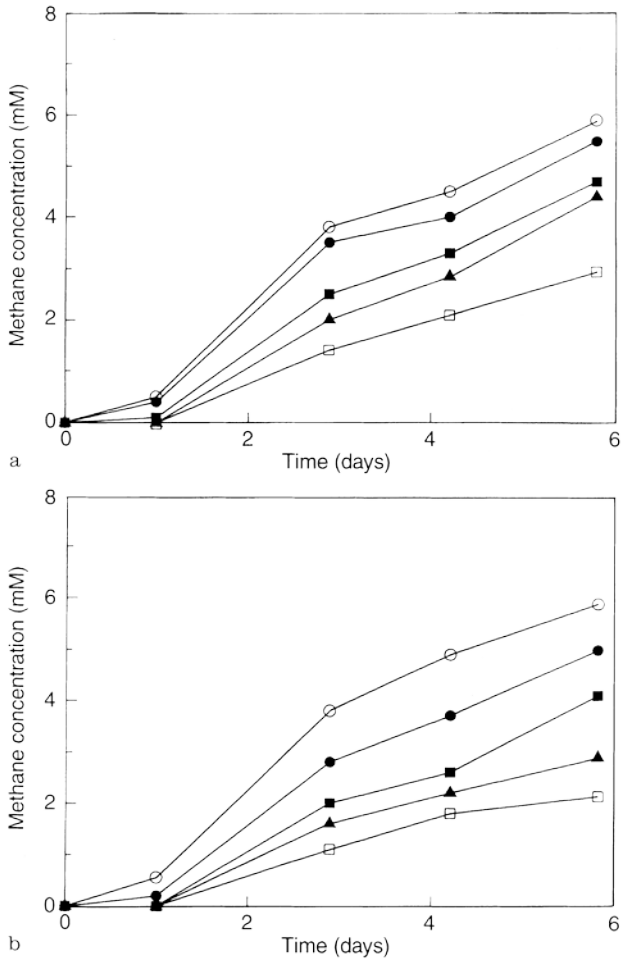

Fig. 3a Cumulative methane production as a function of time with culture B and butyrate at initial C_2Cl_4 concentrations of (μM) 0 (O), 7 (●), 40 (■), 80 (▲), 160 (□). b Cumulative methane production as a function of time with culture B and butyrate at different C_2HCl_3 concentrations of (μM) 0 (O), 40 (●), 120 (■), 200 (▲), 300 (□)

Enumeration and enrichment studies

After several transfers in aqueous medium with C_2Cl_4, samples of the enriched cultures grown on butyrate or on hydrogen/carbon dioxide were serially diluted and poured on lates under strict anaerobic conditions, using either butyrate or a H^2/CO^2 mixture as substrates. C_2Cl_4 was also added on the plates. Different morphotypes were found although a regular short rod was highly dominant in both cases. Aliquots of the dilution series were transferred under sterile conditions to fresh medium containing either H^2/CO^2 alone or a mixture of H_2/CO_2 and C_2Cl_4, to evaluate the ratio of methanogenic bacteria and C2Cl4 dechlorinators in the medium. Each dilution was prepared in triplicate to give a ratio of approximately 107 methanogens to 106 dechlorinators.

Discussion

Under strict anaerobic conditions, the dechlorination of halogenated toxic compounds is dependant on the nature of the electron donors utilized. The inoculum source is another important factor. Both parameters were studied and the present data are compared to other recently published results. As already mentioned, depending on the microbial community and culture conditions used, different primary carbon sources or sources of electron donors may be suitable for C_2Cl_4 dechlorination (Bagley and Gossett 1990; DiStefano et al. 1992; Fathepure and Boyd 1988; Smatlak et al. 1996; Vogel and McCarty 1985; Wu et al. 1993), either in the presence or in the absence of sulphate (Freedman and Gossett 1989). The results reported so far suggest that, in some cases, dechlorinators may represent new species and/or genera (Holliger et al. 1993; DeWeerd et al. 1990), although at least some methanogens and sulphate-reducing bacteria also seem to be able to biodegrade C_2Cl_4 and other chloroaliphatic compounds (Fathepure and Boyd 1988; Egli et al. 1987).

In the present case, butyrate was the most suitable electron donor to sustain the dechlorination of chloroethylenes (Table 1). C_2Cl_4 removal was also possible without any butyrate consumption in the presence of hydrogen. The fact that C_2Cl_4 dechlorination was not inhibited when both butyrate and hydrogen were used as the source of electron donors suggests that hydrogen rather than butyrate is important in dechlorination. The exact dechlorination mechanisms have not been elucidated so far.

Enhancement of biodegradation in the presence of yeast extract indicates the key role of non-identified micro-elements or other compounds in C_2Cl_4 removal. The complete inhibition of the dechlorinating activity when autoclaved cells were used, together with the effect of specific inhibitors, indicate that the dechlorination process is due to the metabolic activity of living cells of specific microbial groups. When single primary substrates were used, methanol, hydrogen and formate were less effcient than butyrate. The slowest biodegradation rates were observed with acetate and almost no C_2Cl_4 removal was found with propionate. These data suggest the important role of hydrogen for C_2Cl_4 dechlorination with the present cultures, which is in agreement with recently reported results (DiStefano et al. 1992; Smatlak et al. 1996). The cultures were enriched in liquid medium, although no pure colonies could be isolated in the presence of C_2Cl_4 on plates.

Limited inhibition of dechlorination was observed with specific inhibitors of methanogenesis or sulphate reduction, suggesting that methanogens or sulphate reducers only play a minor role ± if any ± in dechlorination, or otherwise that inhibitors of methanogenesis and sulphate reduction do also show influence on dechlorination mechanisms. C_2Cl_4 as well as C_2HCl_3 was inhibitory to methanogenic activity even at low concentrations. In contrast, inhibition of dechlorination activity was not detected at such C_2Cl_4 and C_2HCl_3 concentrations, which again seems to indicate that de chlorinating microorganisms are probably not methanogens. Since both methanogens and dechlorinators use hydrogen as electron donor, their competition for hydrogen might explain the inhibition of methanogenesis in the presence of chlorinated ethylenes, as was also recently suggested by Ballapragada and coworkers (Ballapragada et al. 1997). At relatively low C_2Cl_4 and C_2HCl_3 concentrations, Monod kinetics are observed, with Ks values close to 7 lM for both compounds. This has to be taken into account for bioremediation studies since removal rates will significantly decrease below such a concentration. These data were, by the way, obtained during spiking experiments, after adaptation of the cultures to chloroethylenes, from which it can be

concluded that no (further) natural improvement of kinetic parameters might be expected.

Reductive dechlorination of C_2Cl_4 and C_2HCl_3 in the presence of the various primary carbon sources yielded, in all cases, both the cis and the trans isomers of dichloroethylene as well as trace levels of 1,1-$C_2Cl_2H_2$, contrary to other reports where only one isomer was detected (Bagley and Gossett 1990). In all cases C_2Cl_4 and C_2HCl_3 were completely dechlorinated. Degradation of dichloroethylenes to non-chlorinated compounds appeared to be much slower than C_2Cl4 or C_2HCl_3 removal and suggests the potential for using integrated anaerobic/aerobic systems for a complete decontamination of C_2Cl_4 using the anaerobic stage for the initial fast C_2Cl_4 dechlorination and the aerobic stage for further complete biodegradation of less chlorinated ethylenes to non-toxic compounds. It is indeed well known that less chlorinated ethylenes can be degraded completely and rapidly by aerobes (Alvarez-Cohen and McCarty 1991; Dobbins et al. 1995; Fogel et al. 1986; Nelson et al. 1987; Oldenhuis et al. 1991; Wilson and Wilson 1985).

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