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Anaerobic dechlorination and mineralization of pentachlorophenol and 2,4,6-trichlorophenol by methanogenic pentachlorophenol-degrading granules

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Abstract Anaerobic granules developed for the treatment of pentachlorophenol (PCP) completely minearilized ^{14}C-labeled PCP to $^{14}CH_4$ and $^{14}CO_2$. Release of chloride ions from PCP was performed by live cells in the granules under anaerobic conditions. No chloride ions were released under aerobic conditions or by autoclaved cells. Addition of sulfate enhanced the initial chloride release rate and accelerated the process of mineralization of ^{14}C-labeled PCP. Addition of molybdate (10 mM) inhibited the chloride release rate and severely inhibited PCP mineralization. This suggests involvement of sulfate-reducing bacteria in PCP dechlorination and mineralization. Addition of 2-bromoethane sulfonate slightly decreased the chloride release rate and completely stopped production of $^{14}CH_4$ and $^{14}CO_2$ from [^{14}C]PCP. 2,4,6-trichlorophenol was observed as an intermediate during PCP dechlorination. On the basis of experimental results, dechlorination of 2,4,6-trichlorophanol by the granules was conducted through 2,4-dichlorophenol, 4-chlorophenol or 2-chlorophenol to phenol at pH 7.0–7.2.

Dedicated to the memory of Dr. L. Bhatnagar, who died before the manuscript was completed

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Introduction

Pentachlorophenol (PCP) is a toxic biocide. Dechlorination and degradation of PCP by anaerobic consortia and mixed cultures have been observed under anaerobic conditions (Mikesell and Boyd 1985, 1986). A sulfate-reducing bacterium, *Desulfomonile tiedjei*, can perform the partial dechlorination of PCP to 2,4,6-trichlorophenol (2,4,6-TCP) (Mohn and Kennedy 1992). Anaerobic dechlorination of PCP to lesser chlorinated phenols was observed undr methanogenic conditions (Mikesell and Boyd 1985, 1986; Hendriksen et al. 1992; Mohn and Kennedy 1992; Wood et al. 1989). In some cases, dechlorination of dichlorophenols may be coupled to sulfate reduction (Kohring et al. 1989; Häggblom and Young 1990). However, the role of sulfate-reducing bacteria in the mineralization of PCP has not been evaluated by using ^{14}C-labeled PCP.

Anaerobic PCP-degrading granules have been developed in laboratory upflow anaerobic reactors under mesophilic conditions (Bhatnagar et al. 1991; Wu et al. 1993). These granules are self-immobilized anaerobic microbial consortia, which are capable of dechlorinating and then mineralizing PCP to methane and CO_2. When a high PCP concentration (40 mg/l) with a high PCP loading rate (27 mg PCP g volatile suspended solids^{-1} day^{-1}) was applied to the reactor, 2,4,6-trichlorophenol (TCP) and 3,4,5-TCP were detected (Wu et al. 1993). These two TCP are likely intermediates of PCP dechlorination. The complete dechlorination pathway of PCP via 3,4,5-TCP by the granules uses the pathway reported by Miksell and Boyd (1985). However, the complete dechlorination pathway of PCP via 2,4,6-TCP by the granules has not been well studied. The purpose of this study was to examine factors that influence the activities of sulfate-reducing bacteria and methanogens during anaerobic PCP dechlorination and mineralization in order to understand the role of these bacteria, and to investigate the dechlorination pathway of 2,4,6-TPC by the granules.

Materials and methods

Chloride release from PCP, [^{14}C] PCP mineralization, and dechlorination of chlorophenols

The anaerobic PCP-degrading granules used in these assays were developed in a laboratory-scale upflow anaerobic reactor (Wu et al. 1993) and maintained in laboratory reactors at 35°C. These granules were fed with a medium containing PCP, methanol, acetate, propionate and butyrate.

Chloride-release assays were conducted in 158-ml serum vials at 35°C. The PCP-degrading granules were disrupted and centrifuged (10000 *g*, 20 min, 15°C) under strictly anaerobic conditions. They were then washed twice with an anaerobic medium prepared using distilled water and containing 8.0 mM phosphate (pH 7.0) and 1.0 mM $Na_2S \cdot 9H_2O$. The absorbance density (660 nm) was 1.4 after the pellet had been re-suspended in the above medium with added PCP. The vials were incubated at 35°C. Chloride release from PCP was monitored by analyzing samples withdrawn at different times. Duplicates were used in this assay.

Anaerobic phosphate-buffered basal medium (Kenealy and Zeikus 1981) was used for the test of mineralization of chlorophenols. Mineralization of [^{14}C] PCP was performed in 10-ml serum vials with 5.0 ml basal medium reduced with 0.04 ml $Na_2S \cdot 9H_2O$ (2.5%) and buffered with 0.2 ml $NaCHO_3$ (10% w/vol). The medium contained the following carbon sources: 10 mM acetate, 5 mM propionate, 4 mM butyrate, and 10 mM methanol. An initial pH of 7.0 was reached by pressurizing the vials with $N_2 : CO_2$ (70:30). When required 10 mM 2-bromoethane sulfonate (BES), 10 mM Na_2MoO_4, or 3.0 mM $FeSO_4$ was added. The ^{14}C-labeled PCP (2.25×10^5 dpm) and 0.5 mg/l non-labeled PCP was used in the study. Duplicates were used. Mineralization of PCP was monitored by determining the concentration of $^{14}CH_4$ and $^{14}CO_2$ in the handspace at different times.

Dechlorination and degradation of 2,4,6-TCP, 2-chlorophenol (CP) and 4-CP were performed in 158-ml serum vials containing 50 ml basal medium at 35°C. The medium was supplemented with 0.5 ml vitamin solution (Wolin et al. 1963), and 0.5 ml $Na_2S \cdot 9H_2O$ (2.5%). Additional carbon sources were 35 mM acetage, 25 mM propionate, 25 mM butyrate, and 40 mM methanol. The vials were pressurized with the $N_2 : CO_2$ (70:30) gas mixture. PCP-degrading granules were added to each vial. The dechlorination of these chlorophenols was monitored by withdrawing liquid samples periodically and analyzing the chlorophenols and phenol with the HPLC.

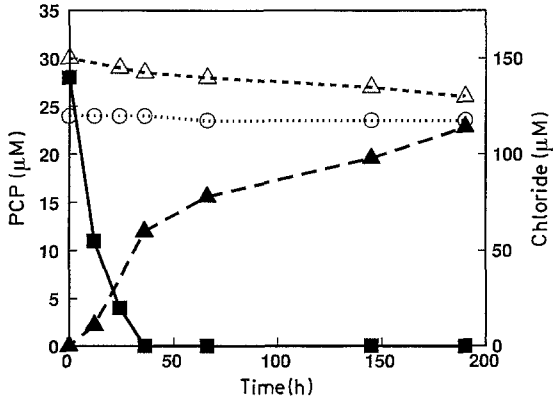

Fig. 1 Pentachlorophenol (*PCP*) removal and chloride ion release by PCP-degrading granules. ■ PCP concentrations with the granules under anaerobic conditions; △ PCP concentrations with the granules under aerobic conditions; ○ PCP concentrations with autoclaved granules under anaerobic conditions; ▲ chloride ion concentrations with the granules under anaerobic conditions

Analytical methods

Chlorophenols and phenol were detected using HPLC with a RP18 reversed-phase column and a UV detector at 300 nm. The mobile phase contained methanol, water and acetic acid (75:23:2). The determination of methane and volatile fatty acids was performed using gas chromatography as described elsewhere (Wu et al. 1991). The ^{14}C-labeled methane and CO_2 were determined using a gas chromatograph connected to a gas proportional counter. Helium was used as the carrier gas (Wu et al. 1993). The chloride ion concentration was detected using an Ion-Chromatography Module/SP (Dionex Corp., Itasca, Ill.) equipped with an A54A anion-exchange column and a Dionex conductivity detector. The protein concentration was estimated by a modified method of Lowry (Smith et al. 1985). Bovine serum albumin was used as standard.

Results

Chloride release during CPC degradation

The release of chloride ions from PCP was tested in serum vials using a cell suspension of disrupted PCP-degrading granules as inoculum under three different conditions: anaerobic conditions with live cells, anaerobic conditions with autoclaved (killed) cells and aerobic conditions (i.e. with air as headspace gas) with live cells. A typical test result is presented in Fig. 1. Neither chloride release nor PCP removal was observed in the bottles containing autoclaved cells. Under aerobic conditions, the removal of PCP was very poor and no significant amount of released chloride ion was detected. Rapid removal of PCP and release of chloride ions occurred under anaerobic conditions with live cells. PCP was removed from the initial 28 μM to a concentration below detectable limits (less than 0.3 μM) within 40 h. At the same time, approximately 56 μM chloride was released to the medium. The release of chloride ions continued and a total 114 μM were released after incubation of 190 h. On the basis of the stoichiometry of chlorine substitutes in the PCP molecule, 80% of the chlorine in PCP was released. This result indicates that removal of PCP and release of chloride ions is performed by microbial activity because no chloride ions were released by autoclaved cells under anaerobic conditions. This result also demonstrates that anaerobic conditions are essential for the PCP removal and release of chloride from PCP. Other experimental results (including reactor operational data) supported the conclusion that the release of chloride ions from PCP was anaerobic dechlorination because dechlorinated intermediates such as trichlorophenols (TCP) and dichlorophenols (DCP) were observed during PCP degradation by the granules (Wu et al. 1993).

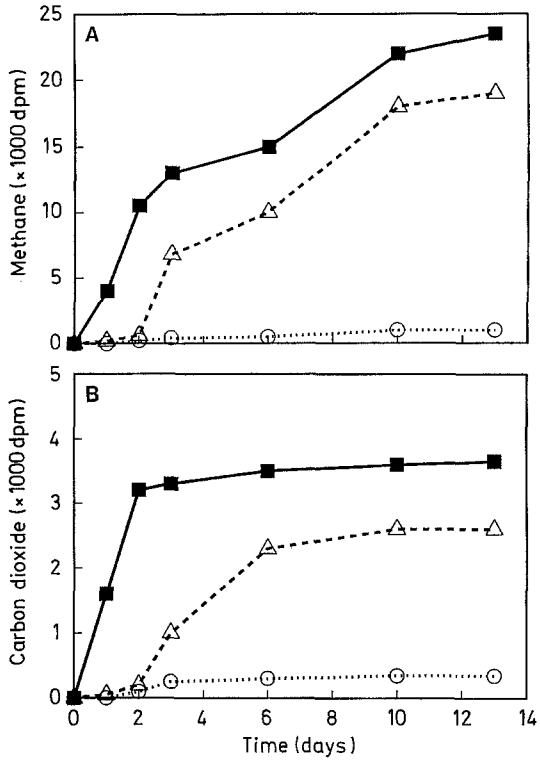

Fig. 2A,B ^{14}C-labeled methane and CO_2 production from ^{14}C-labeled PCP by PCP-degrading granules in serum vials in the presence of 3.0 mM $FeSO_4$ (■), in the absence of sulfate (△), and in the presence of 10 mM of $NaMoO_4$ (○). Neither $^{14}CH_4$ nor $^{14}CO_2$ was detected in the presence of 10 mM 2-bromoethanesulfonate. **A** methane production; **B** CO_2 production

Effect of sulfate, molybdate and BES on PCP dechlorination and mineralization

The role of sulfate-reducing bacteria and methanogens in PCP dechlorination was examined by determining the initial chloride release rates in the serum bottles receiving sulfate ($FeSO_4$, 3.0 mM), sodium molybdate (10 mM) or 2-bromoethanesulfonate (BES, 10 mM) compared with those in control serum vials. Duplicates were used for this assay. The initial chloride release rates were calculated on the basis of the difference in chloride ion concentration at time 0 and after 40 h, the volume of medium (50 ml), and the cell protein concentration in the medium. Molybdate is an effective and relatively selective inhibitor of sulfate-reducing bacteria (Taylor and Oremland 1979; Smith and Klug 1981). BES has been shown to be a potent inhibitor of methylcoenzyme M reductase of methanogens (Gunsalus et al. 1978). The initial PCP concentration was 28 μM. The average initial dechlorination rates were 118, 145, 87 and 96 nmol chloride released mg protein^{-1} h^{-1} in the control vials, with added sulfate, added molybdate, and added BES, respectively. The variation of the values for each duplicate was less than 10%. These results indicate that addition of sulfate increased the initial PCP dechlorination rate and addition of molybdate and BES inhibited dechlorination.

The effect of addition of sulfate, molybdate and BES on PCP mineralization was tested using ^{14}C-labeled PCP. In the control vials, which received only a fatty acid mixture and methanol, more than 98% of the ^{14}C added was recovered in labeled methane, CO_2 and bicarbonate. As shown in Fig. 2, the addition of sulfate leads to production of $^{14}CH_4$ and $^{14}CO_2$ from [^{14}C]PCP without lag. On the other hand, the presence of molybdate (10 mM) severely inhibited PCP mineralization, resulting in a limited amount of $^{14}CCH_4$ and $^{14}CCO_2$ produced. Neither [^{14}C]methane nor $^{14}CCO_2$ was detected in the presence of BES (10 mM). This result indicates a compete inhibition of the production of $^{14}CH_4$ and $^{14}CO_2$ from ^{14}C-labeled PCP by the added BES. By contrast, the presence of BES only slightly inhibited PCP dechlorination, as described previously.

Dechlorination pathway of 2,4,6-TCP

The PCP-degrading granules were incubated in a serum vial receiving 2,4,6-TCP (approx. 25 μM) under anaerobic conditions. The changes in concentration of 2,4,6-TCP and its dechlorinated intermediates are presented in Fig. 3. High levels of 2,4-DCP and low levels of 4-CP were detected on day 11 as dechlorinated intermediates in the medium. Accumulation of 2-CP and 4-CP was observed when 2,4-DCP disappeared on day 19. The continuous increase in concentrations of 2-CP was observed until day 32 when the concentrations of 2,4,6-TCP and 2,4-DCP were below detection limits. This could be due to the production of 2-CP from 2,4,6-TCP or 2,4-DCP which had been sorbed by biomass. Finally, both 4-CP and 2-CP were removed by the granules. In this test, phenol was not detected.

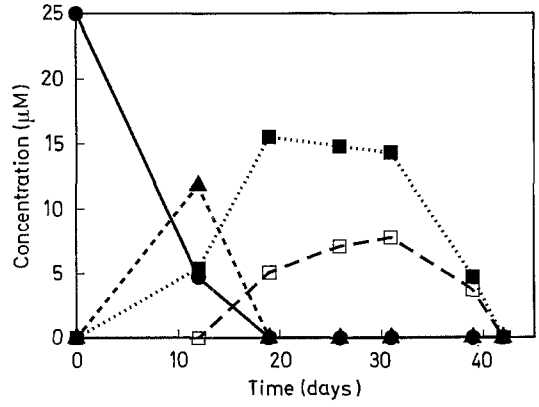

Fig. 3 The concentrations of 2,4,6-trichlorophenol (●) 2,4-dichlorophenol (▲), 4-chlorophenol (■) and 2-chlorophenol (□) detected in medium during a course of dechlorination and degradation of 2,4,6-trichlorophenol by PCP-degrading granules under anaerobic conditions

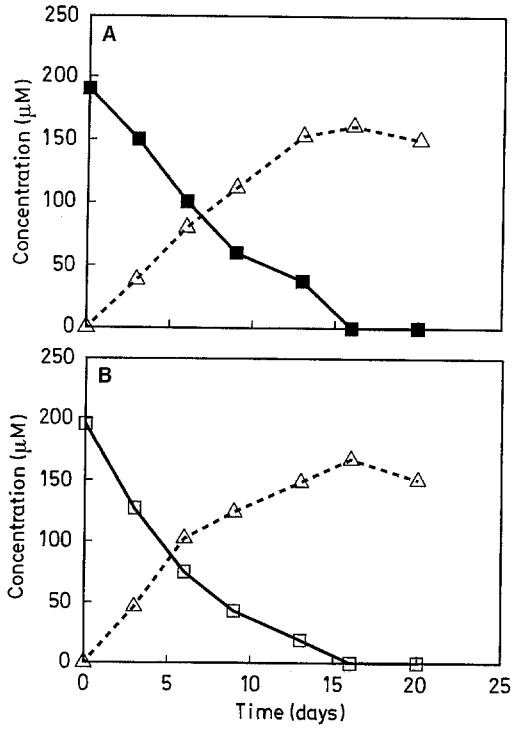

Fig. 4A, B Anaerobic dechlorination of 4-chlorophenol (■) and 2-chlorophenol (□) by PCP-degrading granules under anaerobic conditions. Phenol (△) accumulated as a dechlorinated product in both cases

Further experiments were conducted with high concentrations of 4-CP and 2-CP (approximately 200 μM) in order to examine whether phenol was a dechlorination product. The dechlorination rates of 4-CP and 2-CP were identical (Fig. 4A, B). A significant level of phenol (more than 150 μM) accumulated during anaerobic dechlorination of 4-CP and 2-CP. Benzoate was not detected. During this test, the degradation rate for phenol was low. This may be due to inhibition of phenol-degrading organisms and methanogens (needed for syntrophic phenol degradation) caused by high levels of chlorophenols.

These results indicate that the initial dechlorination of 2,4,6-TCP occurred at the *ortho* position, resulting in the appearance of 2,4-DCP; subsequently the dechlorination of 2,4-DCP occurred at either the *ortho* or *para* position, resulting in the appearance of 2-CP and 4-CP, and phenol was the dechlorinated end-product of 2-CP and 4-CP. In addition, the dechlorination of 2,4-DCP at the *ortho* position appeared prevalent since more 4-CP than 2-CP was produced.

Discussion

The results of tests for release of chloride ions from PCP indicate that anaerobic (or low redox potential) conditions are essential for PCP dechlorination by the PCP-dechlorinating/degrading microorganisms. However, if intact PCP-degrading granules were used, PCP dechlorination could be observed in serum vials where low levels of dissolved oxygen were present in the medium. This is because the granular structure can protect internal anaerobic organisms from exposure to oxygen and provide a low-redox-potential microenvironment. A similar observation was also made during reactor operation. Dissolved oxygen in the reactor influent (approximately 5–7 mg/l) never influenced PCP removal performance because of the rapid consumption of the oxygen in the reactor.

The roles of sulfate-reducing bacteria and methanogens in the reductive dechlorination and mineralization of mono- and dichlorophenols have been reported (Kohring et al. 1989; Sharak Genthner et al. 1989; Häggblom and Young 1990). However, no reports on the effect of methanogenic or sulfate-reducing conditions on the anaerobic mineralization of [^{14}C]PCP have been reported previously. The results reported in the literature are quite different and depend on the origin of the culture and on the compounds studied: sulfate was either inhibitory or stimulatory. Häggblom and Young (1990) demonstrated with an inoculum from an estuarine sediment that the dechlorination of 2,4-dichlorophenol and all the monochlorophenol isomers tested was coupled to sulfate reduction. Earlier, Gibson and Suflita (1986) tested the anaerobic biodegradation of trichlorophenoxyacetate, trichlorophenol, dichloro-benzoates and less chlorinated compounds with samples from different habits. These haloaromatic molecules were dechlorinated in samples from a methanogenic aquifer but not in samples from sulfate-reducing sites. Our studies using ^{14}C-labeled PCP and determining chloride ion release suggested that SRB were involved in both dechlorination and mineralization of PCP. The addition of $FeSO_4$ stimulated both the chloride release rate and the PCP mineralization process. We also found that the addition of $FeCl_2$ did not stimulate [^{14}C]PCP mineralization (data not shown). Addition of molybdate, an inhibitor of sulfate-reducing bacteria, reduced the chloride release rate and inhibited mineralization of ^{14}C-labeled PCP. This suggests that sulfate-reducing bacteria were involved in the dechlorination of chlorophenols and degradation of dechlorinated products. Although a slight decrease in chloride release rate was observed in the presence of BES, this does not support the dechlorination of chlorophenols by methanogens because no dechlorination of chlorophenols has been observed using pure methanogen cultures. However, methanogens are essential for mineralization of PCP to methane and CO_2. For example, methanogens play an important role in the syntrophic degradation of non-chlorinated compounds produced after dechlorination (such as phenol, benzoate, etc.). This was supported by radio-labeled PCP tests; i.e., when BES, a specific inhibitor for meth-

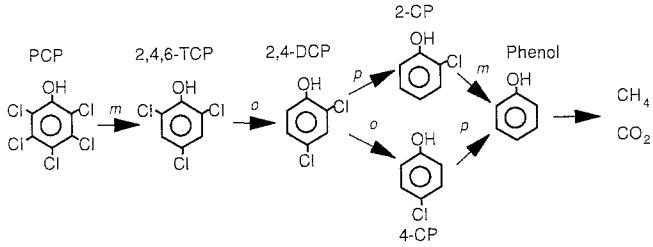

Fig. 5 Proposed a pathway for anaerobic dechlorination and degradation by the PCP-degrading granules. Part of the PCP was dechlorinated through 2,4,6-trichlorophenol (*TCP*), 2,4-dichlorophenol (*DCP*), and 2-chlorophenol (*CP*) or 4-CP to phenol and phenol was further degraded to methane and CO_2 (*o ortho* dechlorination; *m meta* dechlorination; *p para* dechlorination)

anogens, was present, neither [^{14}C]methane nor $^{14}CO_2$ was produced.

Based on the results obtained in this study, an anaerobic PCP dechlorination/degradation pathway is proposed in Fig. 5. Part of the PCP was dechlorinated by the granules through 2,4,6-TCP, 2,4-DCP, and 2-CP or 4-CP to phenol, and phenol was further degraded to methane and CO_2. As previously reported, both 2,4,6-TCP and 3,4,5-TCP were detected when a high level of PCP was applied to the PCP-degrading granules (Wu et al. 1993). Therefore, the presence of other pathways of anaerobic PCP dechlorination with 3,4,5-TCP as an intermediate cannot be ruled out. Both trichlorophenols could be the intermediates during PCP dechlorination by the granules. The production of the *ortho*-dechlorinated intermediate 3,4,5-TCP and *meta*-dechlorinated intermediate 2,4,6-TCP from PCP has also been observed in a PCP-enriched digested sludge (Nicholson et al. 1992). It was difficult to compare the dechlorination rates between 2,4,6-TCP and 3,4,5-TCP to identify which isomer was the more prevalent intermediate during anaerobic dechlorination of PCP by the granules. This is because, among all chlorophenols, 3,4,5-TCP is the most toxic (Wu et al. 1989; Madsen and Aamand 1992) and dechlorination of 3,4,5-TCP was inhibited even in the presence of a moderate level of 3,4,5-TCP (e.g., 5 μM). Methane production from volatile fatty acids was completely inhibited in the presence of 1.3 μM 3,4,5-TCP but not in the presence of 21 μM 2,4,6-TCP (Wu et al. 1989). Apparently, dechlorination of PCP by the anaerobic microbial ecosystem through 2,4,6-TCP is much more favorable than through 3,4,5-TCP.

The results of the 2,4,6-TCP dechlorination test suggest that, first, the granules produced 2,4-DCP from 2,4,6-TCP by reductive dechlorination of the *ortho*-chlorine, and subsequently *ortho*-dechlorinated 2,4-TCP to 4-CP or *para*-dechlorinated 2,4-TCP to 2-CP (Fig. 3). Finally, 4-CP and 2-CP were dechlorinated to phenol (Fig. 4). The *ortho* dechlorination of 2,4,6-TCP to 2,4-DCP has been also reported by other investigators (MJikesell and Boyd 1985; Woods et al. 1989; Nicholson et al. 1992). The 2,4-DCP dechlorination pathway followed by the granules appears to be the same as that observed in freshwater sediments reported by Bryant et al. (1991), i.e., both 2-CP and 4-CP were dechlorinated intermediates. However, this pathway is different from those observed in other anaerobic cultures where only 4-CP was detected as an intermediate of 2,4-DCP dechlorination (Zhang and Wiegel 1990; Nicholson et al. 1992; Armenante et al. 1993).

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