

Influence of a Supplemental Carbon Source on Anaerobic Dechlorination of Pentachlorophenol in Granular Sludge

HANNE V. HENDRIKSEN, SUSAN LARSEN, AND BIRGITTE K. AHRING*

Anaerobic Microbiology/Biotechnology Research Group, Department of Biotechnology, Building 223, The Technical University of Denmark, DK-2800 Lyngby, Denmark

Received 26 June 1991/Accepted 24 October 1991

Anaerobic dechlorination of pentachlorophenol (PCP) was studied in two upflow anaerobic sludge blanket reactors. One reactor received glucose (0.9 g liter^{-1}) as an additional carbon source; the other one served as a control. The concentration of PCP in the medium was 4.5 and $3.0 \text{ mg liter}^{-1}$ in the experimental and control reactors, respectively. The reactors were inoculated with granular sludge previously grown on sugar-containing wastewater. After 10 months of continuous operation, the removal of PCP was 99% in the glucose-amended reactor, whereas the removal in the control reactor varied between 32 and 77%. Furthermore, 94% of the PCP was completely dechlorinated in the glucose reactor compared with a maximum of 20% in the control reactor. In the same period, the amount of biomass in the glucose reactor had increased by approximately 150% compared with that in the control reactor, where no growth of the sludge bed occurred. Batch culture activity tests showed that the addition of glucose had a stimulatory effect on the dechlorination rate of PCP per gram of volatile solids. This indicated that the better performance of the glucose-amended reactor was due to a higher concentration of biomass and a direct stimulatory effect of glucose on the dechlorination rate. The pattern of dechlorination of PCP showed that an initial para cleavage was followed by two ortho cleavages.

The anaerobic degradation of chlorinated aromatic compounds has been studied extensively for the last decade to evaluate the fate of these xenobiotic compounds in the environment. Chloroaromatic compounds are reductively dechlorinated, resulting in less toxic and less recalcitrant compounds. The specificity of the dechlorinating enrichments characterized so far and the fact that the rates of degradation are enhanced after acclimation suggest the existence of a specific biological process (2, 3, 5, 11, 12, 16, 21, 22, 26, 27, 30). In accordance with this, studies of the only anaerobic dechlorinating organism isolated so far, *Desulfomonile tiedjei*, showed that this organism gains energy through dechlorination of 3-chlorobenzoate (4, 6, 8, 23). This mechanism is in contrast to the dechlorination of chlorinated aliphatic compounds, which most often occurs as a nonspecific process catalyzed by low-potential electron carriers (9, 17, 28).

The addition of organic substrates stimulates the dechlorination of chloroaromatic compounds (5, 13, 19, 25). The rate and extent of dechlorination of polychlorinated biphenyl (Arochlor 1242) in anaerobic sediment increase when acetone, glucose, or methanol is present (25). The same pattern was seen in experiments with 2,4,5-trichlorophenoxyacetic acid, in which both short-chain organic acids and alcohols stimulated the onset and rate of dehalogenation (13). Dehalogenation of 3,4-dichloroaniline was stimulated by complex sources, e.g., ruminal fluid, yeast extract, or trypticase, whereas volatile fatty acids or glucose had no effect (19).

Only a few studies on the anaerobic degradation of chlorinated compounds in continuous culture have been undertaken. Guthrie et al. (14) observed more than 99% removal of pentachlorophenol (PCP) during anaerobic digestion of sewage sludge in a continuously stirred tank reactor operated at a hydraulic retention time of 10 to 40 days and a PCP

concentration of 5 mg liter^{-1} . However, the extent of dechlorination was not reported. Experiments in an upflow anaerobic sludge blanket (UASB) reactor (29) with various chloroaromatic compounds showed that these compounds were partially dechlorinated but never totally mineralized. Dechlorination of PCP to 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP) and 3,4,5-trichlorophenol (3,4,5-TCP) indicated that ortho cleavage was favored. In addition to chlorophenols, the reactors received 4.3 g of chemical oxygen demand liter^{-1} of readily degradable organic carbon (29). Krumme and Boyd (18) have shown that treatment of monochlorophenols in an upflow anaerobic filter reactor resulted in approximately 90% conversion of these compounds, but only 40% were completely mineralized to carbon dioxide and methane. The chlorophenols were the sole carbon and energy source. When PCP was used as the substrate in the same reactor, a maximum conversion efficiency of 35% was found. Experiments in our laboratory on the dechlorination of PCP in anaerobic fixed-film reactors inoculated with sewage sludge showed that dechlorination was highly stimulated by the addition of glucose. Total mineralization of the added PCP was, however, not observed (15).

PCP was dechlorinated and completely mineralized in batch experiments using a mixture of sludges previously adapted to dechlorination of the three monochlorophenols as the inoculum (22). Unadapted sewage sludge (21) or anaerobic soil (24) showed some transformation of PCP into lesser-chlorinated phenols. Experiments by Larsen et al. (20) showed a high potential for PCP dechlorination under thermophilic conditions with inocula from natural anaerobic environments.

In the present study, we examined the dechlorination of PCP in UASB reactors. Specific research objectives were (i) to investigate the potential for PCP transformation in granules previously grown on sugars, (ii) to establish and maintain a stable dehalogenation of PCP in the reactors for a prolonged period of time, (iii) to examine the influence of an

* Corresponding author.

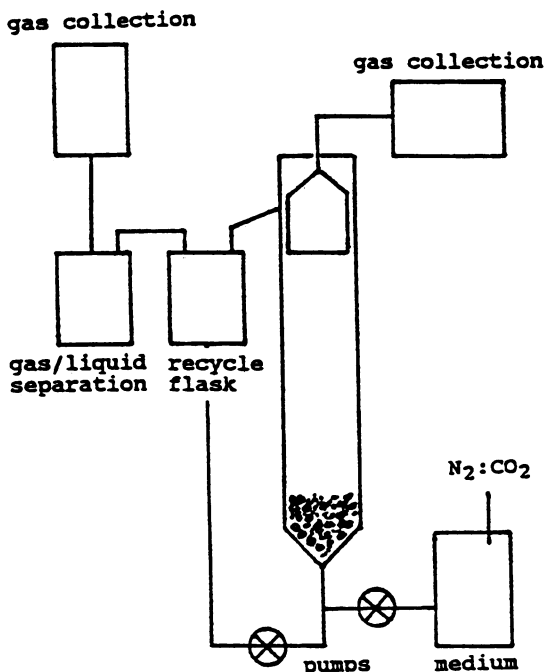


FIG. 1. Diagram of the experimental setup.

easily degradable carbon source on the dehalogenating activity, and (iv) to evaluate the pattern and rate of PCP dehalogenation in the granular sludge used for the reactor experiments.

MATERIALS AND METHODS

Reactor design. Two UASB reactors made of glass with an active volume of approximately 2 liters were used for the experiments. The setup is shown in Fig. 1. To minimize sorption, all tubing used was either Teflon tubes put inside a butyl rubber tube (to prevent oxygen diffusion) or Viton tubes. The reactors were operated for 2 months with a hydraulic retention time of 3 days and a recirculation ratio of approximately 1:4. The hydraulic retention time was then reduced to 2 days, and the recirculation ratio was increased to 1:16. The reactors were placed in a constant temperature room at 37°C. Samples were taken from the top of the reactor once a week and analyzed for chlorophenols, phenol, and volatile fatty acids. The pH was checked regularly, and it stayed at approximately 7.0 to 7.5.

Source of inoculum. The inoculum used was granular sludge grown on sugar-containing wastewater and stored at 10°C for 1.5 years. The volume of granules used as the inoculum was approximately 200 ml. Prior to inoculation, the reactors were flushed with oxygen-free gas, N₂-CO₂, 80:20, and filled with an anaerobic buffer (1.8 mM NaH₂PO₄, 38.7 mM NaHCO₃, 0.5 mg of resazurin liter⁻¹, 0.2 g of Na₂S · 7 to 9H₂O liter⁻¹). To activate the granular sludge, the medium in both the reactors contained 0.9 g of glucose liter⁻¹ during the start-up. After 3 to 4 weeks of operation, PCP and phenol were supplied to the reactors (day 0). One reactor still received glucose in addition to the phenols, while the other reactor (the control) only received PCP and phenol.

Medium. BA medium (1) without cysteine was used. The medium was prepared in 20-liter flasks and flushed with sterile N₂-CO₂ (80:20) gas after autoclaving. NaHCO₃ and Na₂S · 7 to 9H₂O were added to the autoclaved, anaerobic medium from anoxic, sterile stock solutions to a final concentration of 2.6 and 0.25 g liter⁻¹, respectively. PCP and phenol were added from concentrated ethanolic stock solutions of 200 and 625 g liter⁻¹, respectively. The concentration of PCP in the medium was approximately 3 mg liter⁻¹ during the first 2 months of operation and was then increased to approximately 4.5 mg liter⁻¹. The phenol concentrations were 15 and 22.5 mg liter⁻¹, respectively, for the two different periods. In the control reactor, the PCP concentration were lowered again to 3.0 mg liter⁻¹ after 8 months in an attempt to enhance transformation. Also, the phenol concentration was lowered to 15 mg liter⁻¹. Glucose (0.9 g liter⁻¹) was added to the medium of one reactor from a sterile, anoxic stock solution.

Batch experiments. Batch experiments were performed in 50- or 100-ml serum vials containing 20 or 40 ml of BA medium prepared as previously described (1). The vials were sealed with butyl rubber stoppers (Bellco, Inc.) and aluminum caps. A granular sludge suspension (approximately 50% sludge) taken from the bottom of the reactors was used for inoculation. The sludge suspension was gassed with anaerobic gas (N₂-CO₂) and mixed vigorously before inoculation. The vials were inoculated with 10% (vol/vol) of the suspension. PCP was added from an ethanolic stock solution to a final concentration of either 2.5 mg liter⁻¹ in experiments with the glucose-supplemented reactor or 1.5 mg liter⁻¹ for the control reactor. Experiments were made with PCP as the sole carbon source or with the addition of glucose (0.9 g liter⁻¹). The experiments were run in triplicate and incubated at 37°C under slightly shaking conditions. The amount of volatile solids in the vials was determined at the end of the experiment.

Analytical methods. (i) **Chlorophenols.** Chlorinated phenols were quantified by using an HP 5890 series II gas chromatograph equipped with an electron capture detector and a Megabore DB-5 capillary column (J&W Scientific), as previously described (20). Before analysis, the samples were centrifuged and derivatized. A 0.3-ml sample was mixed with 2.55 ml of a 0.0588 mM Na₂HPO₄ solution (pH 9.0) and 0.15 ml of internal standard (2,4,6-tribromophenol). Twenty microliters of acetic anhydride and 1 ml of pentane were added, and the mixture was shaken vigorously for 1 min. Two microliters of the pentane phase was used for injection. Samples were compared with known standards. Monochlorophenols and phenol were determined by high-pressure liquid chromatography using a Waters model 510 pump and a Waters Lambda-Max 480 UV detector set at 275 nm. The column was a Waters C₁₈ reverse phase, and the mobile phase was a 40:15:45 mixture of methanol, ethanol, and water-0.01% acetic acid.

(ii) **Fermentation products.** Gas analysis was made by using a Microlab ML GC 82 chromatograph, equipped with a thermal conductivity detector. Volatile fatty acids were measured with an HP 5890 gas chromatograph, equipped with a flame ionization detector and a Superox II FA capillary column (RSL Chromatography) (1). Glucose was quantified enzymatically by using a glucose plus L-lactate analyzer YSI model 2000. The amount of volatile solids was estimated according to standard methods (10).

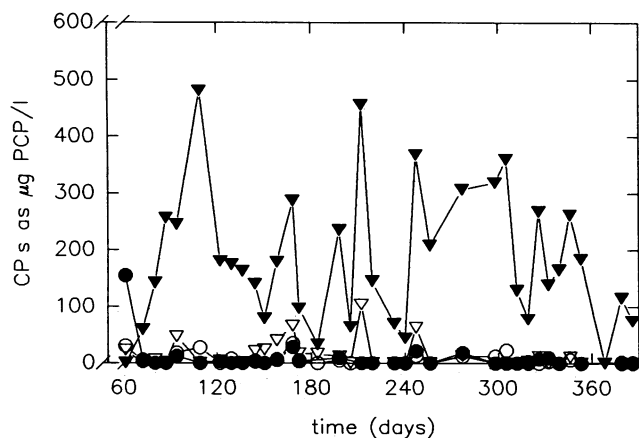


FIG. 2. Effluent concentrations of chlorinated phenols (CPs) from day 61 onward calculated as micrograms of PCP liter⁻¹ in the glucose-amended UASB reactor. The influent concentration of PCP was 4.5 mg liter⁻¹. ○, PCP; ●, total TeCPs; ▽, total TCPs; ▼, total DCPs.

RESULTS

Approximately 75% of the influent PCP (3 mg liter⁻¹) was removed 4 days after PCP was added to the UASB reactors (day 4). Lesser chlorinated phenols could not be detected during the first 6 days of operation, indicating that part of the PCP was removed by sorption. After 10 days, no PCP could be detected in the effluent from the glucose reactor, whereas the concentration in the control reactor was approximately 35% of the influent concentration. The concentrations of PCP and lesser chlorinated intermediates in the effluent of the reactor supplemented with glucose are shown in Fig. 2. The depicted data are from day 61 onward, when the medium concentration of PCP was increased to 4.5 mg liter⁻¹. The total amount of chlorinated phenols leaving the reactor was less than 500 µg liter⁻¹ calculated as PCP, indicating a very efficient removal of PCP. The major intermediates in the effluent were dichlorophenols (DCPs). No monochlorophenols or phenol could be detected. Figure 3 shows the data from the control reactor from day 61 onward. The concentration of PCP leaving the reactor varied between 200 and 5,000 µg liter⁻¹, showing that the process was very unstable. As in the glucose reactor, the influent concentration of PCP was raised from 3.0 to 4.5 mg liter⁻¹ at day 61. However, after 8 months of operation, it was necessary to decrease the concentration again to 3.0 mg liter⁻¹ in an attempt to increase dechlorination. The major intermediate in the effluent of the control reactor was TeCP, and only traces of TCP and DCP were detected. A lag phase of 7 to 8 months of operation was needed in the control reactor before the added phenol was degraded.

No glucose in the effluent of the glucose-supplemented reactor could be detected, indicating that it was completely metabolized. Intermediates like volatile fatty acids were never observed, except for traces of acetate in the glucose reactor. The total methane production in the glucose-supplemented reactor was approximately 200 ml day⁻¹, representing 54% of the theoretical amount. The low yield is probably caused by leakages in the experimental setup (tubings, etc.), which is of importance when the organic loading rate is low. The percentage of methane in the top of the reactor was 30 to 60%, compared with 10 to 30% in the control reactor. No

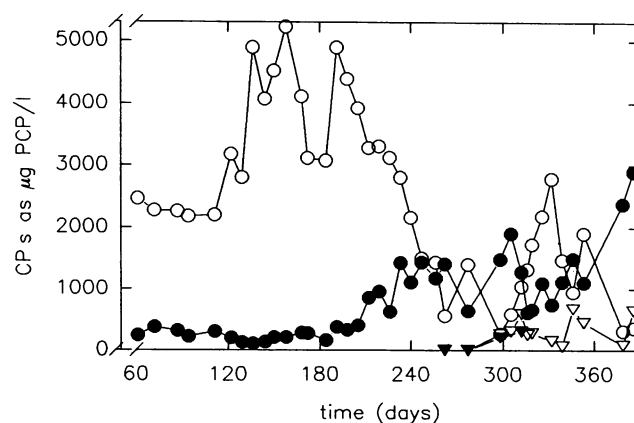


FIG. 3. Effluent concentrations of chlorinated phenols (CPs) from day 61 onward calculated as micrograms of PCP liter⁻¹ in the control reactor. The influent concentration of PCP was approximately 4.5 mg liter⁻¹ for days 61 to 240 and 3.0 mg liter⁻¹ for the rest of the period. ○, PCP; ●, total TeCPs; ▽, total TCPs; ▼, total DCPs.

net production of methane in the control reactor could be measured.

A comparison between the chlorophenols in the effluents of the two reactors between days 277 and 353 is shown in Fig. 4. The removal of PCP was 50 and 99.8% in the control and glucose reactors, respectively, during this period of time. The amount of PCP that was dechlorinated further than DCPs reached 94% in the glucose-supplemented reactor, compared with 0 to 10% in the control. The average PCP removal rate calculated over a period of 3 months (days 240 to 346) was 2.2 mg of PCP liter⁻¹ reactor⁻¹ day⁻¹ and 0.6 to 1.2 mg of PCP liter⁻¹ reactor⁻¹ day⁻¹ for the glucose and control reactors, respectively. Due to the instability of the control reactor, the removal of PCP varied considerably with time in this reactor.

Figure 5 shows the results from representative vials from the batch activity test with the granules from the glucose-amended reactor. Experiments were made with either PCP as the sole carbon source (Fig. 5B) or glucose (Fig. 5A). The major intermediates found were 2,3,5,6-TeCP, 2,3,5-TCP, and 3,5-DCP, representing approximately 95% of the transformed PCP. This indicated a PCP dechlorination pathway, as shown in Fig. 6, path B. The remaining 5% was degraded via 2,3,4,5-TeCP and then probably 3,4,5-TCP and 3,5-DCP (path A). Traces of 3,4-DCP were also detected. Experiments with the addition of 3,5-DCP directly into the vials revealed that this compound was further dechlorinated to 3-chlorophenol (data not shown).

Batch activity tests on the control reactor showed that the dechlorination of PCP proceeded via the same intermediates as in the glucose reactor (Fig. 5C). Experiments were made with PCP as the sole carbon source.

The approximate rates of PCP dechlorination obtained in the various batch experiments are as follows. The addition of glucose to three vials increased the rate significantly (mean, 1,736 µg of PCP per g of volatile solids [VS]; standard deviation [SD], 460) compared with three vials with no glucose (not determined). The basic rates without glucose addition were similar in experiments from the two reactors (451 [65] versus 521 [71], mean µg of PCP per g of VS [SD] for the glucose and control reactors, respectively), which

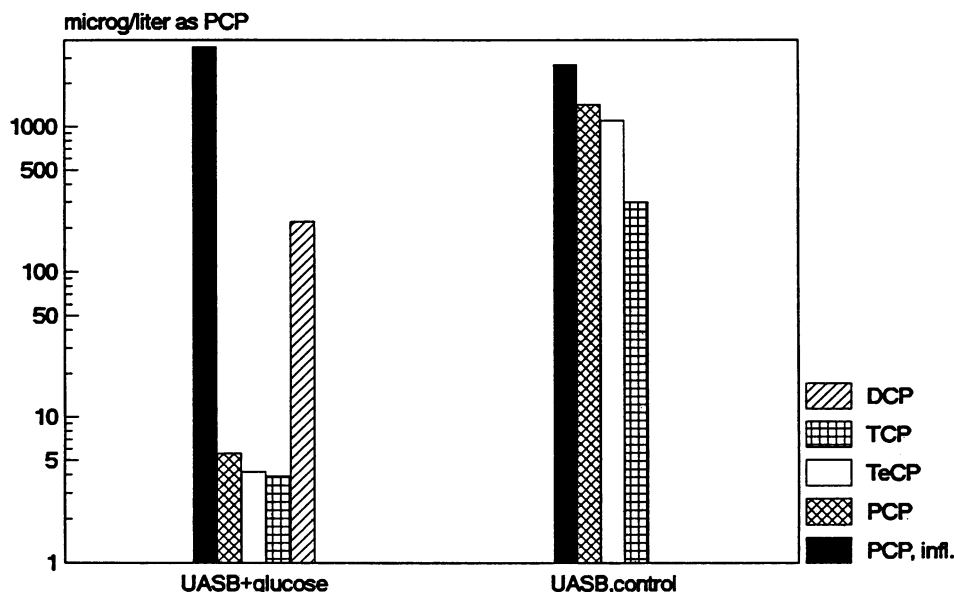


FIG. 4. Comparison of the performance of the two reactors, calculated as the average effluent concentrations of chlorophenols from days 277 to 353.

could indicate that the same microorganisms were active in the two reactors.

DISCUSSION

The results of the present study showed that conventional sugar-grown granules never previously exposed to xenobiotic compounds have a potential for PCP transformation. The dechlorinating activity of these granules suggests that dehalogenation is a common ability in anaerobic environments, since samples from various environments can dechlorinate chlorinated compounds (11, 12, 20, 21). Our results further showed that transformation of a recalcitrant compound can occur concurrently with an easily degradable substrate in a continuous flow system. No washout or loss of activity was seen during approximately 1 year of operation in the glucose-amended reactor, and the stability was superior to the reactor receiving only PCP and phenol.

The PCP dehalogenation rate ($2.2 \text{ mg of PCP liter}^{-1} \text{ reactor}^{-1} \text{ day}^{-1}$) for the glucose-amended reactor was somewhat higher than the rates reported elsewhere. Guthrie et al. (14) reported a dechlorination rate of $0.5 \text{ mg liter}^{-1} \text{ reactor}^{-1} \text{ day}^{-1}$, and Woods et al. (29) reported a rate of $1 \text{ mg liter}^{-1} \text{ reactor}^{-1} \text{ day}^{-1}$. Both reactor systems were supplemented with an extra carbon source. The conversion efficiency found for the control reactor (32 to 77% on days 240 to 346) is similar to results obtained by Krumme and Boyd showing a 35% transformation with PCP added as the sole carbon and energy source during short-term experiments (18).

The results from the batch tests showed that the activity with no glucose added was approximately the same in the two reactors. This suggested that the low transformation of PCP in the control reactor was partly caused by the limited amount of biomass present in this reactor compared with the glucose reactor. After 1 year of operation, the amount of biomass in the glucose-amended reactor was two to three

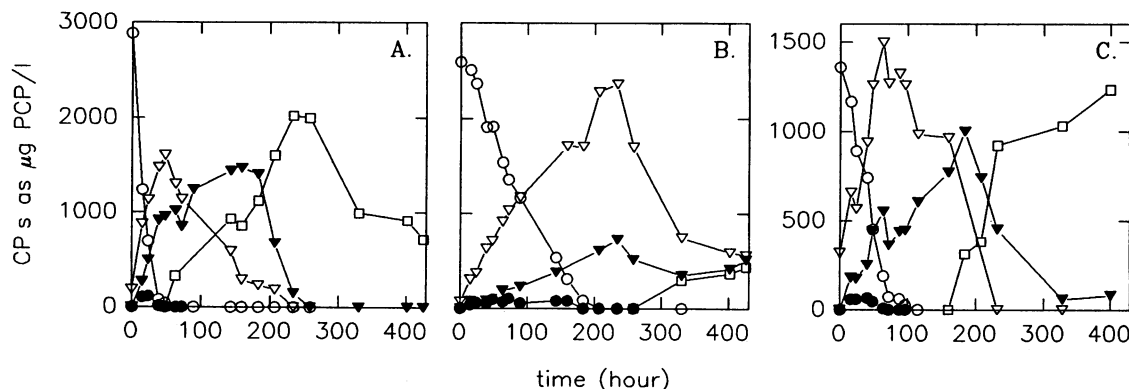


FIG. 5. PCP dechlorination in the batch activity tests. Activity tests from the glucose-amended reactor from one representative vial supplemented with glucose (A), one representative vial with PCP as the sole substrate (B), and one representative vial from the control reactor with PCP as the sole substrate (C). \circ , PCP; \bullet , 2,3,4,5-TeCP; ∇ , 2,3,5,6-TeCP; \blacktriangledown , 2,3,5-TCP; \square , 3,5-DCP.

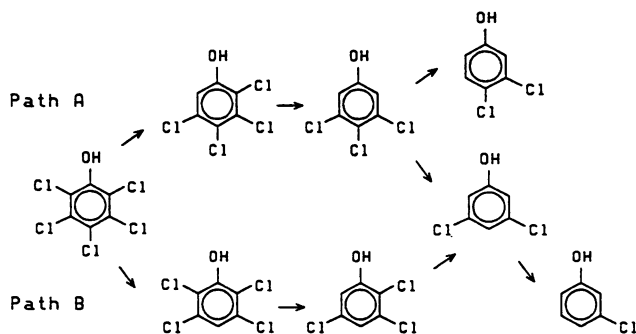


FIG. 6. Dechlorination pathways for PCP.

times as high as in the control, because of the growth of the sludge bed. No growth in the control reactor could be observed, probably because of the low amount of carbon available for microbial growth. The batch activity test from the glucose reactor showed, however, that the rate of dechlorination of PCP per unit of biomass was enhanced significantly by the addition of glucose. These results indicated that the superior performance of the glucose reactor was a combined effect of a higher active biomass concentration and stimulation caused by the glucose.

The stimulatory effect of glucose could be due to several reasons. The dehalogenation process could be a cometabolic process in which glucose acts as a primary substrate. An example of such a process is found in a study by Dietrich and Winter, in which 2-chlorophenol was degraded with butyrate serving as a cosubstrate (5). The cometabolism theory, however, is not supported by the fact that our enrichment cultures could dechlorinate PCP completely without the addition of glucose. The metabolism of glucose by other microorganisms present in the reactor could also generate low-potential electrons that could nonspecifically dechlorinate PCP, similar to what has been found for the degradation of the chlorinated aliphatics (17). Alternatively, production of hydrogen, formate, ethanol, propionate, or acetate could serve as the source of reducing equivalents required for the dechlorination, as has been shown for *D. tiedjei* (6, 7). The presence of a glucose degrading consortium could also supply the dechlorinating organism with essential compounds, e.g., vitamins or cofactors. A cross-feeding pattern of this kind could explain the very strict demand for complex carbon sources like yeast extract, peptone, or ruminal fluid found for all the dechlorinating enrichments characterized so far (5, 30).

The major pathway for PCP dechlorination in both reactors was via 2,3,5,6-TeCP as an intermediate (Fig. 6, path B), and only a minor fraction of PCP was metabolized via 2,3,4,5-TeCP (path A). This result is in contrast to results previously described by Mikesell and Boyd and Woods et al. in which an initial ortho cleavage leading to 2,3,4,5-TeCP was found (22, 29). Dechlorination of PCP in a freshwater sediment under thermophilic conditions has also been shown to proceed mainly via the 2,3,5,6-TeCP intermediate (20).

Results from the operation of two fixed-film reactors previously studied in our laboratory (15) showed a PCP removal of 98% when glucose was supplemented, compared with 61% with no glucose added. The rates of dechlorination per reactor volume per day were, however, considerably lower than the rates observed in the UASB reactors. In addition, only a minor fraction of the added PCP (approx-

mately 20%) was completely dechlorinated in the glucose-supplemented reactor. Except for the source of inoculum, the operational parameters for the two fixed-film reactors were similar to those of the UASB reactors. The fixed-film reactors were inoculated with anaerobic digested sewage sludge from a municipal treatment plant, whereas granular sludge was used for the UASB reactors. The better performance of the UASB reactors suggests that the potential for dechlorination is higher in the granular sludge than the sewage sludge. The dechlorination pathway in the two reactor systems also differed, underlining the microbiological difference between the inocula. In the fixed-film reactors, the major intermediates were 2,3,4,5-TeCP and 3,4,5-TCP (unpublished results), indicating dechlorination exclusively via path A (Fig. 6), which constituted only 5% of the activity in the UASB reactors.

Our study demonstrated that it is possible to effectively remove a highly recalcitrant compound in a UASB reactor with conventional granular sludge. Furthermore, we have been able to maintain the dechlorinating activity for more than 1 year with a defined medium and a defined carbon source. The addition of glucose stimulated the dechlorination, stabilized the process, and ensured the presence of a sufficient amount of active immobilized biomass. Evaluation of the exact nature of the glucose stimulation would be helpful in the design of an optimal treatment process.

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