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Isolation and Characterization of a Pentachlorophenol-Degrading Bacterium

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With a new enrichment protocol, pentachlorophenol (PCP)-degrading bacteria were isolated from soil, water, and sewage. When characterized, all isolates were related and shared characteristics of the genus *Arthrobacter*. Growth rates for strain NC were determined for a number of substrates, including PCP and 2,4,6-trichlorophenol. Changes in PCP concentration affected growth rate and length of the lag phase but not cell yield. Increasing the pH from 6.8 to 7.8 decreased the length of the lag phase for growth on PCP. Cessation of growth, upon incremental addition of PCP, was found to be related to a decrease in pH rather than to a buildup of a toxic metabolite. Degradation of PCP by strain NC was shown to be complete.

Pentachlorophenol (PCP) is a wide-spectrum biocide with applications in agriculture, industry, and public health (6). It is considered to be an environmental pollutant because of its broad toxicity and its persistence in soil and sewage (2, 18, 20). In the United States, approximately 5.0 $\times$ 10^7 pounds (ca. 2.27 $\times$ 10^{10} g) is produced annually (22). The primary use of PCP, accounting for over 80% of all PCP produced, is as a wood preservative (4). Effluents that contain PCP arise not only from its manufacture but also from its use in wood treatment. A 1974 survey disclosed that only 60% of the wood treatment plants surveyed met water pollution standards, and 17% of the plants released their wastewater without any treatment (26). By 1983, all plants must be in compliance with regulations restricting the release of chlorinated phenols in wastewater (23).

One method of removing chlorinated phenols, including PCP, from wastewater is by means of biodegradation in aerated aqueous systems (14). In practice, difficulties are often encountered in such systems due to a paucity of knowledge concerning PCP-degrading populations.

Partial or complete degradation has been observed in soil (21, 30), chicken litter (13), and adapted sewage (15). Two bacterial isolates have been shown to utilize PCP as a sole source of carbon and energy: a coryneform bacterium isolated by Chu and Kirsch (9) and a pseudomonad isolated by Watanabe (29).

In the present study, PCP-degrading bacteria were isolated from soil, water, and sewage. The degraders were characterized, and those kinetic parameters associated with growth rate, length of the lag period, and toxicity were studied. These parameters were chosen for their significance in any application of biodegradation to the removal of chlorinated phenols from wastewater or other polluted environments.

MATERIALS AND METHODS

Habitats. Four habitats were sampled for the presence of PCP-degrading microorganisms. Two of these habitats were soil (one sample was known to be contaminated by PCP), and two were aquatic (one sample had a history of PCP contamination). Soil samples collected 4 and 15 cm from a utility pole were pooled and labeled as strain S1. The soil around the pole was colored dark brown by the seepage of preservative from the pole into the soil. Soil 4 cm from the pole was colored dark brown by the seepage of preservative from the pole into the soil. Soil 4 cm from the pole contained 76 μg of PCP per g of soil. The second habitat contaminated with PCP was Naylor's Run Creek, Haverford, Pa. This stream was sampled by Fountaine et al. in 1975 (16). A sample collected from the creek at Monoa Road contained 5 μg of PCP per ml. This source was designated strain NC. Samples were also collected from habitats with no histories of PCP contamination. One sample, strain S2, was garden soil, and the other sample, strain RS, consisted of clarified raw sewage from the Ithaca, N.Y., municipal treatment plant.

Isolation of PCP degraders. All isolation steps were performed in media with a mineral salts (MS) base of the following composition (in grams per liter): K_2HPO_4, 1.73; KH_2PO_4, 0.68; NH_3NO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.03; and $FeSO_4 \cdot 7H_2O$, 0.03. The calcium, manganese, and iron salts were made up separately as a 100× concentrate, acidified, filter sterilized, and added after autoclaving. The final pH of the MS base was adjusted to 7.4 with ammonium hydroxide.

For isolation, McCloskey-Rott-Fyter solution (McCloskey Varnish Co., Philadelphia, Pa.) or PCP

(99+% PCP; Aldrich Chemical Co., Milwaukee, Wis.) served as the sole carbon source. The McCloskey-Rott-Fyter solution consisted of 5% PCP, 0.6% other chlorophenols, and 94% nonaqueous solvent. MS base plus 0.1% McCloskey-Rott-Fyter solution, added without sterilization, contained 40 to 50 μg of PCP per ml and was designated as commercial PCP medium. PCP, when used as the carbon source, was first converted to its sodium salt by being dissolved in 0.2 N NaOH. Portions of a stock sodium PCP solution (10,000 μg/ml) were then added to MS base, before or after autoclaving, and designated as PCP medium.

Isolation procedure. Samples were inoculated into commercial PCP medium and incubated at 30°C at 200 rpm. Cell growth was monitored by measuring the absorbance at 600 nm, and PCP content was monitored by measuring the absorbance at 320 nm (analytical methods). When the PCP content decreased to 1 to 10 μg/ml, additional McCloskey-Rott-Fyter solution was added to bring the PCP content of the medium back up to 50 μg/ml. A regimen of regular additions of McCloskey-Rott-Fyter solution and weekly transfers to fresh commercial PCP medium was carried out for 2 to 4 weeks. A transition was then made from the addition of McCloskey-Rott-Fyter solution to the addition of pure PCP, and the maximum concentration of PCP in the medium was raised to 100 μg/ml. When the culture repeatedly degraded the PCP that was added, a 10% (vol/vol) inoculum was transferred to PCP medium containing 100 μg of PCP per ml as the sole carbon source. A schedule for monitoring and for PCP additions similar to that described above was followed.

Batch cultures growing in PCP medium were transferred to a New Brunswick Scientific Co. Bioflo model C-30 chemostat and adapted from batch to continuous culture. At steady state, the influent PCP concentration was 500 μg/ml, the dilution rate was 0.016 h^{-1}, the temperature was 30°C, air flow was 6.2 liters h^{-1}, agitation was 300 rpm, and the volume was 350 ml; the pH was maintained at 6.8 by the automatic addition of 1 N NaOH. Portions were removed during steady-state growth, diluted, and surface plated on Trypticase soy broth (TSB) solidified with 1.5% agar (BBL Microbiology Systems, Cockeysville, Md.). Predominant colony types were picked and tested for PCP-degrading capability in PCP batch cultures.

Analytical methods. The amount of PCP in the Naylor's Run Creek (NC) sample was determined by the method of Fountaine et al. (16). The amount of PCP in the 4-cm fraction of sample S1 was determined by the following assay. A-5 g (wet weight) soil sample was extracted for 5 min in a separatory funnel with 50 ml of acidified hexane. The hexane fraction was filtered and then extracted with 0.2 N NaOH. The aqueous phase contained PCP as a precipitate. The hexane fraction was extracted a second time with 0.5 N NaOH. The aqueous phases were combined, the precipitate was dissolved, and the absorbance of the sample was read at 320 nm.

At an alkaline pH, PCP in its sodium salt form, has a characteristic absorbance at 320 nm (29). Samples of 5 ml were made alkaline by the addition of 1 drop of 10 N NaOH. Samples were then centrifuged to remove cells, and absorbances were read at 320 and 360 nm with a Beckman UV-Visible spectrophotometer. The 320-nm absorbance minus the 360-nm absorbance (background) was then converted to micrograms of PCP per milliliter by using a standard curve.

Cell densities were determined by reading the absorbance of 5-ml samples either at 430 nm with a Bausch and Lomb Spectronic 20 spectrophotometer or at 415 nm with a Beckman UV-Visible spectrophotometer. Absorbance readings were converted to dry weights by using a standard curve.

Determination of the extent of mineralization of PCP by strain NC was carried out in a chemostat with PCP as the sole carbon source. The influent PCP concentration was 525 μg/ml, the effluent PCP concentration was 17 μg/ml, the dilution rate was 0.045 h^{-1}, and all other conditions were as previously described. The chloride content was determined by silver nitrate titration (10). Total organic carbon content for cell-free influent and effluent samples was monitored with a Beckman 215A carbon analyzer.

Characterization of PCP degraders. PCP-degrading isolates and Chu and Kirsch's KC-3 strain (9) were compared as to their abilities to assimilate various carbon sources. Carbohydrates were utilized at a concentration of 300 μg/ml in MS base. Phenolic and benzoic carbon sources were tested at a concentration of 200 μg/ml in MS base. When required for growth, 2 μg of yeast extract (Difco Laboratories, Detroit, Mich.) or 0.2 μg of biotin (Sigma Chemical Co., St. Louis, Mo.) per ml was added. Cellular morphology and motility were determined at various stages of growth in TSB. Cytochrome oxidase was assayed by the *N,N*-dimethyl-*p*-phenylenediamine oxalate (Difco) method (17). The color reaction took 3 min to develop.

The cell wall constituents, diaminopimelic acid and arabinose, were determined by ascending paper chromatography. Cells were grown in TSB and then washed three times. Diaminopimelic acid was determined with whole-cell hydrolysates by the method of Becker et al. (5). *Escherichia coli* and DL-α-ε-diaminopimelic acid (Sigma) served as positive controls. For determination of arabinose, cells were extracted for 1 h in 0.1 N NaOH at 100°C, washed, hydrolyzed for 2 h in 2 N HCl at 100°C, and then dried to remove HCl (7). Chromatographs were run in a butanol-lactic acid-water (4:1:5) solvent and developed at 70°C with an aniline hydrogen oxalate developer (24). *Corynebacterium renale* and arabinose (Fisher Scientific Co., Rochester, N.Y.) served as positive controls.

Growth conditions for axenic culture studies. Strain NC was used in all studies. Experiments were conducted in one-quarter-strength (7.5 g/liter) TSB or media with MS base and added carbon sources (50 to 400 μg/ml). PCP and 2,4,6-trichlorophenol (TCP; Chemical Service, Westchester, Pa.) were first converted to sodium salts form by being dissolved in 0.2 N NaOH before being added to MS base.

One-liter creased flasks with working volumes of 500 ml were incubated at 30°C and 200 rpm. The pH was controlled to within 0.1 unit by the manual addition of NaOH while the effect of pH on growth rate was studied. A Bioflo model C-30 chemostat, without metered addition of nutrient, was used as a stir tank with pH control for growth of the organism with selective addition of PCP.

PCP toxicity. The inhibition of cellular growth by PCP was studied with batch cultures. Glucose or acetate was added to MS base to give a final concentration of 300 μg/ml. Biotin, when required, was added to give a final concentration of 1 μg/ml. PCP was

TABLE 1. Characterization of PCP-degrading bacterial strains by growth on various carbon sources

Strain	Growth on indicated carbon source										
	Glucose	Cellobiose	Acetate	Succinate	Malate	Cellulose	Benzoate	Trichloro-benzoate	Phenol	TCP	PCP
KC-3	+	+	+	−	−						
NC	+[a]	−	+	−	−	−	−	−	−	+	+
S1	+	−	+	+[b]	−	−	−	−	−	+	+
RS	+	−	+	−	−	−	−	−	−	+	+
S2	+	−	+	−	+[b]	−	−	−	−	+	+

[a] Biotin was required for growth.
[b] Yeast extract was required for growth.

added in sodium salt form. Incubation was carried out at 30°C and 200 rpm. The pH was adjusted to 7.4 with NaOH or HCl. Strain NC or a mixed population of soil microorganisms was used as the inoculum.

RESULTS

Isolation of PCP-degrading bacteria. Closely related bacteria capable of degrading PCP were isolated from both aquatic and soil habitats. A history of prior PCP exposure was not required for the isolation of PCP degraders.

The degradation of PCP in the commercial PCP medium commenced after a lag of 1 to 2 weeks. There was no correlation of PCP degradation with the extent of initial bacterial growth. After the degradation of the initial load of PCP, subsequent additions of commercial PCP medium resulted in degradation after lag periods of only 1 to 3 days. Transfer of the mixed population from the commercial PCP medium to MS base with pure PCP (PCP medium) resulted in continued PCP degradation. Attempts to isolate the PCP-degrading microorganisms from these batch cultures by plating on TSB with 1.5% agar were unsuccessful. Bacteria which failed to degrade PCP were still numerically predominant in the batch cultures several weeks after the transfer to PCP medium even though the only carbon source added during this time span was PCP.

Mixed growth from each batch culture was used to inoculate a chemostat. At steady state, the cell density was 25 mg (dry weight)/liter at a feed concentration of 500 μg of PCP per ml. At steady state, PCP-degrading bacteria predominated numerically. In one instance, they accounted for 93% of the steady-state population; however, after 13 days of chemostatic growth, the population remained mixed. When the mixed population was plated on TSB with 1.5% agar, small yellow colonies routinely predominated after 3 days of incubation. These colonies possessed the ability to utilize PCP as a sole carbon source.

Characterization of PCP-degrading bacteria. From each habitat, a different strain of PCP-degrading bacterium was isolated. The strains could be differentiated from one another and from Chu and Kirsch's KC-3 strain (9) by nutrition tests (Table 1). When grown in TSB, the isolates exhibited a rudimentary coccus-to-rod-to-coccus cell cycle. The isolates and the KC-3 strain contained diaminopimelic acid but not arabinose in their cell walls (Table 2). These are characteristics of the genus *Arthrobacter*.

No *Pseudomonas* species capable of utilizing PCP as a sole carbon source was isolated. The isolation technique did not select against pseudomonads since pseudomonads were present at

TABLE 2. Characterization of PCP-degrading bacterial strains by physiological and morphological criteria

Strain	Characterization by:										
	Gram stain[a]	Cellular morphology[b]	Motility[c]	Pigmentation[d]	Colony size (mm)[d]	Colony consistency[d]	Glucose fermentation	Catalase	Cytochrome oxidase	Diaminopimelic acid	Arabinose
KC-3	+,−	Rods and cocci		Yellow	1–3	Butyrous	−	+	+	+	−
NC	+,−	Rods and cocci	−	Yellow	1–2	Caseous	−	+	+	+	−
S1	+,−	Rods and cocci	−	Yellow	1–2	Butyrous	−	+	+	+	−
RS	+,−	Rods and cocci	+	Yellow	1–2	Butyrous	−	+	+	+	−
S2	+,−	Rods and cocci	+	Yellow	1–2	Butyrous	−	+	+	+	−

[a] Early growth in TSB was predominantly Gram positive (+); later growth was predominantly Gram negative (−).
[b] Growth in TSB was accompanied by a coccus-to-rod-to coccus change in morphology.
[c] Motility was determined by phase-contrast examination of wet mounts prepared from TSB cultures.
[d] Growth was observed on plates containing TSB with 1.5% agar.

TABLE 3. Comparative growth rates for strain NC metabolizing various substrates

Substrate[a]	Doubling time (h)	Growth rate (h^{-1})	Cell yield (g per g of substrate)
TSB (7.5 g/liter)	2.5	0.28	
Sodium acetate (500 μg/ml)	3.0	0.23	0.21
TCP (150 μg/ml)	5.5	0.13	0.26
Glucose (500 μg/ml)	12	0.06	0.35
PCP (50 to 300 μg/ml)	7–14	0.1–0.05[b]	0.15

[a] pH, 7.1 to 7.2. Initial substrate concentrations are given in parentheses.
[b] This value varied with PCP concentration.

all stages of isolation up to and including chemostatic enrichment. The absence of PCP-degrading pseudomonads must thus be considered to be a reflection of the habitats sampled.

Growth of the isolates in chemically defined medium with PCP as the sole carbon source was prima facie evidence of at least some dissimilation and assimilation of PCP. Evidence of the complete mineralization of PCP was provided by chemostatic growth of strain NC. At steady state in PCP medium, 97% of the PCP and 89% of the carbon in the medium disappeared, whereas chloride was released into the medium in an amount which accounted for 95% of the chlorine added as PCP.

In shaker flasks, growth rates of 0.05 to 0.28 h^{-1} were recorded for a number of carbon sources. The highest growth rate was recorded with TSB, whereas the lowest growth rates were recorded when PCP or glucose was the sole carbon source (Table 3). Although glucose was associated with one of the lowest growth rates, its metabolism resulted in the highest cell yield (y) for a sole carbon source. The lowest cell yield was for PCP, based on grams of cells (dry weight) per grams of PCP (y = 0.15). This low cell yield was to a great extent a reflection of the chlorine content of PCP (66.56% [wt/wt]), when based on carbon content of PCP (y = 0.55).

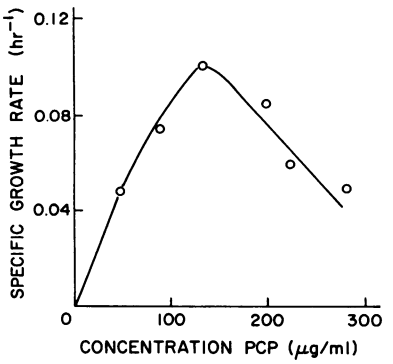

FIG. 1. Specific growth rate of strain NC determined in batch culture in PCP medium at a PCP concentration of 50 to 300 μg/ml. Flasks were inoculated with PCP-adapted cells to an initial absorbance of 0.02 at 600 nm. The initial pH was 7.2, the temperature was 30°C, and agitation was 200 rpm.

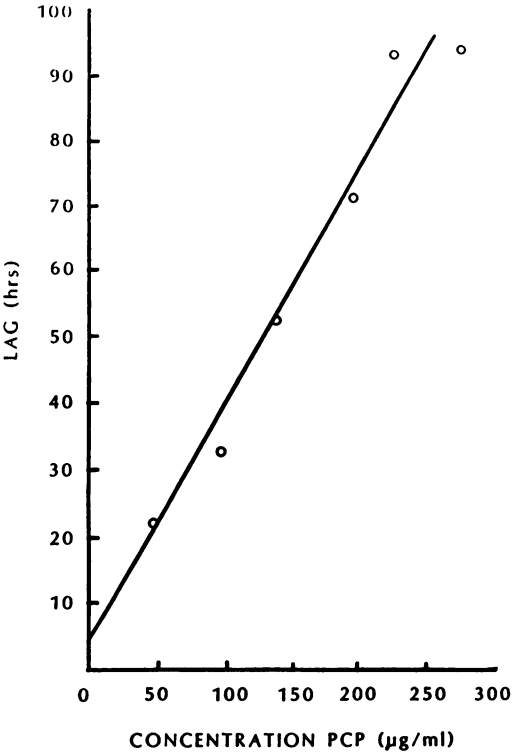

FIG. 2. Effect of PCP concentration on lag time in batch culture. Conditions were as described in the legend to Fig. 1.

The effects of substrate (PCP) concentration on the growth rate, length of the lag phase, and yield were investigated for strain NC grown in batch culture. Figure 1 shows the effect of increased concentrations of PCP on the growth rate of strain NC. The growth rate increased with increasing PCP concentration up to 130 μg/ml (4.9×10^{-4} M). Concentrations of PCP above this level significantly decreased the growth rate.

Increases in PCP (substrate) concentration resulted in directly proportional increases in lag time (Fig. 2). The size of the initial inoculum (0.12, 0.24, or 0.34 μg/ml) had no discernable effect on the length of the lag period. Figure 3

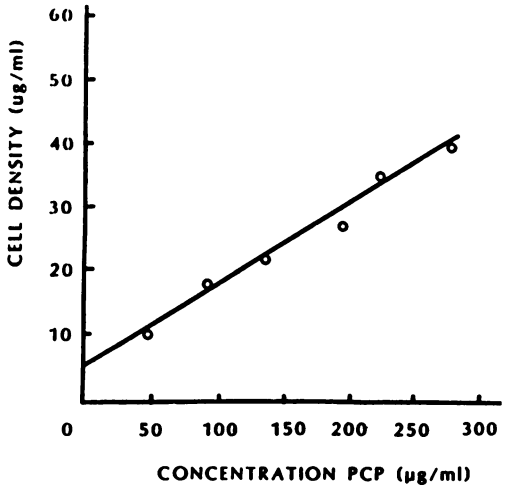

FIG. 3. Dry cell mass versus PCP concentration in batch culture at $y = 0.15$. Conditions were as described in the legend to Fig. 1.

shows the relationship between final cell density and initial substrate concentration. Increases in PCP concentration had no effect on cell yield in batch culture.

pH and PCP toxicity. When strain NC was grown in a shaker flask with repeated additions of PCP (to give a maximum concentration of 100 μg/ml), the PCP was degraded, the cell mass increased, and the pH dropped until a point was reached at which PCP degradation ceased. At that point, an amount of PCP equivalent to 600 μg/ml had been degraded, the cell density was 50 μg/ml, and the pH had fallen to 6.15 (in MS base plus glucose strain NC grew well at a pH of 6.0). Adjustment of the pH back up to 7.1 resulted in degradation of the residue PCP and of subsequent additions of PCP. When the strain was grown in a stir tank with controlled pH (6.9 to 7.1), an amount of PCP comparable to a final concentration of 1,500 μg/ml was consumed in incremental additions of 100 μg/ml.

No effect of pH (6.8 to 7.8) on cell yield at a PCP concentration of 130 μg/ml was observed for strain NC. Decreasing pH exerted an influence on the duration of the lag phase comparable with that observed for increasing PCP concentrations.

On the presumption that toxicity is related only to the acid (undissociated) form of PCP, a series of growth experiments was conducted at various pH levels and total PCP concentrations. The results, shown in Table 4, suggested that concentration of the free acid form of PCP did correlate with the toxic effect. Growth of strain NC was inhibited by concentrations of undissociated PCP above approximately 2 μg/ml.

Some bacteria incapable of degrading PCP were found to be more resistant to the toxic effect of PCP than was strain NC. One such organism isolated from MS base plus glucose and 400 μg of PCP per ml was a cytochrome oxidase-negative bacterium identified as a member of the genus *Acinetobacter*.

DISCUSSION

PCP is a recalcitrant (1, 18, 28) molecule capable of being biodegraded by only a limited number of bacteria (9, 29). Based on its susceptibility to biodegradation, PCP would be related to those chemicals found in Alexander's category iv, namely "chemicals that are suitable substrates for populations in axenic culture or in one or more microbial habitats but which occasionally are quite persistent" (2). The persistence of PCP would then be due to such factors as lack of microorganisms in the habitat capable of decomposing PCP, too high or too low a PCP concentration, too low a pH, or anaerobic conditions.

Although the number of sites sampled was small, it is significant that PCP-degrading bacteria were isolated from all sites. There appeared to be no indication of selection pressure afforded by prior exposure to PCP for the isolation of PCP-degrading microorganisms. All strains isolated were similar to Chu and Kirsch's KC-3 strain (9) and possessed cell wall characteristics common to the genus *Arthrobacter*. No *Pseudomonas* species capable of decomposing PCP, as isolated by Watanabe in Japan (29), was encountered.

Based on cell yield, PCP proved to be a poor substrate for the growth of strain NC. To a certain extent, this is a reflection of the high chlorine content of PCP, but even based on carbon weight, the yield of PCP was lower than the yield of the closely related TCP. The effect

TABLE 4. Effect of pH on PCP toxicity for strain NC

Growth	Undissociated PCP (μg/ml)[a]	Sodium PCP added (μg/ml)	pH
+[b]	0.39	50	7.0
+	0.79	100	7.0
+	1.52	50	6.5
+	1.58	200	7.0
+,−	1.97	250	7.0
−	2.36	300	7.0
−	3.03	100	6.5
−	4.55	50	6.0

[a] The amount of undissociated PCP was calculated from the Henderson-Hasselbalch equation: log (salt/acid) = pH − pK_a. The pK_a of PCP is 5.0.

[b] +, Turbidity in all tubes; +,−, turbidity in 50% of the tubes; −, turbidity in none of the tubes.

of PCP concentration on the length of the lag phase and the exponential growth rate for strain NC catabolizing PCP was characteristic of toxic substrate catabolism (19, 27). One model predicts that lag time can be shortened by increasing the size of the initial inoculum (3). Other experimental results conflict with this hypothesis (25). For strain NC, the size of the initial inoculum had no discernable effect on the length of the lag period. Inoculum history, i.e., maintenance in PCP medium or TSB, had a profound effect on the length of the lag period. Lag periods were longer when strain NC was maintained in media without PCP. Growth on a nonselective medium may not only increase the length of the lag period but can result in loss of the ability to catabolize the substrate (8).

PCP, as an industrial waste, is primarily associated with wood treatment plants (4). Biological treatment of the effluent from these plants is practiced (14). Incorporation of the following points will hopefully improve the efficiency of PCP biodegradation in such treatment systems. (i) To date, only a limited number of bacteria have been found to possess the capacity to mineralize PCP, and this bacterial mineralization of PCP occurs only aerobically. (ii) A significant lag, 10 or more h, occurs before PCP mineralization commences. The length of the lag period is directly proportional to the PCP concentration. Because of the extended lag time, continuous culture methods would be more suitable to PCP biodegradation than batch culture methods. (iii) Bacteria capable of mineralizing PCP are themselves susceptible to PCP toxicity. (iv) Yields are low when bacteria utilize PCP as the sole carbon source. This may necessitate the sporadic or continuous addition of PCP-adapted (log phase in PCP) cells to the treatment system. (v) Changes in pH affect PCP toxicity. In addition to the present study with bacteria, an effect of pH on PCP toxicity has been shown for fish (11) and fungi (12). Therefore, the pH should be maintained at the highest level possible commensurate with adequate bacterial growth.

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