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Journal of Hazardous Materials 39 (1994) 19–31

JOURNAL OF HAZARDOUS MATERIALS

Cometabolism of pentachlorophenol by microbial species

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Received 15 January 1994; accepted in revised form 24 March 1994

Abstract

Pentachlorophenol (PCP) cometabolism in the presence of dextrose was investigated with a pure culture *Pseudomonas cepacia* and an acclimated mixed culture developed from a local activated sludge plant. The *Ps. cepacia* cells degraded PCP up to 50 mg/l without any significant lag, but at higher PCP concentrations (> 50 mg/l and up to 100 mg/l) there was a significant lag period (up to 30 h) before the degradation started. Overall, PCP degradation was about 80% in these systems. PCP was found to be a non-competitive inhibitor of *Ps. cepacia* with an inhibition constant (K_i) of 222 mg/l. The *Ps. cepacia* cells were unable to use PCP as a sole carbon and energy source.

The mixed culture cometabolized PCP in the presence of dextrose at rates somewhat similar to that of *Ps. cepacia* up to PCP concentrations of 100 mg/l. These cells, acclimated to PCP at a particular concentration, degraded PCP up to the acclimation concentration fairly rapidly, but at PCP concentrations higher than the acclimation concentration, the degradation rates were much slower. The mixed culture cells could be progressively acclimated to using up to 100 mg/l PCP as sole carbon and energy source with degradation rates similar to those when dextrose was present as a primary metabolite. The PCP and dextrose utilization by the mixed culture was characterized by a diauxic growth pattern.

1. Introduction

Pentachlorophenol (PCP) has been used in the past as a pesticide, herbicide, antifungal agent, bactericide and molluscicide [1]. In addition, in the US it is most widely used as a wood preservative and slime controller in pulp and paper industries [2]. With its wide use, it is not surprising to find PCP in soil and ground water near wood treatment facilities. Surface soil samples near a wood treatment plant have been reported to have several thousand mg PCP/kg soil [3]. The ground water in the

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SSDI 0304-3894(94)00044-H

vicinity of such sites had PCP concentrations between 0 and 5 mg/l. In addition, a survey of ground water quality throughout the US found that 5% of the 600 samples taken at 153 locations in 43 states showed detectable levels of PCP. The average concentration of PCP in these samples was only 0.13 ppb [4]. In view of its acute toxicity to many mammals [2], the maximum contaminant level in drinking water has been set at 200 ppb [5].

Much research work has been done to evaluate the biodegradation of PCP in aqueous and soil environments. Many researchers have isolated pure cultures that are capable of utilizing PCP as sole carbon and energy source [3, 6, 7]. Some of these organisms were capable of utilizing PCP at concentrations up to 300 mg/l [8]. Also, there has been a report indicating that addition of another easily degradable carbon source can accelerate the degradation rate of PCP [9] and also reports on cometabolic degradation of PCP [10]. The study reported herein evaluated the cometabolism of PCP by a pure culture *Pseudomonas cepacia* and a mixed culture developed from a local activated sludge wastewater plant.

1.1. Literature review

Ledbetter and Foster [11] in 1959 were the first to introduce the term cooxidation to describe the process where a microorganism oxidizes a compound without using the energy derived from the oxidation for its growth. Later, a more general term, cometabolism, was coined to include other processes such as dehalogenation reaction carried out by some microorganisms. Many so-called recalcitrant compounds such as DDT, 2,4,5-T and 2,3,6-TBA have been shown to be degraded under cometabolic conditions [12]. Thus, the process of cometabolism is an important natural process by which many recalcitrant and toxic organic compounds are biodegraded and made less toxic.

PCP biodegradation studies involving cometabolism have also been conducted by several investigators. Brown et al. [10] studied the PCP degradation by a pure culture of *Flavobacterium* using a continuous flow system in the presence of cellobiose as a primary substrate. Results showed that an influent stream containing 600 mg/l PCP could be continuously and completely degraded by the cells in the presence of cellobiose. Both cellobiose and PCP were utilized simultaneously not sequentially. Topp et al. [9] reported on the effect of a readily degradable carbon source on the PCP degradation of *Flavobacterium*. In the presence of compounds like glutamate, aspartate, succinate, acetate, dextrose and cellobiose, the lag period preceding PCP removal was greatly reduced, but the PCP removal rates in terms of PCP/g cell/h were also reduced. Combination of glutamate and dextrose or glutamate and cellobiose appeared to suppress the PCP metabolism.

Moos et al. [13] acclimated a mixed bacterial culture to 20 mg/l PCP using raw sewage and dog food extract as the primary substrate. Kinetic experiments were conducted using four continuous stirred-tank reactors with solids retention times (SRT) of 3.2, 7.8, 12.8 and 18.3 d. The data suggested that an SRT of at least 7.8 d was necessary to maintain a consistent PCP degradation. A non-interactive model was developed to predict the PCP concentration in the system. The results indicated that

regardless of the reactor SRT the minimum PCP concentration attainable was 27 mg/l. Jacobsen et al. [14] studied the degradation of PCP and lindane in an activated sludge system. Synthetic sewage was added to the system as the primary substrate. PCP and lindane mixed with three other model compounds at concentrations ranging from 1 to 200 μg/l were the secondary substrates. In the reactor where synthetic sewage and PCP only were added, the degradation rate of PCP increased with increasing SRT, which indicated that the degradation was taking place by catabolic growth by a specific fraction of the biomass. The first-order biodegradation rate constant for PCP, at an SRT greater than 8 d, was 2.5×10^{-3} l/mg MLSS/d at 15 °C.

2. Materials and methods

2.1. Microorganisms

The bacteria *Pseudomonas cepacia* AC was chosen for the study. This organism was obtained from American Type Culture Collection (ATCC), Rockville, Maryland, and maintained on nutrient agar slants at 4 °C. The cells were subcultured every two months.

A mixed culture capable of degrading PCP concentration up to 100 mg/l was developed slowly (over three months) by acclimating an activated sludge sample from the wastewater treatment plant at Columbia, Missouri. The culture medium consisted of: dextrose 1 g/l, ammonium sulfate 0.5 g/l, potassium dibasic phosphate 5.8 g/l, and potassium monobasic phosphate 4.5 g/l and tap water. Table 1 provides an analysis of the tap water used. The PCP concentration in the growth medium initially was 10 mg/l which was progressively increased to 100 mg/l.

2.2. Media

Ps. cepacia cells were grown in a medium described by Kilbane et al. [15], which consisted of the following items: dextrose 4 g/l, potassium dibasic phosphate 5.8 g/l, potassium monobasic phosphate 4.5 g/l, ammonium sulfate 2 g/l, magnesium chloride 0.16 g/l, calcium chloride 20 mg/l, sodium molybdate 2 mg/l, ferrous sulfate 1 mg/l; and manganous sulfate 1 mg/l. The media was prepared in ultrapure water. Stock solutions of $CaCl_2$, $MgCl_2$ and dextrose were autoclaved separately from the rest of the salt solution. After cooling, the solutions were mixed under sterile conditions in order to avoid the formation of a precipitate.

PCP was obtained from Sigma Chemical Company, St. Louis, Missouri. The chemical was 99.3% pure. A solution of PCP was prepared in 0.05 *N* NaOH, and its pH adjusted to pH 6.8–7.0 before adding to the medium.

2.3. Analytical methods

Cell dry weights were determined by the procedures outlined in *Standard Methods* Section 208C [16]. Cell dry weights of *Ps. cepacia* were measured by observing the

Table 1
Concentrations of various elements and ions in tap water at the College of Engineering, University of Missouri, Columbia, Laboratory

Elements and ions	mg/l
Ag	< 0.01
Al	< 0.05
As	0.1
B	0.3
Ba	0.057
Be	< 0.001
Ca	59.0
Cd	< 0.01
Co	< 0.01
Cr	< 0.01
Cu	< 0.01
Fe	< 0.02
K	4.0
Li	0.04
Mg	26.0
Mn	0.006
Na	48.0
Ni	< 0.01
P	0.11
Pb	< 0.05
Se	< 0.01
Si	5.2
Sn	0.08
Sr	0.75
Ti	< 0.01
V	< 0.01
Zn	0.45
Cl^-	—
SO_4^{-2}	30.0
Alkalinity, as $CaCO_3$	225.0
Total dissolved solids	326.0
pH (no units)	8.0

optical density (OD) of the aqueous suspensions at 540 nm. The readings were converted to dry weight units using a calibration curve developed for *Ps. cepacia* cells.

Dextrose analysis was performed using a high-performance liquid chromatograph (HPLC) using a refractive index detector [17]. Some samples were analyzed by an enzymatic method also using a glucose oxidase/peroxidase kit obtained from Sigma Chemical, St. Louis, Missouri.

PCP analyses were performed by three methods. These methods were gas chromatography (GC), liquid chromatography (HPLC) and spectrophotometry. For most on-spot PCP analyses, the spectrophotometry method reported by Edgehill and Finn [18] was used. The GC analysis (EPA method 604) was used occasionally to

verify the PCP concentrations determined by the spectrophotometry. For several aqueous phase samples, PCP analyses were done by HPLC method. The HPLC procedure involved the chromatographic separation of PCP on a C-18 reverse-phase column (Supelco 25 cm × 4.6 mm i.d.). The mobile phase consisted of acetonitrile containing 1% v/v glacial acetic acid and acetonitrile/water (50/50) containing 1% v/v glacial acetic acid. The absorption peaks were determined using a Spectraflow 783 programmable UV detector at 254 nm. There was good agreement between the three methods for PCP determination.

Chloride ion was measured by a chloride ion electrode connected to an Orion pH/ISE meter.

3. Results and discussions

3.1. PCP degradation by Ps. cepacia

In the experiments conducted, dextrose was used as a primary metabolite, while PCP was the cometabolite. The growth of *Ps. cepacia* in the presence of dextrose and PCP was evaluated. The PCP levels were varied from 0 to 100 mg/l, and the dextrose concentration was kept at about 4 g/l. Fig. 1 shows the growth pattern of the cells on dextrose in the presence of 50 mg/l PCP. It can be seen that *Ps. cepacia* growth occurred with virtually no lag. The cells reached a stationary phase at about 22 h at which time about 80% of the influent PCP was degraded. The growth pattern at 100 mg/l PCP was different as indicated in Fig. 2. There was a considerable lag in the growth rate, and the stationary phase was reached after 50 h. The PCP degradation did not start until cell growth occurred. In both cases, at the end of the growth phase some 10–20 mg/l PCP remained unmetabolized in the liquid phase. Others have observed similar results during PCP degradation where it was a sole carbon and energy source for the organism [19]. Radehaus and Schmidt [19] reported the biodegradation of PCP at 40 mg/l as sole carbon and energy source by a *Pseudomonas* sp. strain RA2 with no lag and attainment of stationary phase in about 48–50 h. With increased PCP concentration to 150 mg/l, there was a marked lag period of more than 48 h before the cells started to biodegrade the molecule. They reported that the higher the concentration of PCP, the longer the lag phase. This *Pseudomonas* strain was one of the few that can use PCP as a sole carbon and energy source. In their system there was also some residual PCP left after the end of the cell growth phase. Stanlake and Finn [8] reported the isolation of a strain NC capable of degrading PCP which had characteristics similar to the genus *Arthobacter*. The lag phase for this organism also increased as PCP concentration increased.

It should be noted that the cometabolic degradation of PCP (as reported herein) was somewhat faster compared to its utilization as sole carbon and energy source [19]. Thus, there may be some kinetic advantage to the cells degrading PCP in the presence of an easily degradable carbon source (dextrose).

The specific growth rates for *Ps. cepacia* cells were estimated at different PCP levels from the data using semi-logarithmic plots. These values are shown in Table 2. The

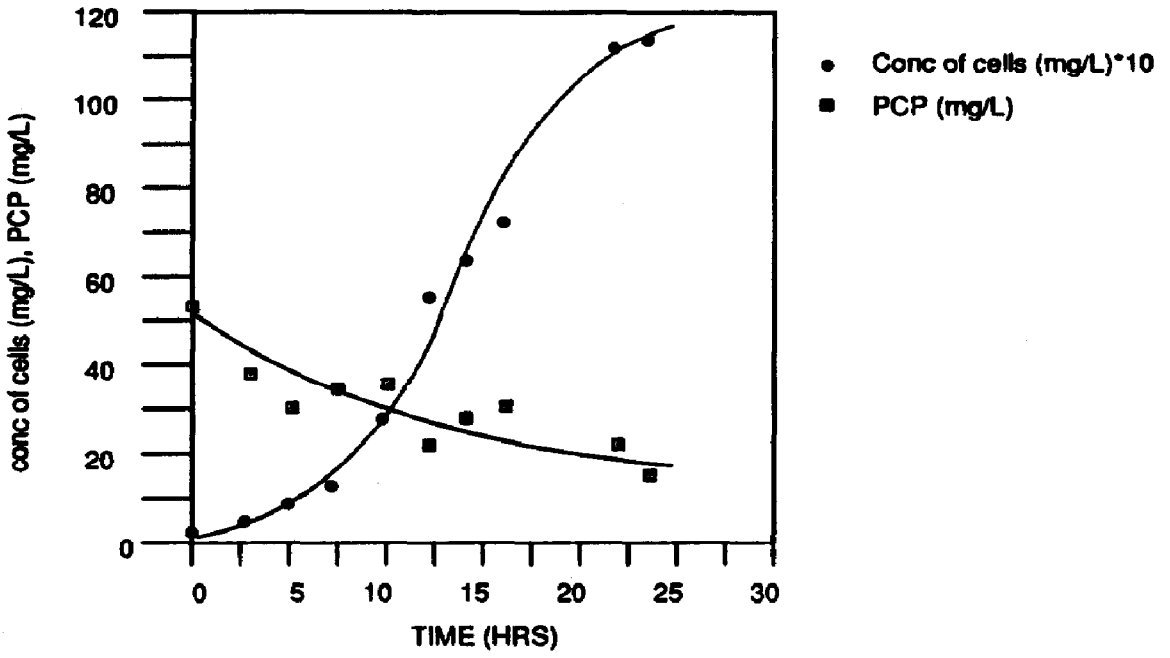

Fig. 1. Kinetics of growth of *Ps. cepacia* on dextrose in the presence of 50 mg/l PCP.

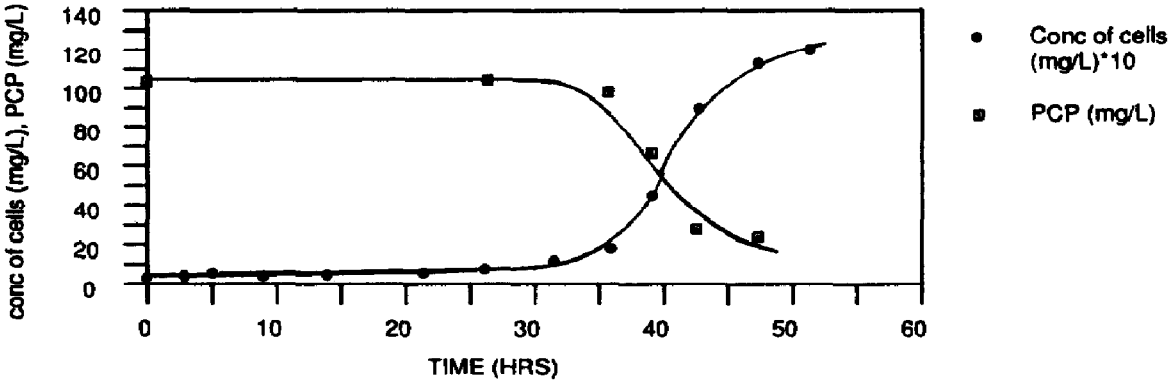

Fig. 2. Kinetics of growth of *Ps. cepacia* on dextrose in the presence of 100 mg/l PCP.

specific growth rates declined as the PCP concentration increased, which was caused by the co-metabolite inhibition. Using a non-competitive inhibition model for the growth of the organism in the presence of PCP, the inhibition constant K_i can be calculated from a linear plot of Eq. (1) (Fig. 3):

$$\frac{1}{\mu} = \frac{1}{\mu_m} + \frac{p}{\mu_m K_i}$$

where μ is the specific growth rate, h^{-1}; μ_m is the maximum specific growth rate, h^{-1}; p is the PCP concentration, mg/l; and K_i is the substrate inhibition constant, mg/l.

Table 2
Specific growth rates of *Ps. cepacia* at different PCP levels

Initial PCP concentration (mg/l)	Specific growth rates (h^{-1})
0	0.36
20	0.33
50	0.25
100	0.25

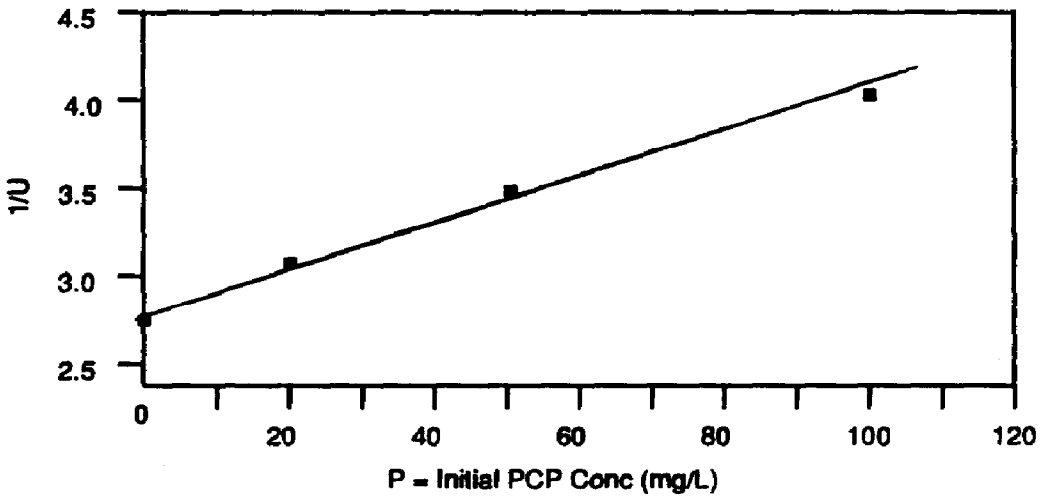

Fig. 3. Effect of PCP concentrations on specific growth rates of *Ps. cepacia* cells with dextrose as the primary substrate.

The inhibition constant K_i was found to be 222 mg/l. Topp et al. [9] reported a value of K_i of 86.8 mg/l for *Flavobacterium* grown on sodium glutamate in the presence of 70–150 mg/l of PCP. The difference in the K_i values could be due to different tolerance levels of these organisms to PCP.

Efforts to use PCP as a sole carbon source with *Ps. cepacia* cells were not successful. The cells were initially grown on dextrose (4 g/l) and PCP (50 mg/l). The cells were harvested at their late log growth phase and inoculated into flasks containing salts and PCP at three concentrations of 100, 200 and 300 mg/l, respectively. The control flask did not contain any PCP. There was no growth or loss of any PCP in 350 h. Karns et al. [20] had also observed similar results with this organism during an experiment lasting 66 h. It was felt that 66 h may not have been enough acclimation time; hence, the prolonged incubation period of 350 h was used in the experiment.

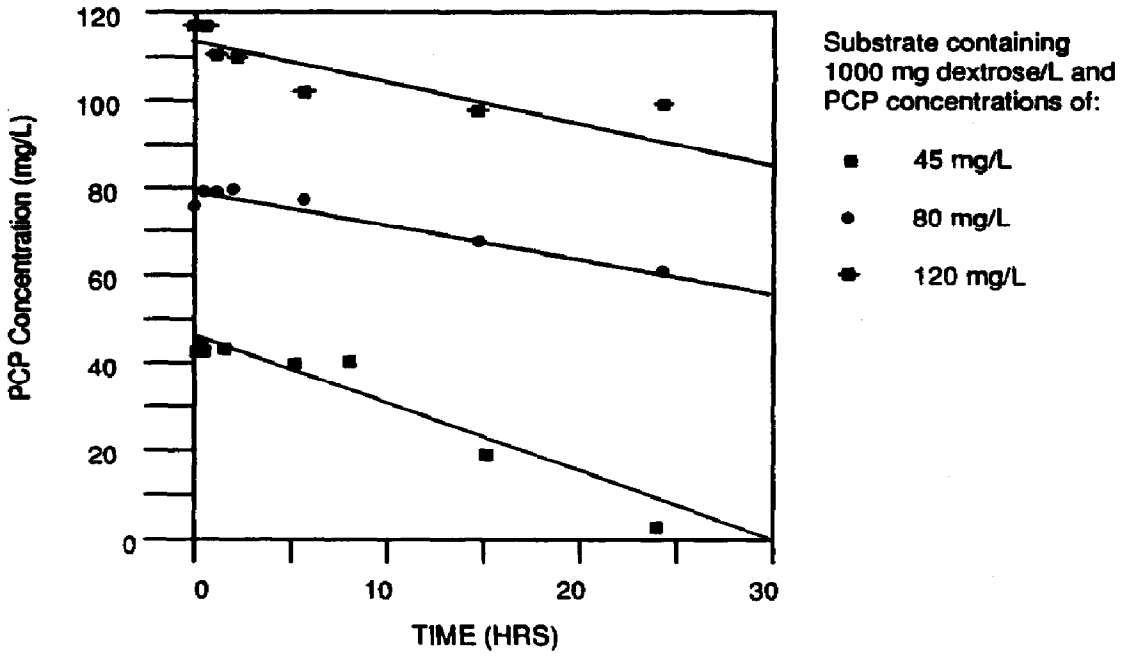

Fig. 4. PCP removal by the mixed culture acclimated to 60 mg/l PCP with dextrose as the primary substrate.

3.2. PCP degradation with mixed culture

The mixed cultures were progressively acclimated to two PCP concentrations, 60 and 100 mg/l with dextrose at 1000 mg/l as the carbon and energy source. Experiments were conducted to evaluate the behavior of these acclimated cells at PCP above and below the acclimated concentrations. Fig. 4 shows the PCP removal kinetics of cells acclimated at 60 mg/l PCP, with 45, 80 and 120 mg/l PCP concentrations. The PCP removals for systems having the initial PCP concentrations greater than the acclimated value were quite slow. When the PCP concentration was below the acclimated value, the PCP removal rate was rapid, achieving complete degradation ($>90\%$) within 30 h. The mixed liquor suspended solids increases are shown in Fig. 5. The presence of higher PCP concentration (120 mg/l) than the acclimated value (60 mg/l) caused growth inhibition and delayed the arrival of the stationary phase. Similar results were observed with the system acclimated to 100 mg/l PCP. The presence of PCP in these systems had no significant impact on the dextrose removal rate of these cells.

Experiments were conducted to determine if the PCP degrading mixed cultures grown on dextrose as the carbon and energy source could be acclimated slowly so that instead of dextrose, PCP could be used as the sole carbon and energy source. In these experiments in the stock cultures, the dextrose feed was reduced progressively from 1000 mg/l in stages to 0 mg/l, while the PCP concentration was kept at 100 mg/l. Fig. 6 shows the PCP removal pattern at different dextrose levels. In the presence of

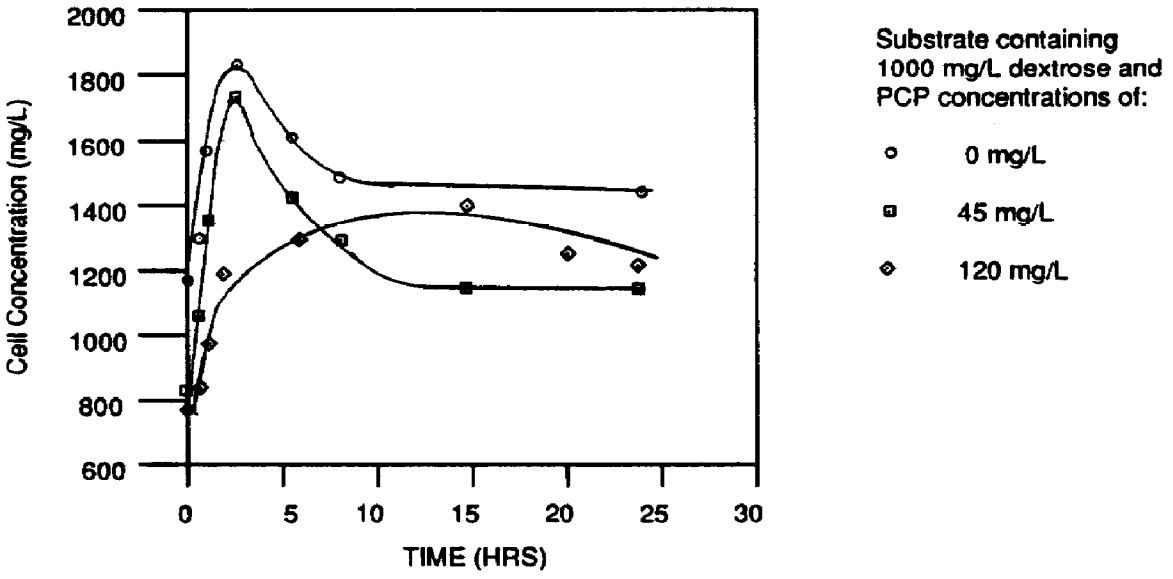

Fig. 5. Growth rates of the mixed culture acclimated to 60 mg/l PCP with dextrose as the primary substrate.

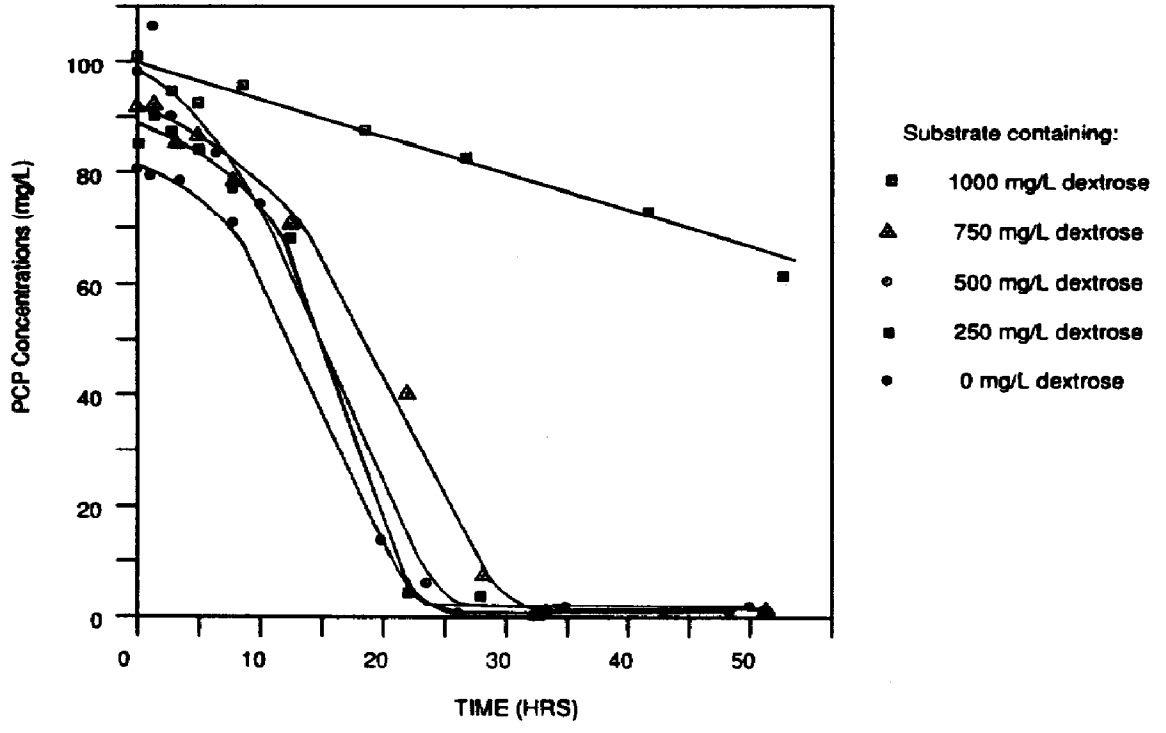

Fig. 6. PCP removal pattern by the mixed culture with decreasing dextrose concentrations.

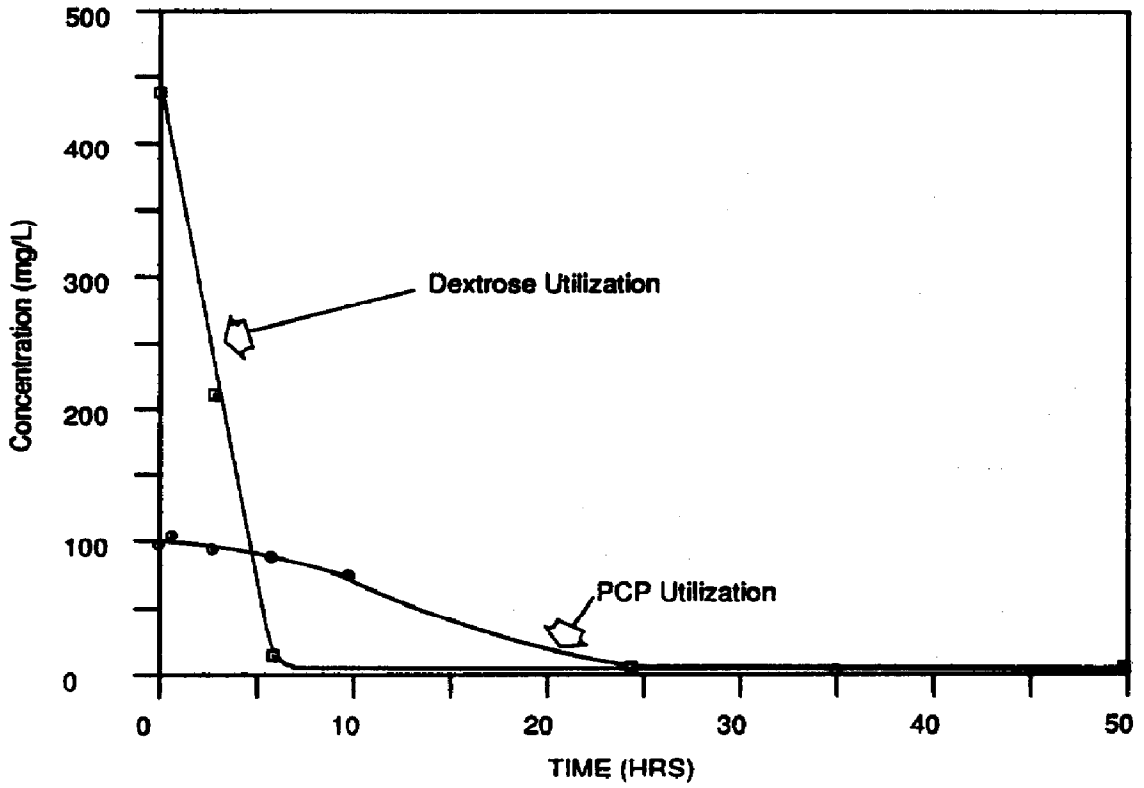

Fig. 7. Substrate utilization behavior of the mixed culture with 100 mg/l PCP and 500 mg/l dextrose.

1000 mg/l of dextrose, about 40% PCP was degraded in 52 h. When the dextrose concentration was reduced to 500 mg/l, greater than 95% PCP degradation occurred in about 24 h. From these results, it seems that dextrose concentrations greater than 500 mg/l hindered the PCP removal behavior by these cells. Liu et al. [21] also found that glucose at 500 mg/l suppressed the rate of PCP degradation by a mixed culture. Rozich and Colvin [22] observed similar results when phenol degradation by heterogeneous culture was reduced by the presence of glucose. Metabolism of PCP as a sole carbon source was possible by this mixed culture and the PCP removal rate was comparable to the cometabolic systems with dextrose. Research performed by other investigators on pure culture bacteria [18, 23] obtained similar biodegradation kinetics when PCP was used as a sole carbon source.

Fig. 7 shows the removal rates of the primary (dextrose) and secondary (PCP) substrate by the mixed culture cells. There was a rapid initial utilization of dextrose with a slower concomitant PCP removal. Rozich and Colvin [22] observed a similar pattern with phenol degradation in the presence of glucose. It appears from the figure that the PCP and dextrose were being metabolized concurrently to some extent, but it was more likely that diauxic utilization was occurring; i.e., PCP was not removed much while the primary substrate was present, but once the primary substrate was removed, PCP degradation took place. In this case, perhaps PCP was not cometabolized but metabolized in a diauxic fashion.

Comparing the results of PCP degradation by cometabolism by pure culture *Ps. cepacia* and the acclimated mixed culture, it can be seen (Figs. 1 and 4) that the kinetics of removal of PCP at about 50 mg/l for both the systems were quite similar. However, the mixed culture could slowly be acclimated to use PCP as sole carbon and energy source up to a concentration of 100 mg/l PCP, whereas the pure culture was unable to do so.

4. Conclusions

1. PCP was cometabolized up to a concentration of 100 mg/l by a pure culture *Ps. cepacia* in the presence of dextrose as the primary substrate. At concentrations of PCP below 50 mg/l, there was practically no lag in the growth of the cells, but at higher concentrations (up to 100 mg/l PCP) significant lag occurred (> 30 h) in the cell growth and PCP degradation. In all the systems, some residual PCP (10–20 mg/l) remained after the cell growth had ceased.
2. PCP was found to be a non-competitive inhibitor of the growth of *Ps. cepacia* cells with an inhibition constant (K_i) of 222 mg/l.
3. *Ps. cepacia* cells were unable to use PCP as sole carbon and energy source.
4. A mixed culture developed from a local activated sludge plant cometabolized PCP in the presence of dextrose at rates somewhat similar to the PCP degradation rate of *Ps. cepacia* cells up to PCP concentrations of 100 mg/l.
5. Mixed culture cells acclimated to PCP at a particular concentration (in the presence of 1 g/l dextrose) degraded PCP up to the acclimation concentration fairly rapidly, but at PCP concentrations higher than the acclimation concentration, the degradation rate was much slower.
6. Mixed cultures could be progressively acclimated to using up to 100 mg/l PCP as sole carbon and energy source with no dextrose addition. The degradation rate of PCP in these systems was as rapid as when dextrose was present as a primary metabolite.
7. PCP and dextrose utilization by the mixed culture was characterized by a diauxic growth pattern. The readily degradable carbon was metabolized rapidly initially followed by a slower degradation of PCP.

Acknowledgments

The research reported herein was funded in part by the USEPA under assistance agreement R-815709 to the University of Missouri through the Hazardous Substances Research Center for the USEPA regions 7 and 8, headquartered at Kansas State University. It has not been subjected to the Agency's Peer and Administrative Review and, therefore, may not necessarily reflect the views of the Agency and no official endorsement should be inferred.

The authors acknowledge the assistance of graduate students R.V. Rayavarapu and D.W. Currence of the University of Missouri-Columbia who conducted the experimental work reported.

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