# BIODEGRADATION OF PCP-CONTAINING WASTEWATER BY FREE AND IMMOBILIZED CELLS

By

Chun-Mei Zhu

B. Sc. Wuhan University, 1982

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

DEPARTMENT OF BIO-RESOURCES ENGINEERING

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September, 1995

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Bio-Resources Engineering

The University of British Columbia
Vancouver, Canada

Date October 11, 1995

## Abstract

Free and immobilized *flavobacterium sp.* were tested and confirmed for their degradability of synthetic PCP-containing wastewater. Laboratory scale batch and continuous reactors were developed for the research. Free *flavobacterium sp.* in batch reactors could completely degrade PCP into non-toxic chemicals at PCP concentrations of 30, and 50 ppm. Only partial degradation was found at PCP concentrations higher than 65 ppm. Activated sludge showed its incapability of PCP degradation, but activated sludge mixed with free *flavobacterium sp.* in the batch reactors showed the same degradation capability as the ones with only free *flavobacterium sp.* Free *flavobacterium sp.* had limitations for PCP degradation. *Flavobacterium sp.* immobilized in alginate were tested in the continuous reactors and indicated their ability to degrade 65 ppm PCP efficiently. The levels of PCP degradation by immobilized *flavobacterium sp.* cells decreased as the influent PCP loading rate increased, or as the hydraulic retention time (HRT) decreased. Immobilized *flavobacterium sp.* can tolerate higher concentrations of PCP than free ones. The research results indicate the possibility of scale-up and design of reactors for treating PCP-containing wastewater by using immobilized *flavobacterium sp.* cells.

## Table of Contents

## List of Tables

## List of Figures

**Acknowledgement**

I could never have completed this without the guidance and support of my committee: Dr. K.V. Lo, Dr. R.M.R. Branion, and Dr. A. Lau. My sincere thanks for all of the help they gave me. I would also like to thank other BioE members: Dr. S. T. Chieng for his spiritual support; Dr. P. Liao for his help in the laboratory procedures; and fellow graduate student, Mr. G. Wu, for sharing his vast computer experience.

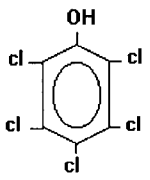
I also wish to thank my husband, X. Liu; my mother-in-law, S. Cheng, and my daughter, Y. Liu, for their effort and support given to me during the study period.

# Chapter 1

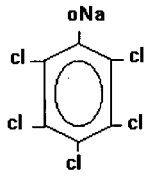
## Introduction

The principle purpose of this research is to study the possibility of degrading pentachlorophenol by bacteria under aerobic conditions. Pentachlorophenol, also called penta or PCP, is a phenolic compound carrying five atoms of chlorine. A schematic molecular diagram of PCP and its sodium salt is shown in Figure 1.1. PCP and salt (Na-PCP) have antimicrobial, antifungal, herbicidal, insecticidal and molluscidal properties, which have lead to its widespread application. However they are mainly used for the preservation and treatment of wood.

The large amounts of PCP used for industrial and agriculture applications have brought a significant introduction of PCP into the environment, including soil, surface water, groundwater, and living organisms. Depending on the soil type, PCP can be very mobile, potentially leading to contamination of groundwater and hence, of drinking-water. Because application in agriculture has been reduced, soil contamination will, for the most part, be confined to those areas where treatment PCP is applied. PCP concentrations in surface water are usually in the range of 0.1 – 1 $\mu$g/litre, with maximum values of up to 11 $\mu$g/litre, though much higher levels can be found near point sources or after accidental spills. A study concerning contamination of the Fraser River estuary in British Columbia by chlorophenols has shown that the North Arm of the estuary contained several chlorophenols including 2,4-dichlorophenol, 2,4,6-trichlorophenol, 2,3,4,5-tetrachlorophenol, and pentachlorophenol (Carey and Hart, 1988). The concentration of PCP increased significantly along the North Arm near lumber mills using fungicides, mainly based on PCP compounds, for surface treatment against sapstain.




Pentachlorophenol
PCP




Sodium Pentachlorophenol
Na-PCP

Figure 1.1: Schematic Molecular Diagram of PCP and Na-PCP

PCP is an uncoupler of oxidative phosphorylation and thus is lethal to a widely variety of plants and animals, and is highly toxic for aquatic organisms. As little as 1 $\mu$g PCP/litre can have adverse effects on very sensitive algal species, and low concentrations ($\mu$g/litre) may lead to substantial alterations in the community structures of an ecosystem. Moreover, PCP appears to accumulate in the food chain, and is considered to be comutagenic (Rao, 1978).

PCP has been identified as a chemical of great concern in Canada and U.S.A. because of its widespread use, its toxic properties and its potential release to the environment. The regulatory status of pentachlorophenol is under review in Canada (Canada, 1989). Background and regulatory options are presented in a Discussion Document released by Agriculture Canada (1987). How to treat the wastes arising from PCP-using sites has been intensely studied for the past two decades.

Basically, physical and chemical treatments for PCP removal are very effective in treating PCP-containing waste. Adsorption of PCP by activated carbon used as a final cleanup step was

found to remove 100% PCP (Richardson, 1978). Chemical oxidation techniques such as ozonation and hydrogen peroxide addition in the presence of UV light are in the experimental stage. Incineration has also been used to dispose of PCP wastes. A controlled air incinerator destroyed greater than 99.99% of PCP in treated wood at combustion temperatures of between 910 and 1025°C, and yielded no measurable TCDD or TCDF in the offgas (Stretz, 1984). However, the cost of chemical treatment and incineration is relatively high. Adsorption treatments can not destroy PCP but merely transfer it to another medium from which it must be disposed of. The degree of airborne contamination resulting from incineration processes has not been fully quantified.

Biological degradation of PCP probably could be a cost effective solution to treat contaminated waters. Several laboratory and treatment plant studies have shown that PCP can be degraded by activated sludge treatments (Dustand and Thompson, 1973; Kirsch and Etzel, 1973; Etzel and Kirsch, 1974; Hickman and Novak, 1984; Berard and Tseng, 1986). However, such treatments are often subject to sudden loading and may not be efficient with all types of PCP-containing wastewater. The US EPA surveyed 14 municipal treatment plants and found that 8 did not remove any of the PCP load, while the remainder were considered to remove PCP (6-87%) primarily by adsorption onto solids (Hickmn and Novak, 1984).

PCP is believed to be resistant to biodegradation due to its high chlorine content and acute toxicity. However, some organisms have been found to be able to degrade the PCP molecule completely to carbon dioxide and chloride. Among these are bacteria and fungi, in both pure and mixed cultures (Chu and Kirsch, 1972; Cserjesi, 1967; Cserjesi and Johnson, 1972; Ide *et. al.*. 1972; Kirsch and Etsel, 1973; Suzuki, 1977; Suzuki and Nose, 1971). Most of these works have concentrated on the study of purification, isolation and kinetics of microorganisms which can degrade PCP, but little information exists on the development of biological treatment systems. Numerous, isolated strains of *Flavobacteria* have been said to be most efficient in degrading

PCP at substantially higher concentrations than other microorganisms.

The overall objective of this study is to investigate the capability for biological degradation of toxic PCP by *Flavobacteria* cells under aerobic conditions. Specific objectives are to study the capability of free *Flavobacteria* cells for degrading PCP in fed-batch reactors; and to study the capability of immobilized *Flavobacteria* cells for treating synthetic PCP-containing wastewater in continuous reactors.

The research study consisted of 2 stages. In the first stage, fed-batch reactors were set up to find out suitable conditions for PCP degradation by *Flavobacteria* species. Changes in PCP concentration, pH, Cl concentration and turbidity in the supernatant were monitored. In the second stage, bench-scale continuous reactors, which were fed with *Flavobacteria* species immobilized in alginate, were developed to treat a synthetic wastewater. The removal efficiencies of PCP by these immobilized *Flavobacteria* species were measured.

# Chapter 2

# Literature Review

## 2.1 Introduction To PCP

### 2.1.1 The History Of PCP Application

PCP was first introduced for use as a wood preservative in 1936. Because of their effectiveness against a wide spectrum of target organisms and their low cost, PCP and Na-PCP have since been used as herbicides on ornamental lawns, golf courses, aquatic areas, and rights-of-way; or for control of subterranean termites, as anti-microbial agents in cooling towers, adhesives, latex paints, paper coating, cements used with food can ends and seals, coatings in reusable bulk food storage containers, photographic solutions, leather tanneries, pulp and paper mills, and as disinfectants.

PCP is mainly produced by the stepwise chlorinating of phenols in the presence of catalysts. Basically, chlorinating of phenol occurs in two stages. In stage one, chlorine is bubbled through phenol at 105°F to yield tri- and tetrachlorophenols. In stage two, the temperature is gradually increased to 130°F to keep the reaction mixture molten and to further chlorinate the tri- and tetrachlorophenols to form pentachlorophenol. The process, however is incomplete. Technical grade PCP contains from 4 to 12 percent tetrachlorophenols, which are toxic in their own right. In addition, the high temperatures used in manufacturing PCP produce several contaminants including hexachlorobenzene, dioxins, and furans. Na-PCP is produced by dissolving PCP flakes in sodium hydroxide solution (World Health Organization, 1987).

World production of PCP is estimated to be of the order of 30,000 tonnes per year. In the USA, approximately 20,000 tonnes of PCP is produced annually, about 80% of this PCP is used for commercial wood treatment, 6% is in use for slime control in pulp an paper production, and 3% accounts for non-industrial purposes, such as weed control, fence-post treatment and paint preservation (Crosby *et al.*, 1981). Because of the toxicology of PCP, the U.S. Environmental Protection Agency (EPA) canceled all uses of PCP except for its use as a wood preservative in the USA in 1988. According to the most current year for which statistics are available, 10,000 tonnes of PCP were used as wood preservatives in United States in 1988 (Fisher, 1991).

PCP had been produced in Canada, with an estimated production of 1,300 tonnes annually before 1982. However, domestic production ceased as of July 1983. Since then Canada has imported large quantities of PCP primarily from the USA and Europe. In 1985, sales of PCP and Na-PCP registered under the Pest Control Products Act were 2155 tonnes (Health and Welfare Canada, 1989), mainly used for wood preservation. Other applications of Na-PCP in Canada are to inhibit algae and fungal growth in boiler waters, and in cooling water at electrical plants.

### 2.1.2 Physical and Chemical Properties Of PCP

PCP and its salt, Na-PCP, are the most important forms of pentachlorophenol in terms of production and use. Pure PCP consists of light tan to white, needlelike crystals, which have a pungent odor when heated. It is soluble in most organic solvents, but practically insoluble in water. However, its salt Na-PCP, is readily soluble in water. At the approximately neutral pH of most natural water, Na-PCP is more than 99% ionized. General information and properties of PCP and Na-PCP are included in Table 2.1.

PCP may exist in two forms: the anionic phenolate, at neutral to alkaline pH, and the undissociated phenol at acidic pH. At pH 2.7, PCP is only 1% ionized; at pH 6.7, it is 99% ionized.

Table 2.1: Physical and Chemical Properties Of PCP And Na-PCP

| Properties | PCP | Na-PCP |
|---|---|---|
| Molecular formula | $C_6HCl_5O$ | $C_6Cl_5ONa$ |
| Molecular weight | 266.34 | 288.3 |
| Physical state | Dark colored flakes | cream-colored beads |
| Boiling point | 309 - 310°C | |
| Melting point | 1191°C (anhydrous) | |
| Density (g/ml) | 1.987 | 2 |
| Vapor pressure | 0.00011 mm Hg at 20°C | |
| Specific gravity | 1.978 at 22°C | |
| Water solubility | 14 mg/L in water at 20°C | > 200 in water at 20°C |
| Odor threshold | 1600 $\mu$g/L | |
| Taste threshold | 30 $\mu$g/L | |

PCP is non-inflammable and non-corrosive in its unmixed state, whereas a solution in oil causes deterioration of rubber (Mercier, 1981). Because of the electron withdrawal by the ring chlorides, PCP behaves as an acid, yielding water-soluble salts such as sodium pentachlorophenol (Na-PCP). Na-PCP is non-volatile, its sharp PCP odor results from slightly hydrolysis (Crosby *et al.*. 1981).

### 2.1.3 Environmental Fate Of PCP

#### Environmental Contamination by PCP

Algae, bacteria, fungi, insects, and marine borers are the major biological agents for wood degradation. In order to be effective against these organisms over a long period of time, wood preservatives, like PCP, must be persistent pesticides. This means that the potential of PCP to cause environmental damage is very high.

PCP is a significant contaminant of soil, surface water, and groundwater especially around sawmills and wood preserving facilities. Preservative material containing PCP may be transported into streams and lakes by soil runoff or by direct discharge of contaminated effluents into waterways. Generally, municipal sewage discharges contain PCP concentrations at levels comparable with those in surface waters. However, wood-treating factories may contribute substantially to the PCP load on surfaces water. The PCP levels of up to 10,500 $\mu$g/L reported by Fountaine *et al.* (1976) were found in a highly polluted stream near an industrial area in the vicinity of Philadelphia, USA.

In general, the sediments of a water body contain much higher levels of PCP than the overlying waters. Leaching is an important means of transport for PCP in some instances. Substantial quantities of PCP may be found in waters leaching from contaminated sites. Thompson *et al.* (1978) found that 2.05 and 3.35 mg PCP/litre were detected in groundwater from a wood preservation plant near Lake Superior, and PCP in the mg/litre range was detected in water seeping from a landfill (Kotzias *et al.*, 1975).

## PCP Impact On Aquatic Systems

Evaporation of PCP from aquatic systems is most likely minimal. Kloppfer *et al.* (1982) determined a half-residence time for PCP in a laboratory system of 3120 hours at a pH of 6.0, and detected no losses at all at pH 8.0. PCP is highly toxic to fish, with a mean 96 h $LC_{50}$ value to salmonids of 85 $\mu$g/L. Sublethal PCP concentrations in the range of 2 to 34 $\mu$g/L not only inhibit the feeding and growth of salmonids, but also reduce the embryonic survival and egg hatchability of the fish. PCP lethality to various fish is shown in Table 2.2.

The toxicity of PCP to fish changes as a function of environmental pH and temperature, which is shown in Table 2.3. We can see from Table 2.3 that an increase in pH from 4 to 8 is

Table 2.2: PCP Lethality To Various Fish

| **Fish** | **96-hr $LC_{50}$($\mu$g/L)** |
|---|---|
| Salmonids | 85 |
| Juvenile rainbow trout (*Oncorhynchus mykiss*) | 115 |
| Chinook salmon (*Oncorhynchus tschawytscha*) | 68 |
| Lake trout (*Salmo trutta*) | 54 |
| Juvenile atlantic salmon | 150 |

Table 2.3: The Toxicity of PCP as a Function of pH and Temperature

| **pH** | **T°C** | **$EC_{50}$($\mu$g/L)** |
|---|---|---|
| 4 | 15 | 526 |
| | 25 | 384 |
| | 35 | 253 |
| 6 | 15 | 782 |
| | 25 | 465 |
| | 35 | 415 |
| 8 | 15 | 2,046 |
| | 25 | 2,052 |
| | 35 | 1,263 |

associated with a dramatic decrease in toxicity. This change in toxicity is attributable to ionization of PCP as pH goes up. At higher pH the dissociated species of PCP is less lipophilic than the unionized form and it is less likely to bioaccumulate. As a result, toxicity is reduced as pH goes up. As temperature rises from 15°C to 35°C, the toxicity of PCP increases significantly at all 3 pH levels.

### PCP Impact On Human Beings

The International Agency for Research on Cancer has classified PCP as a Group 3 chemical for carcinogens. EPA classified it as a Class B2 carcinogen based on the 1988 National Toxicology Program study (Fisher, 1991).

Exposure of the general population to low levels of PCP is common. PCP has been found in food and drinking-water at concentrations ranging from 1 to 50 $\mu$g/L, and was also detected in domestic well water (Wong and Crosby, 1981). PCP levels in Florida drinking-water supplies ranged from 0.003 to 0.34 $\mu$g/L (Morgade *et al.*, 1980).

The acute toxicity of PCP on human is believed to derive from its ability to interfere with the production of energy at the cellular level. A person experiencing systemic poisoning by PCP would show symptoms of profuse sweating and intense thirst, rapid heart rate, fever, abdominal pain, weakness, dizziness, anorexia, and nausea.

## 2.2 Biological Degradation of PCP

PCP is believed to be somewhat resistant to biodegradation due to the high chlorine content of the molecule. However, some bacteria and fungi, in both pure and mixed cultures, under both aerobic and anaerobic conditions have been found to be able to degrade the PCP molecule to nontoxic chemicals: carbon dioxide and chloride. Aerobic treatment was used in my study, thus only information of PCP degradation by microorganism under aerobic condition is referred to in this thesis.

Numerous reports have appeared in the literature describing the microbial degradation of PCP in soil (Mueller *et al.*, 1991; Seech *et al.*, 1991) and water (Chu and Kirsch, 1972; Moos *et*

*al.*, 1983; Radehaus *et al.*, 1992; Mileski *et al.*, 1988; Saber and Crawford, 1985; Topp and Hanson, 1990) and in systems simulating aerobic wastewater treatment (Etzel and Kirsch, 1985). Moreover those microorganisms isolated from variety of sources have been shown to be able to use PCP as their sole carbon source. These results suggest that PCP-degrading bacteria are widely distributed in the environment. On the whole, most former research focused on purification, isolation and kinetic studies of microorganisms that could degrade PCP (Brown *et al.*, 1986; Chu and Kirsch, 1972; Edgehill and Finn, 1982; Saber and Crawford, 1985; Suzuki, 1977; Topp and Hanson, 1990).

### 2.2.1 Degradation of PCP in Pure Culture

Attempts to isolate and characterize microorganisms which can metabolize PCP as a sole source of organic carbon and energy were made by Chu *et al.* (1972), Edgehill *et al.* (1982), Lin *et al.* (1990), Klecka *et al.* (1985), Kirsch et al (1973), Suzuki (1977), Topp (1990), Haggblom *et al.* (1988), Lamar *et al.*(1990) and Radehaus *et al.* (1992). Not very many microorganisms can degrade and detoxify PCP to the nontoxic form as $CO_2$ or chloride. Those microorganisms which can biodegrade PCP aerobically include *Arthrobacter, Phanerochaete, Pseudomonas, Rhodococcus, Mycobacterium, and Flavobacterium* species.

#### *Arthrobacter*

The isolation, characterization and growth kinetics of an *Arthrobacter* strain metabolizing PCP were studied through batch culture and in a chemostat by Edgehill and Finn (1982). *Arthrobacter*, designated strain ATCC 33790, is a soil bacterium capable of utilizing PCP as a sole source of carbon and energy. The batch culture data showed that the growth rate of *Arthrobacter* on PCP increased rapidly at low concentrations and was apparently constant between about 10 and

135 mg/L. The maximum specific growth rate recorded in batch culture was 0.154 $h^{-1}$. Inhibition of the growth rate was observed at substrate concentrations greater than about 135 mg/L.

The effect of pH on the growth rate of the *Arthrobacter* sp. was studied in batch culture. The maximum specific growth rate could be reached over a pH range of 7.0 to 7.5, above pH 7.5 and below pH 6.4 the growth rates fell sharply. The monitoring of chloride ion in the effluent indicated that nearly 100% of the calculated amount of chlorine in PCP was released into the effluent as chloride. This result suggested complete biodegradation of PCP by the *Arthrobacter* strain.

On the presumption that toxicity of PCP is related only to the acid (undissociated) form of PCP, a series of *Arthrobacter* growth experiments was conducted at various pH levels and PCP concentrations. (Stanlake and Finn, 1982). The results suggested that concentration of the free acid form of PCP did correlate with the toxic effect. Changes in PCP concentration affected growth rate and length of the lag phase but not cell yield. No effect of pH between 6.8 to 7.8 on *Arthrobacter* cell yield at a PCP concentration of 130 ppm was observed, but increasing the pH from 6.8 to 7.8 decreased the length of the lag phase for growth of *Arthrobacter* on PCP. Decreasing pH exerted an influence on the duration of the lag phase comparable with the observed for increasing PCP concentrations.

Chu and Kirsch(1972) also isolated the PCP-degrading microorganism named KC-3, which later was identified to be an *Arthrobacter* species. KC-3 could utilize PCP as its sole carbon source and completely mineralized the PCP. In their studies, the substrate used was $^{14}C$ labeled PCP. The utilization of PCP as a carbon and energy source for KC-3 was demonstrated by analyzing the $CO_2$ liberated. The radioactive carbon dioxide release accounted for nearly 80% and the chloride release accounted for 97% of PCP added.

Chu and Kirsch (1973) then used KC-3 culture to investigate its degradation feasibility for other halogenated phenols. Various chlorophenols, such as PCP, 2,3,4,6-tetrachloro-phenol,

2,3,5,6-tetrachloro-phenol, 2,3,6-trichloro-phenol, 2,4-dichloro-phenol, 2,6-dichloro-phenol, were used as sole organic carbon sources for KC-3. Results indicated that all selected chlorophenols including PCP appeared to be readily degraded by the KC-3 microorganism with the release of high levels of chloride, and the quantitative disappearance of substrate. PCP metabolism was shown to be highly responsive to enzyme induction with PCP as the inducer. Partial induction of the PCP-degrading system occurred when 2,4,6-trichlorophenol was employed as an inducer.

### *Pseudomonas*

*Pseudomonas* species are PCP-mineralizing bacteria, normally isolated from PCP polluted soil. Watanabe (1973) examined PCP degradation in soil perfused with 40 ppm of PCP and observed the typical soil enrichment type phenomena. After an eight day lag period during which essentially no degradation occurred, chloride ion liberation was initiated, and was essentially complete within three weeks. Subsequent additions of PCP were degraded more rapidly with no lag period. A species of *Pseudomonas* was subsequently isolated which was capable of utilizing PCP as sole source of carbon with complete liberation of the chloride ion. The dechlorination process corresponded approximately with PCP disappearance. The effect of medium composition on PCP degradation by *Pseudomonas* species was examined. Yeast extracts accelerated degradation, whereas glucose at 100 ppm suppressed degradation. The substitution of ammonium sulfate for sodium nitrate as a nitrogen source also suppressed degradation. PCP degradation and microbial growth at 40 ppm were greater than at 100 ppm. Neither degradation nor growth occurred at 200 ppm PCP. Pentachloroanisol and the dimethylether of tetrachlorohydroquinone were identified as PCP degradation products.

Suzuki (1977) isolated *Pseudomonas* species from a soil collected from a field in Japan, by using an enrichment culture technique with PCP. *Pseudomonas* cell suspensions, mixed with

PCP-$^{14}$C and mineral salt medium, were then incubated. The bacteria showed very rapid metabolization of PCP-$^{14}$C releasing $^{14}CO_2$ which corresponded to 46.8% of the added PCP-$^{14}$C in 60 hours. The release of $^{14}CO_2$ confirmed the metabolism of PCP by *Pseudomonas* species. The fact that $^{14}CO_2$ is released from PCP-$^{14}$C, would suggest the possible release of chlorine atoms from PCP molecules at the stage of PCP degradation, therefore, the amount of chloride ions in the incubation mixture was determined. The amount of chloride ion detected actually, corresponded to 89.5% of the calculated amount. The results of amino acid analysis of the bacterial cells indicated that the $^{14}$C derived from PCP-$^{14}$C was incorporated rapidly into the cell constituents, and that the pattern of $^{14}$C-amino acids in the cell constituents was not significantly different from 15-minute and 24-hour incubation periods. Tetrachlorocatechol and tetrachlorohydroquinoe were identified as PCP degradation products.

Radehaus and Schmidt (1992) also studied a *Pseudomonas* species and determined the range of PCP concentrations that this *Pseudomonas* species could mineralize. He also tested the effects of glucose additions on PCP degradation, and studied the inhibition of PCP degradation at higher PCP concentrations. *Pseudomonas* sp. strain RA2 was isolated from a soil at the Broderick Wood Products site near Denver, Colo. (Radehaus and Schmidt, 1992). The soil at this site is heavily contaminated with wood-preserving wastes. The mineralization of PCP by the cells was determined by measuring the release of $^{14}CO_2$ from PCP-$^{14}$C degradation and the release of chloride ion. Under batch culture conditions, the destruction of radiolabeled PCP accompanied by $^{14}CO_2$ evolution and release of chloride indicated that these *Pseudomonas* species were capable of mineralizing PCP. On average, 70% of the labeled carbon was recovered as $^{14}CO_2$, 14% was assimilated into cells, and 3% remained in solution at the end of the experiment, approximately 13% of the initial $^{14}$C was not recovered (the reason was unknown). The concentration of PCP also affected the lag phase before the onset of mineralization by these *Pseudomonas* species. The higher the concentration of PCP, the longer the lag phase lasted before the start of

measurable PCP mineralization. It was hoped that glucose would increase the growth rate of *Pseudomonas* sp. strain RA2. Contrary to expectations, however, glucose had no effect on the rate of PCP mineralization. *Pseudomonas* sp. strain RA2 was capable of completely mineralizing PCP at a concentration of 160 mg/liter but was unable to mineralize PCP at a concentration of 200 mg/L.

Trevors (1983) observed the effect of temperature on the degradation of PCP by *Pseudomonas* species. Three *Pseudomonas* species were isolated from an agricultural soil and a fresh water stream, and used to treat PCP in water at different temperatures of 0°C, 4°C and 20°C. *Pseudomonas* isolate 1 grew the most rapidly at both 4°C and 20°C. Isolate 2 and 3 grew very slowly at 4°C, degrading only 23.1 and 11.9% of the PCP respectively, after 100 days. Also the final yields of cells were markedly different. But all the isolates brought about no degradation of the PCP when incubated at 0°C. These results indicated that the temperature influenced the capabilities of *Pseudomonas* species to degrade PCP.

***Rhodococcus***

*Rhodococcus* was isolated by Haggblom *et al.* (1988) independently from soil contaminated with chlorophenol wood preservative and from sludge of a wastewater treatment facility at a bleached Kraft pulp mill. Mineralization of PCP-$^{14}$C by *Rhodococcus* species was monitored for both by trapping evolved $^{14}CO_2$ and by following the concentration of PCP in the culture by GLC. The results showed that 10 $\mu$M PCP was completely removed and 45 and 70% of PCP-$^{14}$C was recovered as $^{14}CO_2$ by *Rhodococcus* sp. cells in less than 1 day. This *Rhodococcus* sp. strain was thus capable of mineralizing PCP.

Apajalahti (1987) isolated a novel actinomycete *Rhodococcus chlorophenolicus* PCP-I from

a PCP-degrading mixed bacterial culture and tested its degradation and mineralization ability on several chlorophenols (PCP, 2,3,4,5-, 2,3,4,6-, and 2,3,5,6-tetrachloro-phenol, and 2,3,5- and 2,3,6-trichloro-phenol) under different temperatures. *Rhodococcus chlorophenolicus* removed 10 $\mu$M of all polychlorinated phenols including PCP completely from the cultures in 6 hours at 41°C, but none of polychlorinated phenols were degraded at 44°C. In contrast to this, tetrachlorohydroquinone was metabolized at a high rate at 50°C, but was not metabolized at 55°C. While the degradation proceeded, two major metabolites, tetrachlorohydroquinone and trichlorohydroquinone, appeared in the medium.

### *Mycobacterium*

*Mycobacterium* strain was another microorganism isolated by Haggblom *et al.* (1988) respectively from chlorophenol-contaminated soil at a sawmill timber-treating facility and from the sludge of a wastewater treatment facility at a bleached Kraft pulp mill through tetrachloroguaiacol enrichment. The results of an experiment designed to assay the ability of *Mycobacterium* species to mineralize PCP showed that 45 to 70% of $^{14}CO_2$ was recovered from PCP-$^{14}C$ and thus this strain was able to mineralize PCP.

### *Phanerochaete*

*Phanerochaete sp.* is a white rot fungus, isolated from soil, which has been believed to degrade PCP. Lamar (1990) measured the ability of several *Phanerochaete sp.* strains to tolerate and degrade PCP in an aqueous medium and in soil. The strains of *Phanerochaete chrysorhiza*, *Phanerochaete levis*, *Phanerochaete sanguinea*, *Phanerochaete Chrysosporium*, *Phanerochaete filamentosa*, *Phanerochaete sordida*, and *Inonotus circinatus* were selected for the research. Temperature growth optima and growth rates for selected strains were determined. The temperature

optimum for most strains fell in the range of 22 to 32°C. Two strains of *Phanerochaete sordida* had optima above 32°C. One of these, *Phanerochaete sordida* 8 had the widest temperature optimum range and grew well up to 40°C. This strain also grew at 44°C, although the rate of growth was significantly less than that at 40°C. Rank of species by growth rate was as follows: *Phanerochaete Chrysosporium* > *Phanerochaete sordida* > *Phanerochaete laevis* > *Phanerochaete chrysorhiza* > *Phanerochaete sanguinea* > *Phanerochaete filamentosa*. The tested strains varied greatly in their sensitivity to PCP, *Phanerochaete chrysosporium* demonstrated the most rapid growth rate at all concentrations of PCP except at 25 ppm. *Phanerochaete sordida* strains were less sensitive to PCP than were strains of the other *Phanerochaete sp.* except for *Phanerochaete chrysosporium*. Depletion of PCP by these fungi occurred in a two-stage process. The first stage was characterized by a rapid depletion of PCP that coincided with an accumulation of pentachloroanisole (PCA). At the end of the first stage, about 64 and 71% of the PCP was converted to PCA in *Phanerochaete Chrysosporium* and *Phanerochaete sordida* cultures, respectively. In the second stage, PCA was mineralized by cells in an aqueous medium. However, the amounts of PCP mineralized by *Phanerochaete Chrysosporium* and *Phanerochaete sordida* grown in a liquid culture were low, only 1 - 11% PCP mineralized to $CO_2$, and in fact these amounts were lower than a previously reported value of 23% mineralization by *Phanerochaete Chrysosporium* after 30 days (Meleski, 1988).

Extracellular enzymes and cell mass obtained from pregrown *Phanerochaete Chrysosporium* culture were used to investigate PCP degradation (Lin *et al.*, 1990). The effects of PCP, extracellular enzyme and cell mass concentrations on the disappearance and mineralization of PCP were examined, and as well, kinetics describing PCP degradation were developed. At initial PCP concentrations of less than 12 $\mu$mol/L, PCP mineralization could be adequately described by the combination of first-order reaction. When initial PCP concentrations were higher

than 30 $\mu$mol/L, PCP mineralization was not dependent on the initial PCP concentration but displayed approximately zero-order reaction kinetics. The results also revealed that the process of PCP degradation by *Phanerochaete Chrysosporium* was mediated by two separable factors: the extracellular enzymes and the cell mass, which were obtained after pregrowth of the culture. Increase in extracellular enzyme concentration or cell mass concentration significantly enhanced PCP disappearance and mineralization. When both cell mass and extracellular enzymes were added into the system, PCP disappearance rates were much more rapid than using only cell mass. By using higher concentrations of extracellular enzymes and cell mass, as high as 70% of added PCP could be mineralized. The disappearance of PCP in the presence of the extracellular enzymes and cell mass is a competitive process. Based on experimental results, the degradation scheme included two mechanisms for PCP mineralization by *Phanerochaete Chrysosporium*. First, extracellular enzymes degraded PCP into some intermediates with subsequent conversion of the intermediates to $CO_2$ by the cell mass. Second, direct mineralization of PCP is also catalyzed by the cell mass.

Mileski (1988) tested the PCP-biodegrading ability of *Phanerochaete Chrysosporium* in nutrient nitrogen-limited culture and nitrogen-sufficient culture. Biodegradation of PCP by *Phanerochaete Chrysosporium* was demonstrated by mineralization of PCP-$^{14}$C to $^{14}CO_2$. After 30 days of incubation in nutrient nitrogen-limited cultures of *Phanerochaete Chrysosporium*, the amount of $^{14}CO_2$ evolved from PCP-$^{14}$C was about 23% of the PCP-$^{14}$C. The mineralization of PCP-$^{14}$C was suppressed (ca. 10% mineralized) when nutrient nitrogen was sufficient. The PCP toxicity experiment showed the PCP concentrations of 4 ppm or higher prevented growth when cultures of *Phanerochaete Chrysosporium* were initiated with spores. However, it was found that if cultures were allowed to establish a mycelial mat before the addition of PCP, the lethal effects of PCP could be circumvented. With this procedure, the fungus was able to grow and mineralize PCP-$^{14}$C at concentrations as high as 500 ppm.

Table 2.4: The Concentrations of PCP Which Inhibit Microorganisms Growth

| **Microorganism** | **Inhibition Concentration of PCP** (mg/L) | **Sources** |
|---|---|---|
| *Arthrobacter sp.* | 135 | Edgehill *et al.* (1982) |
| *Pseudomonas sp.* | 40 | Suzuki (1977) |
| *Pseudomonas* RA2 | 40 | Radehaus (1992) |
| *Rhodocossus sp.* | 2.6 | Haggblom *et al.* (1986) |
| *Mycobacterium sp.* | 2.6 | Haggblom *et al.* (1986) |
| *Phanerochaete sp.* | 25 | Lamar* (1990) |
| | 4 | Mileski (1988) |
| *Flavobacterium sp.* | 200 | Saber and Crawford (1985) |
| | 70 | Xun and Orser (1991) |
| | 600* | Brown (1986) |

* supplement carbon source was also provided besides PCP. Other bacteria were using PCP as sole carbon source and energy source.

***Flavobacterium sp.***

*Flavobacterium sp.* are relatively widely-used PCP-degrading bacteria, which have been isolated from soil. They were selected for my thesis research because of their superior abilities to degrade PCP (see Table 2.4). More detail about *Flavobacterium sp.* is given later in Section 2.3 of this chapter.

In all the research done on the degradability of PCP by different microorganisms, inhibition of PCP at high concentrations of PCP was always found to exist. Table 2.4 demonstrates the inhibition concentration of PCP on several microorganisms.

### 2.2.2 Degradation of PCP in Mixed Culture

A few studies have described the ability of mixed cultures to degrade PCP under aerobic conditions. These studies are prerequisites to the development of commercial systems to treat PCP-contaminated waters.

Two mixed bacterial cultures, capable of utilizing PCP as a sole carbon source, were isolated from samples of industrial sewage, using a continuous culture enrichment technique (Klecka and Maier, 1985). Kinetics of PCP degradation by the mixed culture were investigated through batch and fed-batch experiment. Batch culture experiments showed that the rate of degradation was proportional to the concentration of cell mass used in the test, and also was related to the PCP concentration. However, high substrate concentrations (800 to 1,600 $\mu$g/liter) were inhibitory. The specific growth rate increased with increases in PCP concentration and reached a maximum ($\mu_{max}$=0.074h$^{-1}$) at approximately 300 $\mu$g/liter. PCP concentrations above this level caused a decrease in the specific growth rate as the effects of substrate inhibition become more pronounced. A fed-batch technique was used to examine the kinetics of PCP utilization under conditions resembling continuous-culture operation. Computer analysis indicated that the kinetic coefficients determined in batch experiments also describe the behavior of fed-batch cultures.

Kirsch and Etzel (1973) examined the PCP oxidative capacity of a mixed population of soil microorganisms growing in a fill-and-draw, completely mixed aerator with daily increments of dilute nutrient broth and PCP. They observed that the PCP oxidative capacity reached a maximum of 68% in 25 days, remained stable for approximately 17 days and then began to diminish to a negligible level during the next 14 days. The reason for the sharp decrease in PCP-oxidizing capacity was not known.

Etzel and Kirsch (1975), as well, investigated the biological treatability of sodium PCP-containing wastewater in a unique continuous flow biological treatment unit named a fibre-wall reactor which was a modified activated sludge unit permitting separate control of hydraulic detention time and sludge age without the constraints of external sludge settlers and limitations on cell recycle ratio. The unit was inoculated with a mixed culture taken from a "fill and draw" system previously described by Kirsch and Etzel (1973) and shown to contain PCP metabolizing bacteria. Synthetic wastewaters, prepared by using both a very high purity PCP (referred to as: reagent grade) and a commercial PCP (referred to as: commercial grade), were delivered continuously to the reactors. An authentic, PCP-containing, wood-preservative waste was obtained from a wood treatment site and delivered to the unit as well. A 15-day transient period was used to establish good cell growth in the reactors and to acclimate the cell mass to the type of PCP being used. An additional 30-day operational period was used for treating synthetic wastewaters containing 20, 40 and 60 mg/L PCP respectively. When "reagent grade" PCP was supplied, the effluent PCP levels were on the average very low; all treatment parameters indicated a removal efficiency of PCP in excess of 97%. "Commercial grade" PCP while not being treated quite as effectively as "reagent grade" PCP; for a hydraulic retention time (HRT)=6 hours treatment, PCP removal was only 89%; for HRT 12 hours, all treatments reached 98% removal of PCP. The study of the treatability of authentic wastewater containing 17.8 mg/L PCP indicated that for a 4-week period the average reduction in PCP was greater than 99%, COD removal was about 84%, the effluent was clear but discolored, suggesting that lignin or other colored components might have passed through the system without destruction and contributed to the COD. It appeared that an authentic wood-treating waste is amenable to activated sludge treatment provided that appropriate organisms capable of degrading PCP have developed and are maintained in the system.

Brown *et al.* (1986) studied the potential PCP removal capabilities of PCP-adapted, natural

rock-colonizing microorganisms - epilithic communities, which were collected from rock surface consortia. The rocks were from an artificial stream that had been dosed for 88 days with Na-PCP at a rate of 144 $\mu$g of PCP per liter of stream water. These rocks and their attached microbial populations were maintained in a continuous-flow reactor at constant temperature, nutrient concentrations, and growth medium flow rates. The results showed that rock surface consortia (epilithic communities) appeared to be very efficient at removing PCP from waters. The rates of PCP disappearance generally increased with increasing PCP concentrations, but appeared to approach saturation (ca. 250 mg/liter PCP). Degradation of PCP to inorganic components ($CO_2$, $Cl^-$) or cell mass was observed and correlated with PCP disappearance; 60 to 80% of the total PCP carbon was mineralized. The rate of PCP disappearance was independent of pH in the range from 6.8 to 8.2, but the rate slowed below about pH 6.8; as well the rate was sensitive to the dissolved $O_2$ concentration.

### 2.2.3 Degradation Metabolites of PCP

Several aerobic bacterial strains were shown to mineralize or degrade PCP, but up to now little has been known about the individual dechlorination or dearomatization reactions. Is the benzene ring cleaved prior to total dechlorination, or do the degrading bacteria possess a particular mechanism for removing all five chlorides prior to ring cleavage ?

Although PCP degradation aerobically by several microorganisms in aquatic system has been reported, there is some disagreement over the reaction mechanisms involved in PCP degradation. Generally it is accepted that in the metabolism of PCP by microorganisms in aqueous systems the conversion of PCP to *ortho* or *para* dihydroxyphenol derivatives occur prior to the cleavage of the benzene ring. Limited information exists on the PCP microbial degradation pathways. Table 2.5 describes the different degradation metabolites which have been detected when PCP is aerobically degraded by microorganisms.

Table 2.5: Degradation Intermediates of PCP in Aqueous Systems

| **Chemical name** | **Microorganisms** | **Reference** |
|---|---|---|
| tetrachlorocatchol<br>tetrachlorohydroquinone | *Pseudomonas* | Suzuki 1977 |
| 2,6-dichlorohydroquinone<br>2,3,5,6-tetrachllorohydroquinone<br>2,3,5,6-tetrchlorobenzoquinone | *Arthrobacter* | Reiner *et al.* 1978 |
| 2,3,5,6-tetrachloro-<br>-2,5-cyclohexadione-1,4-dione<br>-2,5-cyclohexadione-1,4-dione | *Phanerochaete chrysosporium* | Mileski 1988<br>Lin *et al.* 1990 |
| pentachloroanisole (PCA) | *Phanerochaete Sordida*<br>*Phanerochaete chrysosporium* | Lamar *et al.* 1990 |
| tetrachlorohydroquinone | *Rhodococcus* | Haggblom 1988<br>Apajalahti 1987 |
| tetrachlorohydroquinone | *Mycobacterium* | Haggblom 1988 |
| tetrachlorohydroquinone<br>trichlorohydroquinone<br>2,6-dichlorohydroquinone | *Flavobacterium sp.* | Steiert *et al.* 1986 |
| tetrachlorohydroquinone | *Flavobacterium sp.* | Xun *et al.* 1991 |

Suzuki (1977) has identified tetrachlorohydroquinone (TeCHQ) and tetrachlorocatechol (TCC) from the incubation medium of a PCP-degrading *Pseudomonas* species. The production of TeCHQ and TCC increased with incubation time, but amounts of these metabolites were extremely small, that is, TeCHQ was found in yields of 0.2 to 0.5% based on the original PCP concentration, smaller yields of TCC from 0.005 to 0.02% were also observed. When PCP was incubated with the sterilized bacterial suspension, TCC and TeCHQ were not detected. Therefore it is concluded that the production of these metabolites was not spontaneous transformation of PCP, but microbial conversion. The release of $^{14}CO_2$ was confirmed, which suggested cleavage of the benzene ring. It is believed that the reason for the small yields of intermediates is because the TCC and TeCHQ are rapidly degraded as soon as they are produced.

Reiner *et al.* (1977) studied the characteristics of KC-3 bacterial attacking PCP. They were convinced that the mechanism of breakdown of PCP involved the conversion of PCP to partially dechlorinated hydroquinone intermediates which then underwent ring breakage. PCP metabolites were extracted from the culture filtrate and were identified as chlorinated hydroquinones or benzoquinones, the critical intermediates appeared to be 2,6-dichlorohydroquinone, 2,3,5,6-tetrachlorohydroquinone and 2,3,5,6-tetrachlorobenzoquinone. Evidence was obtained for the probable participation of 2,6-dichlorohydroquinone and tetrachlorohydroquinone or tetrachlorbenzoquinone as intermediates in the catabolism of PCP. According to their results a hypothetical pathway for the metabolism of PCP by culture KC-3 was suggested as shown in Figure 2.2. But further studies must be completed before this pathway can be firmly established. It is essential that the enzymes responsible for this sequence of reactions be isolated and characterized.

Lamar (1990) investigated the mineralization of PCP by fungi (*Phanerochaete Chrysosporium* and *Phanerochaete Sordida*) in an aqueous medium respectively, an attempt to find intermediates during PCP degradation was made as well. The levels of PCP decreased by 82 to 96%. The rapid depletion of PCP in aqueous medium coincided with an accumulation of

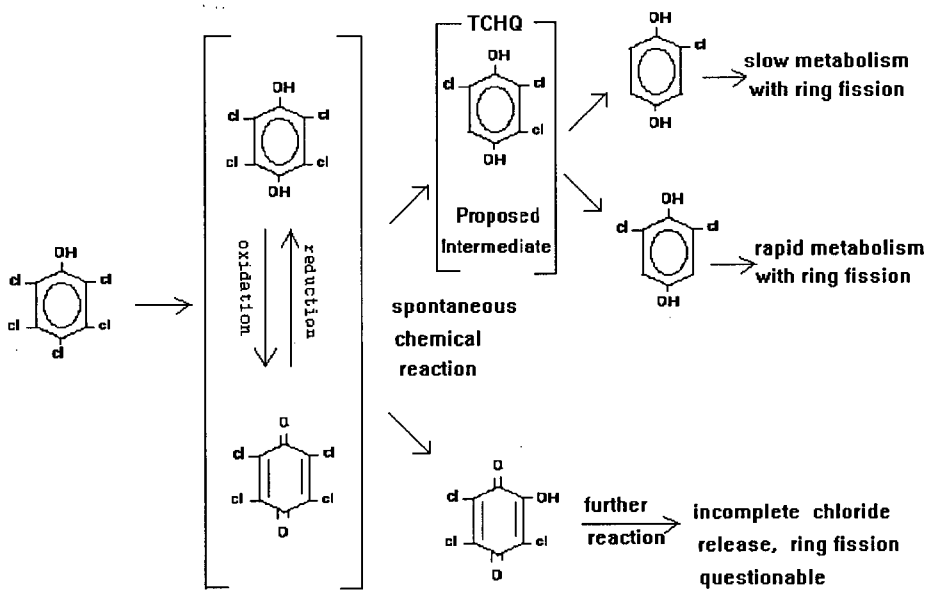



Figure 2.2: Hypothetical Pathway for the Biodegradation of PCP by the Bacterial Culture, KC-3

pentachloroanisole (PCA), which was believed to be an intermediates because no PCA was recovered in extracts from the control culture. Therefore, it is believed that a two-stage process occurred for degradation of PCP by *Phanerochaete Chrysosporium* and *Phanerochaete Sordida*. The first stage was that PCP was converted by fungi with an intermediate accumulation of PCA. In the second stage, PCA was converted to nontoxic $CO_2$. But according to the results of Mileski *et al.* (1988) who used a *Phanerochaete Chrysosporium* culture to biodegrade PCP, a product, TCHD (2,3,5,6-tetrachloro-2,5-cyclohexa-diene-1,4-dione), was noted in the culture, but no PCA appearance was reported. The same results were confirmed by Lin et al. (1990) who used extracellular enzymes and cell mass from the pregrown *Phanerochaete Chrysosporium* culture for the degradation of PCP, the action of the crude extracellular enzymes led to the formation of a degradation intermediate of TCHD.

Haggblom *et al.* (1988) studied the treatability of PCP and other phenols by *Rhodococcus* and *Mycobacterium* strains. The metabolites produced were identified by their mass spectra and retention times in a GLC, with authentic compounds as a reference. The formation of tetrachlorohydroquinone was considered to be a metabolite of PCP degradation by the bacteria. The results suggested that the degradation of PCP by *Rhodococcus* and *Mycobacterium* strains was initiated by *para*-hydroxylation, producing chlorinated *para*-hydroquinone, which was then further degraded. This result was also found by Apajalahti *et al.* (1987), who showed that the *Rhodococcus* strain initially attacked PCP via a tetrachlorohydroquinone-producing *para*-hydroxylation reaction. The metabolite of tetrachlorohydroquinone was further degraded by bacteria.

## 2.3 *Flavobacterium sp.*

### 2.3.1 Characteristics of *Flavobacterium sp.*

*Flavobacterium sp.* is a group of bacteria belonged to the genera of family *Achromobacteraceae*, including *F. aquatile* (Bread, 1957). *Flavobacterium sp.* are gram-negative, rod-shaped bacteria; motile by means of peritrichous flagella or non-motile; characteristically producing yellow, orange, red, or yellow-brown pigmentation, the hue often depending upon the nutrient medium. *Flavobacteria*'s fermentative metabolism usually is not conspicuous; acid reactions commonly do not develop from carbohydrates when available nitrogen-containing organic compounds are in the medium; gas is not produced from carbohydrates according to the usual cultural tests; nutritional requirements usually are not complex. *Flavobacterium sp.* are commonly proteolytic, aerobic to facultatively anaerobic, and occur in water and soil.

### 2.3.2 Isolation and Characterization of *Flavobacterium sp.*

There have been numerous reports of *Flavobacterium sp.* degrading a variety of chlorinated compounds and herbicides. Steenson and Walker (1957) described the dissimulation of 2,4-dichlorophenoxyacetic acid through 2,4-dichlorophenol and 4-chlorocatechol, and MPCA (4-chloro-2-methylphenoxy-acetic acid) through 4-chloro-methylphenol by *Flavobacterium peregrinum.* Bollag *et al.* (1967) confirmed that *Flavobacterium peregrinum* degraded MPCA to 4-chloromethylphenol with full release of chlorine as chloride and conversion of the carboxyl carbon to volatile products. Burger *et al.* (1962) isolated a *Flavobacterium sp.* that metabolized phenoxybutyric acids having chlorine on the aromatic ring; the organic chlorine was liberated, and the aromatic ring was cleaved. MacRae *et al.* (1963) isolated a *Flavobacterium sp.* which degraded the pesticide 4-(2,4-dichlorophenoxy)butyric acid. A *Flavobacterium sp.* isolated from paddy water by Sethunathan and Yoshida (1973) decomposed diazinon to 2-isopropyl-6-methyl-4-hydroxypyrimidine which was then converted to $CO_2$. This bacterium also converted parathion to *p*-nitrophenol.

Saber and Crawford (1985) isolated *Flavobacterium sp.* by selective enrichment from PCP-contaminated soil from three sites in Minnesota. 85 strains of pure cultures of PCP-degrading bacteria were isolated and tested for their ability to degrade PCP in liquid cultures containing PCP. All 85 of the strains proved to be positive for PCP degradation. All strains were differentiated from each other by extensive characterization with a wide variety of biochemical and biophysical tests, but all were identified as being of the genus *Flavobacterium* . Of the strains, five representative strains were tested for their ability to mineralize PCP. PCP-$^{14}$C with the radiolabeled carbon was used in the study. The results indicated that the *Flavobacterium sp.* used could utilize PCP as a sole source of carbon and energy, and that between 73 to 83% of all radiolabeled carbon in PCP-$^{14}$C was returned as $^{14}CO_2$ with full liberation of chlorine as chloride. This suggested that 17 to 27% total carbon was assimilated into cell mass. Mineralization rates

were very consistent, ranging between 3.7 and 7.2% of total PCP-$^{14}C$ returned as $^{14}CO_2$ per hour.

Topp and Hanson (1990) tested the growth of *Flavobacterium sp.* in continuous culture to determine the growth limiting amount of ammonium, phosphate, sulfate, glucose, glucose + PCP, or PCP. The PCP concentration and the viable cell density were determined periodically. Cells that were grown under phosphate, glucose, or glucose + PCP limitation were sensitive to PCP and took longer to degrade 50 mg/L PCP than did cells that were grown under ammonium, sulfate, or PCP limitation. Cells grown under nitrogen or sulfate limitation degraded PCP the most rapidly. Glucose stimulated viability and PCP degradation in all cases except when the cells were grown under carbon limitation with glucose and PCP added together as the carbon source. The results indicated that the sensitivity and degradation of PCP by *Flavobacterium sp.* were influenced by nutrient limitation and phenotypic variation. This suggested that the nature of the nutrient limitation in a certain environment might influence the sensitivity of the *Flavobacterium sp.* to PCP and therefore might influence *Flavobacterium sp.* preceding the degradation of PCP.

Brown *et al.* (1986) also examined the PCP degradation by pure *Flavobacterium sp.* in continuous cultures when cellobiose and PCP simultaneously limited. In the presence of cellobiose, *Flavobacterium sp.* could utilize influent containing up to 600 mg of PCP per liter, while the measured rate of PCP utilization began to slow at influent concentrations of 808 mg/liter PCP. The specific rates of PCP carbon degradation reached as high as 0.15 (dry weight) of C per hour at a specific growth rate of 0.045 $h^{-1}$.

### 2.3.3 Possible Metabolites of *Flavobacterium sp.*

As mentioned above, PCP degradation by *Flavobacterium sp.* and the release of $^{14}CO_2$ were confirmed, which were an implicit proof of the cleavage of the benzene ring. However, the degradation process of PCP has not been completely elucidated by isolating intermediates or products. Little information is available.

Steiert and Crawford (1986) studied the pathway probably employed for aerobic PCP degradation by chemically derived mutants of a *Flavobacterium sp.* strain, which were blocked in their ability to completely degrade PCP. The results demonstrated that PCP degradation by *Flavobacterium sp.* was initiated by conversion of PCP to tetrachloro-p-hydroquinone (TCHQ). Further experiments using $H_2^{18}O$ and $^{18}O_2$ suggested that the first dechlorination, where a hydroxyl replaces the chlorine at PCP ring position number 4, involved a hydrolytic reaction, rather than an oxygenase-catalyzed mechanism. Then two reductive dechlorinations of TCHQ followed to yield first trichlorohydroquinone (TeCHQ) and then 2,6-dichlorohydroquinone (DCHQ). Thus, it was concluded that the pathway probably used by *Flavobacterium sp.* is the one shown in Figure 2.3. These results are in agreement with some pathway intermediates proposed earlier in some of the papers mentioned above. Suzuki (1977) isolated and identified tetrachlorohydroquinone from culture fluids of a PCP-degrading *Pseudomonas* species. Reiner *et al.* (1978) identified tetrachlorobenzoquinone, tetrachlorohydroquinone and 2,6-dichlorohydroquinone from culture media of a PCP-degrading *Arthrobacter* species.

Xun *et al.* (1991) isolated and purified a PCP hydroxylase, a flavoprotein from a *Flavobacterium sp.* culture, which was with a molecular weight of 63,000. This enzyme completely converted PCP to TCHQ in the presence of NADPH, the reaction was confirmed to be enzymatic

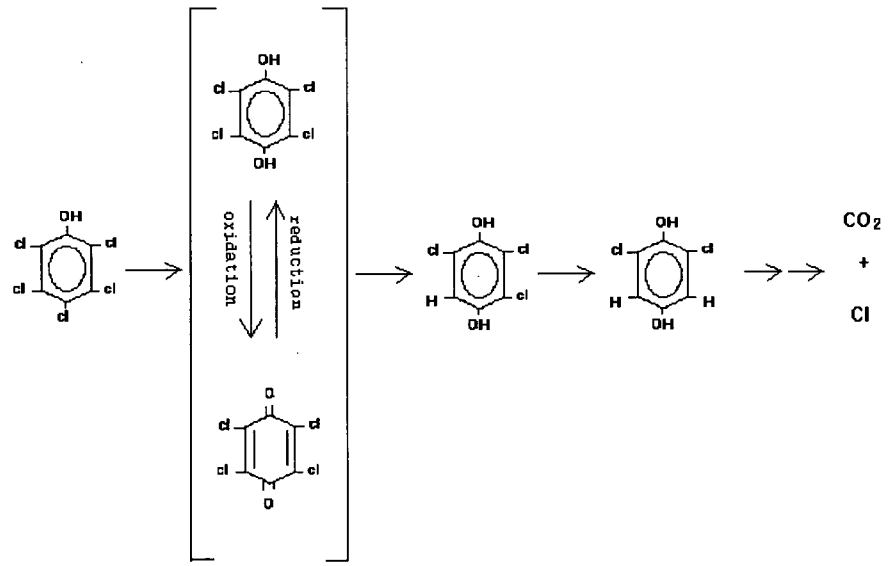



Figure 2.3: Proposed Pathway of PCP Degradation by *Flavobacterium sp.*

because controls without enzyme or with boiled enzymes exhibited no change in PCP concentration after 1 hour of incubation. This result confirmed that TCHQ is the first intermediate during PCP degradation by *Flavobacterium sp.*. Later, Xun (1992) did $^{18}O$ labeling experiments for confirming the oxidative dehalogenation of PCP by pentachlorophenol hydroxylase derived from *Flavobacterium*. The purified enzyme incorporated $^{18}O$ from $^{18}O_2$ but not from $H_2^{18}O$ into the reaction end product TCHQ. The results clearly demonstrate that PCP is oxidatively converted to TCHQ by a monooxygenase type enzyme from a *Flavobacterium sp.* strain.

### 2.3.4 Immobilized *Flavobacterium sp.*

Immobilized cells are defined as cells that have been entrapped within or associated with an insoluble matrix. Many microorganisms exist in the environment in an immobilized state since

they grow attached to surfaces such as stones, plants and even other microorganisms. It is also possible to immobilize bacteria in the laboratory. Under many conditions, immobilized cells have advantages over free cells. Immobilization allows a high cell density to be maintained in a bioreactor at any flow rate. Also, catalytic stability can be greater for immobilized cells (Kutney *et al.* 1985), and some immobilized microorganisms are able to tolerate higher concentrations of toxic compounds than their free counterparts because of the inhibition of toxic compounds diffusion into the matrix (Dwyer *et al.* 1986).

Immobilization of microbial cells can have disadvantages. One common disadvantage of immobilization is the increased diffusional resistance of substrates and products through immobilization matrices. Because of the low solubility of oxygen in water and the high local cell density, oxygen transfer often is the rate-limiting factor in the performance of aerobic, immobilized cell system.

The technique of immobilization has frequently been used for the microbial production of specialty chemicals and for biological wastewater treatment, but few studies have been reported regarding the utilization of artificially immobilized cells to degrade PCP.

Some work has been done by using immobilized cells to degrade 2-chlorophenol, 4-chlorophenol or other forms of phenol (Arvin *et al.* 1991; Faghani-Shoja *et al.* 1988; Prasad and Joyce 1992; Pignatello *et al.* 1983; Tokuz 1989). Rehm's group studied the degradation of phenol (Bettmann and Rehm 1985) and 4-chlorophenol (Westmeier and Rehm 1985) by *Alcaligenes*, and *Pseudomonas* immobilized in alginate and polyacrylamide beads, and on activated carbon. Compared to free cells, immobilized cells were able to tolerate higher concentrations of the toxic substrates. while degrading them at faster rates.

An epilithic microbial consortium capable of degrading PCP was developed in artificial freshwater streams that had been dosed continuously with the biocide (Pignatello *et al.* 1983). After a three week acclimation period, biodegradation had become the primary method of PCP

loss from the system. Tests of the ability of free and attached bacteria within the system to mineralize PCP indicated that most of the activity resulted from those microorganism either attached to surfaces (eg. rocks and macrophytes) or associated with surface sediments (Pignatello *et al.* 1985).

An investigation of PCP degradation by *Flavobacterium sp.* cells immobilized in calcium alginate was made by O'Reilly *et al.* (1988). The *Flavobacterium sp.* was grown in a minimal salt medium and then immobilized in Ca-alginate beads. PCP concentrations up to 150 ppm could be completely degraded in bench-scale batch reactors. Partial degradation occurred in reactors with 200 or 250 ppm PCP, while negligible degradation occurred at higher PCP concentrations. The addition of pure oxygen gas to the batch reactors did not lead to an increase in the PCP degradation rate, indicating the system was not limited by oxygen under the conditions tested.

# Chapter 3

# Materials and Methods

## 3.1 Experiment Design

The growth of *Flavobacterium sp.* is subject to several factors, including pH, temperature, and nutrient limitation.

The optimum pH for removal of PCP from water by *Flavobacterium sp.* is between pH 7.0 and 9.0 as reported by Martinson *et al.* (1985) . The *Flavobacterium sp.* was still active as low as 6.5, but removal rates slowed considerably below that pH. No removal was observed at pH 6.0 according to Martinson's report. The bacteria performed poorly at pH 7.0 or lower, or pH 9.5 and higher. Therefore all reactors, either fed-batch or continuous reactors, were maintained at pH around 7.4 during the degradation process. Any pH change in the reactors was adjusted back to pH about 7.4 by adding 0.1 N NaOH or 0.1 N $H_3PO_4$.

Temperature is an important variable affecting PCP removal rates by *Flavobacterium sp.* Martinson *et al.* (1986) reported that *Flavobacterium* was most effective between temperatures of 15°C and 30°C, and removal rates slowed at 35°C, with no removal at 40°C. Because the optimum temperature for removal of PCP by *Flavobacterium sp.* is between 15°C and 30°C, and the room temperature in our laboratory is from 15°C to 30°C, no temperature control was necessary. Thus all experiments of degradation of PCP by *Flavobacterium sp.* conducted in the Bio-Resource Engineering laboratory were done at room temperature.

The degradation of PCP by *Flavobacterium sp.* is influenced by nutrient limitation (Topp

and Hanson, 1990). The nature of the nutrient limitation in reactors can affect the sensitivity of the bacteria to toxic concentrations of PCP, so nutrient limitation is a very important parameter which should be optimized in order to improve the efficiency of PCP degradation by *Flavobacterium sp.* Sufficient nutrients necessary for *Flavobacterium sp.* growth were provided throughout the experiment, which included certain concentrations of these nutrients as $K_2HPO_4$, $KH_2PO_4$, $NaNO_3$, $MgSO_4$, and $FeSO_4$.

The purpose of the experiments was to investigate the capability for biological degradation of toxic PCP by bacteria. As mentioned in the literature review, several microorganism have been proven to decompose PCP under aerobic conditions. *Flavobacterium sp.* (ATCC 39723) was selected in this research due to its capability of degrading relatively high concentrations of PCP as found from previous work (see Table 2.4).

The work was mainly done in two stages: a fed-batch reactor stage and a continuous reactor stage.

### 3.1.1 Fed-batch Reactor

The fed-batch reactors were used to treat synthetic wastewaters containing PCP. The seed used was either pure *Flavobacterium sp.* or *Flavobacterium sp.* mixed with activated sludge. Activated sludge was taken from the UBC sewage treatment pilot plant. Eight flasks (2 L) with working volumes of 1 L were used as fed-batch reactors. The seeding of each flask was done as shown in Table 3.6. Flasks #1 and #2 were seeded with activated sludge only; #3 and #4 were seeded with both activated sludge and *Flavobacterium sp.*; #5 and #6 used *Flavobacterium sp.* only. Certain concentrations of nutrients, which were necessary to maintain bacterial growth, were added to each flask beforehand, and supplemented later as necessary. PCP was added as the sole carbon and energy source; no supplemental carbon source was added at the same time.

Table 3.6: Seeding of Fed-batch Reactors

| NO. of Flasks | Seeding | PCP Applied (mg/L) | | |
|---|---|---|---|---|
| | | Set 1 | Set 2 | Set 3 |
| #1 and #2 | Activated Sludge only | 30 | 50 | 65 |
| #3 and #4 | *Flavobacterium sp.* only | 30 | 50 | 65 |
| #5 and #6 | Activated Sludge and *Flavobacterium sp.* | 30 | 50 | 65 |

The initial PCP added to each fed-batch reactor was 10 mg/L PCP. Whenever PCP disappeared from the supernatant, 100 ml of the supernatant was removed, and a higher concentration of PCP and new medium were added to the reactors. Three different PCP concentrations were used, 30 mg/L, 50 mg/L, and 65 mg/L PCP. pH was adjusted to around 7.4 by using either 0.2N NaOH or 0.1N $H_3PO_4$. The changes in PCP concentration, pH, $Cl^-$ concentration and turbidity of the supernatant in the fed-batch reactors were monitored.

The schematic layout of the equipment utilized for the fed-batch experiments is illustrated in Figure 3.4, Figure 3.6 is a photo of these reactors.

### 3.1.2 Continuous Reactor

Bench scale, continuous reactors were also set up to treat synthetic PCP-containing wastewater. One reactor was used as control without seeding with any bacteria. The other two reactors worked as duplicates after seeding with immobilized bacteria. The seed was immobilized *Flavobacterium sp.* Alginate was used as a biofilm to immobilize the *Flavobacterium sp.* A synthetic wastewater containing PCP with nutrients essential for bacteria growth was fed to the reactors continuously. The only carbon and energy source for the bacteria was PCP. No supplementary carbon source, such as glucose, was added to the reactor. The relationships among

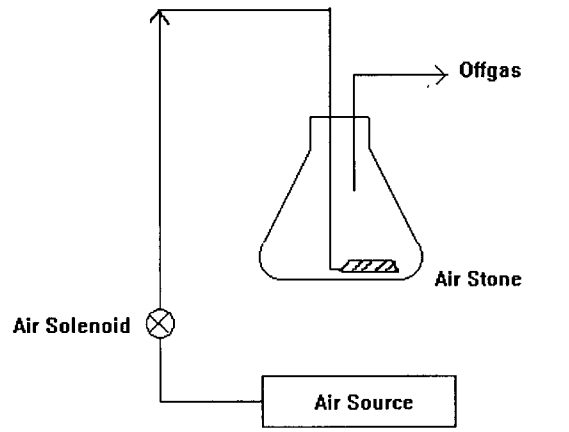



Figure 3.4: The Schematic Layout for Fed-batch Reactors

removal efficiency, hydraulic retention time and organic loading rate were investigated.

The continuous reactor is illustrated in Figure 3.5. Figure 3.7 is a photo of the continuous reactors.

## 3.2 Equipment

Both the fed-batch reactors and the continuous reactors were assembled in the laboratory. All the equipment, including the seals, tubing, air stones, manifolds and pumps were laboratory scale in size.

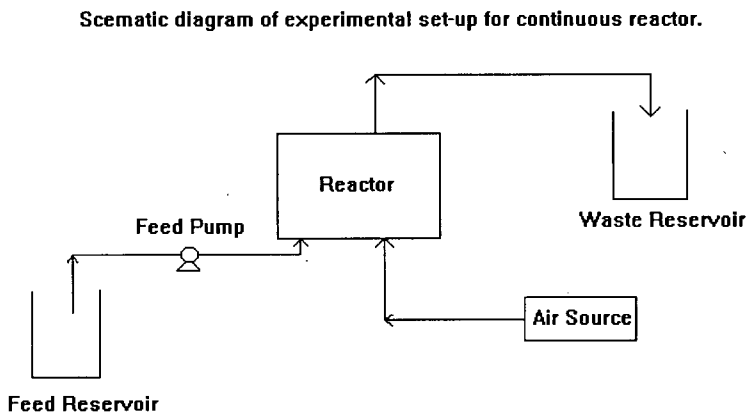



Figure 3.5: The Schematic Layout for Continuous Reactors

### 3.2.1 Reactors

**Fed-batch Reactor** Six two-liter (with 1 liter working volume) Erlenmeyer flasks were used as fed-batch reactors. The flasks and their air stones were thoroughly cleaned and sterilized. The assembling of a fed-batch reactor involved the insertion of the cleaned air stone diffuser into the flask, connecting an air line to the air stones through the manifold, attaching the reactor lid and installing the off-gas line. PCP solution and the media for bacterial growth were spiked into the flasks whenever a new set was started.

**Continuous Reactor** Two types of columns, which had different configurations, were used as upflow continuous reactors to treat PCP-containing wastewater. The different configurations were chosen to determine if there was an unaccounted scale-up factor (such as a wall effect) on the performance of a packed-bed reactor. Column A was a glass tube with working volume 180 ml ( about 4.8 cm inside diameter x 60 cm long ). Column B had a working volume of 1500 ml

Figure 3.6: View of the Fed-batch Reactor Set-up in the Laboratory

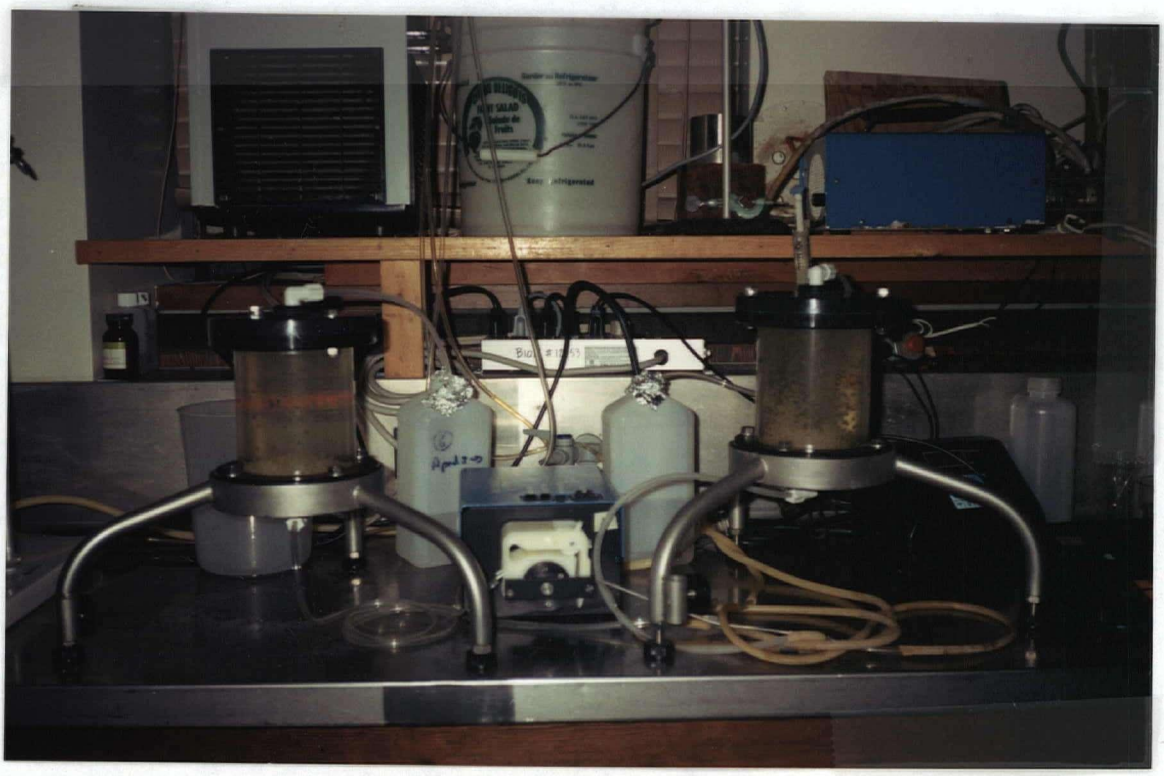


Figure 3.7: View of the Continuous Reactor Set-up in the Laboratory

(approximately 100 $cm^2$ in cross sectional area and 15 cm in height).

Round, inoculum beads with entrapped *Flavobacterium sp.* were placed into the reactor before introducing the PCP and media. A feed stream containing a certain concentration of PCP in induction media was introduced into the reactor at the bottom of reactor at various flow rates. As well air was introduced into the reactor from the bottom. An Ismatec Peristaltic Pump ( Cole-Parmer, Chicargo, USA). was used to introduce influent and pump out effluent from the continuous reactors. An overflow was used to maintain a constant liquid volume.

### 3.2.2 Aeration System

**Fed-batch Reactor** Aeration in the fed-batch reactor was achieved using a 3 cm aquarium air stones, which was connected to an air manifold through rubber tubing. The air flow from a 6 line manifold went to the air stones. The manifold allowed the control of the air flow to the individual reactors. The flow rates in the various lines were controlled to be the same, about 0.5 L/min. The manifold in practice could not control the air flow in the various lines at exactly the same rate.

**Continuous Reactor** No air stones were used for the continuous reactors. Aeration to the continuous reactor was finished by air-diffusing through a thin porous membrane inside the reactors. The membrane contained a lot of fine hoses which could disperse air to the liquid phase in a very similar way to an air stone. Influent was also flowing through this thin membrane to the reactor.

### 3.2.3 Feed Composition

The synthetic wastewater containing the substrate was prepared in the laboratory. Table 3.7 presents the contents and concentrations of the wastewater. PCP is the substrate, the only carbon

Table 3.7: Composition of the Synthetic Wastewater (The desired PCP concentration in the feed solution was diluted from the stock solution of PCP for each experiment at the needed concentration)

| COMPONENT | mg/L | g/L STOCK |
|---|---|---|
| $K_2HPO_4$ | 500 | 50 |
| $KH_2PO_4$ | 650 | 65 |
| $MgSO_4.7H_2O$ | 100 | 10 |
| $NaNO_3$ | 395 | 39.5 |
| $FeSO_4.7H_2O$ | 1.07 | 0.107 |
| PCP | as required | 2 |

source and energy source for *Flavobacterium sp.* Other contents besides PCP are the nutrients necessary for the growth of *Flavobacterium sp.* 2000 ppm PCP stock solution was prepared by dissolving 2 g PCP in 1000 ml 0.02 NaOH solution. All of the media solutions were refrigerated at 4°C.

### 3.2.4 Inoculum

**Dehydration of *Flavobacterium sp.***

A freeze-dried culture of *Flavobacterium sp.* (ATCC 39723) was purchased from American Type Culture Collection which was freeze-dried culture. The media formulation for initial revival and preservation of *Flavobacterium sp.* is Medium 18 - Trypticase Soy. Trypticase Soy Broth was purchased from Canlab (11738 BT). Solutions and slants of Trypticase Soy Broth were prepared and autoclaved at 121°C for 15 min and refrigerated at 4°C. The solution was for initial revival and subculture of *Flavobacterium sp.* The slant was for preservation of *Flavobacterium sp.* which had to be subcultured every month.

The freeze-dried *Flavobacterium sp.* culture was stored in a double vial. The vial was opened as recommended in the supplier's instructions. 0.3 to 0.4 ml of liquid medium were added aseptically to the vial containing freeze-dried *Flavobacterium sp.* by using a sterilized Pasteur pipette, mixed well, and then most of the mixture were transferred to a test tube containing 6 ml Trypticase Soy Broth. The last few drops of the suspension were transferred to an Trypticase Soy agar slant. The tubes and slants were placed in an incubator and incubated at 30°C.

Freeze-dried *Flavobacterium sp.* culture grew in about 4 days. The grown broth culture was used for preparing the inoculum for either the fed-batch reactor or the continuous reactor. The slants were used for preservation of *Flavobacterium sp.* for later subculture. The slants were stored at 4°C and subcultured every month.

**Inoculum for the Fed-batch Reactor**

For the fed-batch reactors three types of inoculum were used: activated sludge only; *Flavobacterium sp.* only; and activated sludge mixed with *Flavobacterium sp.* For each set of inoculum duplicate flasks were used. An uninoculated reactor with salt medium and 10 ppm PCP was used as a nongrowth control to check for possible chemical or physical changes in PCP in the medium.

The aerobic sludge was taken from the pilot scale water treatment plant operated by the Civil Engineering Department of UBC. The water treatment reactors at the facility are fed on municipal sewage via a dosing tank. Four liters of sludge were collected in a 5 liter plastic bottle and returned to the lab where an air stone was used to provide aeration to the entire bottle. All experiments were inoculated with the sludge not older than 24 hours.

The *Flavobacterium* culture was prepared from dehydration of freeze-dried *Flavobacterium*

*sp. Flavobacterium sp.* grown in Trypticase Soy Broth was inoculated and maintained in the mineral salt medium containing glucose as carbon source, which was then used as inoculum for fed-batch reactor.

## Inoculum for the Continuous Reactor

The inoculum for the continuous reactor was immobilized *Flavobacterium sp.* The cells were immobilized in alginate following a method modified from the one used by Sofer (1990), who immobilized activated sludge in calcium alginate to study its degradation of 2-chlorophenol. Likewise, alginate was used as biofilm to immobilize *Flavobacterium sp.*.

**Growth of *Flavobacterium sp.*** *Flavobacterium* was inoculated and grown in the synthetic wastewater as has been noted (Table 3.7), but some changes were made for the continuous reactors in that the PCP concentration used was about 60 ppm for all the continuous reactors run, and the concentrations of $K_2HPO_4$, $H_2PO_4$ were 4.5 mg/L and 5 mg/L respectively. The growth of cells occurred in flasks on a shaker at 30°C. After 2-3 days growth, the *Flavobacterium* cells were collected by centrifugation at 10,000 rpm and 5°C to obtain concentrated pellets for further bead-making.

**Immobilization of *Flavobacterium sp.*** Generally, the characteristics required for a matrix to immobilize microorganisms are: a) to be water soluble, and able to gel at ambient temperatures; b) to have a low toxicity to the immobilized microorganisms during and after gelling; c) to have a high dispersion coefficient for the substrate to be treated in the matrix; d) to have low biodegradability and to be physically strong and durable. Alginate is natural polymer resin which has those required characteristics and is popular for use as a matrix for immobilizing microorganisms. Alginate was selected as the matrix for immobilizing *Flavobacterium sp.* in this study.

The procedure for making beads for the immobilization of *Flavobacterium* cells in alginate is as follows. The collected cells were mixed with cold, sterile, 2% sodium alginate solution (the sodium alginate solution was sterilized in an autoclave at 120°C and stored at 4°C before use). The mixing ratio was 5 grams (wet weight ) of *Flavobacterium* cells with 100 ml of sodium alginate solution. The suspension of cells was blend-mixed well to obtain a homogeneous cell suspension. The suspension was then extruded as discrete droplets by pumps at the rate of 6 ml/min into a 0.2 M calcium chloride solution with continuous, slow stirring at room temperature. On contact with the calcium chloride solution, the droplets hardened to form beads about 3 to 3.5 mm in diameter. These beads with the trapped *Flavobacterium sp.* were collected by filtration. The beads were then cured in 0.4 M calcium chloride solution for 12 hours at 4°C before use.

For the control reactor, beads without cells were also made, as noted above for making *Flavobacterium sp.*-containing beads. The only difference was that sterile 2% alginate was not mixed with any *Flavobacterium* cells, instead it was directly pumped into the calcium chloride solution to make the beads. The beads formed in this way did not contain any cells.

## 3.3 HPLC Analysis of PCP and Other Phenols

### 3.3.1 Introduction

Numerous techniques have been developed for the qualitative and quantitative analysis of pentachlorophenol. The earliest analytical methods used colorimetric techniques in which PCP was reacted with such compounds as nitric acid or 4-aminoantipyrin. These were neither very specific, nor sensitive (Bevenue, 1967). They are no longer widely used. Other procedures for the separation and determination of PCP include gas chromatography (GC) (Brown, 1986), gas-liquid chromatography (GLC) (Borsetti, 1980, Suzuk, 1977), and high-performance liquid

chromatography (HPLC) (Markowski, 1990, Bigley, 1985). GC is well-established and very popular-used technique for determining PCP concentrations in a diverse range of sample types. A GC with an electron capture (EC) or flame ionization (FI) detector is specific and capable of detecting PCP in the part-per-trillion range. The shortcoming of GC and GLC is sample preparation needed, usually involving acidification of the sample to convert PCP to its non-ionized form (or molecular form), and extraction into organic solvent etc. The organic solvents often used for extraction are hexane, benzene, methylene chloride, or ether. These procedures for sample preparation are very tedious and time-consuming. The high temperature maintained in the injection ports and GC column may decompose PCP and its isomers, thus causing an analytical error.

On the other hand, high-performance liquid chromatography has seen increasing application as a combined clean-up, separation and detection system for PCP over the last decade. The advantage of HPLC over GC are that it minimizes sample preparation, most water samples can be directly injected. The derivatization used for GC analyses of PCP very often can be eliminated. It can separate and determine PCP and other chlorophenols at near ambient temperature. No decomposition occurs in the column or injection port. The reported methods of PCP analysis by HPLC have all made used of either isocratic elution or post-column reaction detection. Only one paper has mentioned the gradient elution for HPLC analyzing phenolic pollutants (Makoski, 1990). In this work HPLC gradient elution was used and discussed in detail.

### 3.3.2 Material and Method

#### Chemicals

Methanol: HPLC grade solvent with UV cut off (BDH company). Acetic acid: HPLC grade solvent (BDH company). HPLC grade water: A cartridge (Norganic, Millipore Corp.) with

0.45 $\mu$m pore size membrane used to filter deionized water to make HPLC grade water. Stock standards (1000 ppm) of PCP and other phenols were prepared by accurately weighing 100 mg of each of the phenol standards into separate 100ml volumetric flasks and diluting to volume with methanol, and stored in a 4°C refrigerator. Working standards were prepared by diluting each of these standards to 100ml with methanol or water as needed. PCPs were HPLC grade from Hewlett Packard. Other phenols were obtained from Aldrich or Eastman Kodak.

**Apparatus**

A Hewlett Packard HPLC system (Series 1050) equipped with solvent cabinets, injection valve, quaternary pump and a variable-wavelength UV detector was used. The chromatographs were recorded and analyzed by a Hewlett-Packard chemstation running HPLC software. The stainless-steel column was a LC-8 reversed phase column, 15.0 cm x 4.5 mm I.D. with 5 $\mu$m packing, supplied by Supelcosil company. The eluting solvents (methanol and water) were degassed prior to and during all runs. 20 $\mu$l injections were used throughout this work. A schematic diagram of the HPLC system is presented in Figure 3.8.

The variable-wavelength UV detector allows the programming of the detecting wavelength and bandwidth, as well as the reference wavelength and bandwidth. The detecting wavelength used for PCP analysis was 280 nm, with a reference wavelength of 320nm. A flowrate of 1 mL/min was used. The compositions of eluting solvents were pure methanol with 1% acetic acid, and HPLC grade water with 1% acetic acid. They were in the gradient run from 35:65 to 100:0 over 25 minutes, returning to 35:65 over 10 minutes, afterwards with 10 minutes column stabilization by running eluting solvents. This resulted in 45 minutes for one sample run. The retention times and peaks areas were recorded and compared to authentic standard compounds in order to determine the concentration of phenols.

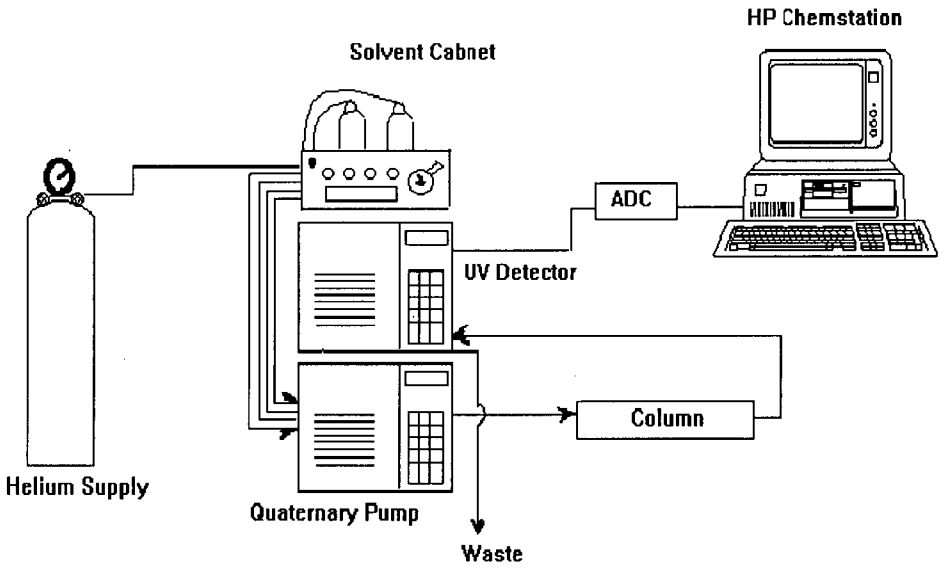



Figure 3.8: A Schematic Diagram of the HPLC System

# Chapter 4

# Results and Discussion

## 4.1 HPLC Analysis of PCP and Other Phenols

### 4.1.1 Results and Discussion

As mentioned in Chapter 2, attempts to find possible intermediates accumulating during PCP degradation were made, six different phenols of possible intermediates were selected in this work (see Table 4.8). HPLC separation and determination of PCP and the other six phenols were carried out by gradient elution using a UV detector. Preliminary experiments were carried out to determine the optimal gradient times which would give best separation and lowest baseline drift. The gradient times of $t_g$=35 minutes with solvents of methanol/acetic acid (1%) : water/acetic acid (1%) running from 35:65 to 100:0 over 25 min, returning to 35:65 over 10 minutes was found to be the optimal condition, giving relative stability of the baseline, and best separation. A series of PCP standard solutions were prepared and applied to the HPLC column at 1.0 ml/min. The typical separation chromatograms of all phenols standards by HPLC under the chosen optimum condition are shown in Figure 4.9.

The known concentration of phenols plotted against the area obtained on the chromatogram for each standard could be used to make a standard curve for each phenol. The standard curve typical for PCP analysis is shown in Figure 4.11, and the resulting regression equation of the standard curve for PCP was found to be:

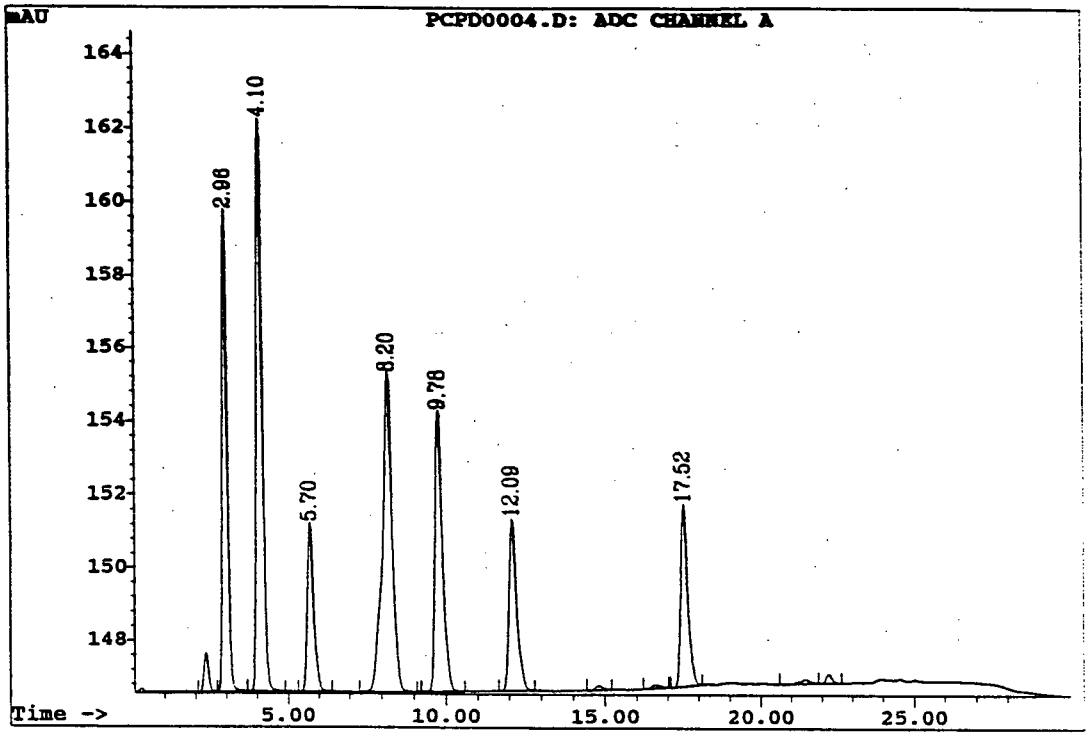



Figure 4.9: Typical Separation Chromatogram of Seven Phenols by HPLC

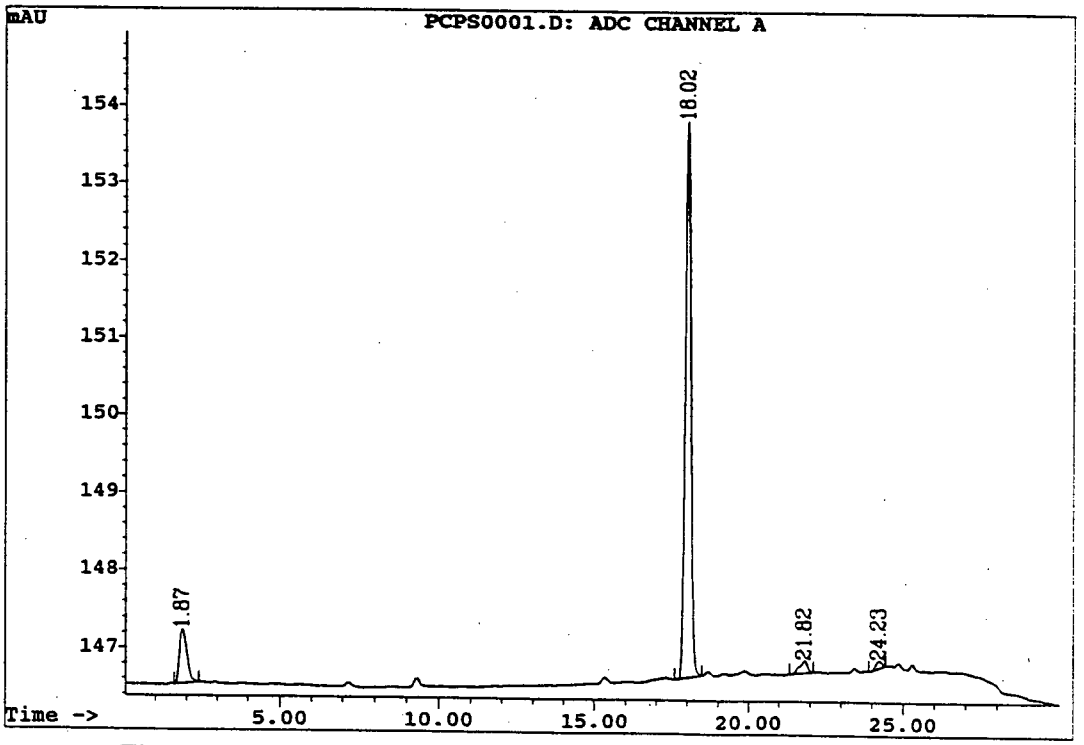



Figure 4.10: Typical HPLC Chromatogram of Sample Analysis

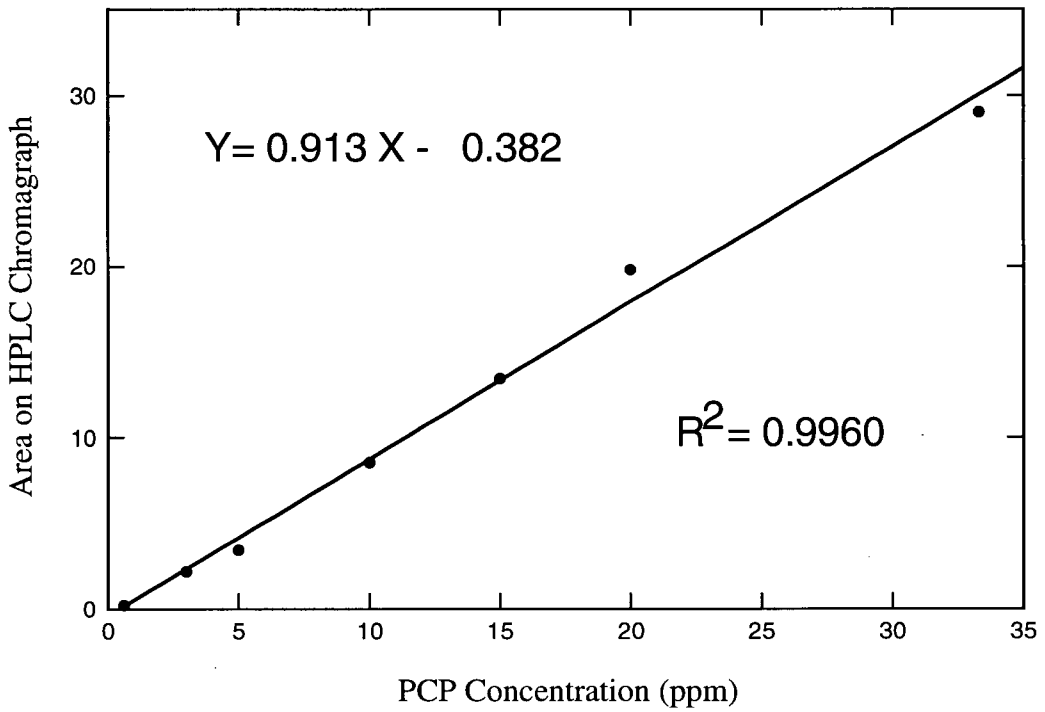



Figure 4.11: Typical Standard Curve for PCP Analyzed by HPLC

$$Y = 0.913 * (Area) - 0.382 \tag{4.1}$$

Where Y is the PCP concentration in ppm and (Area) is the area of the PCP peak on the chromatogram. the regression coefficient $R^2$ is 0.9960. This equation could then be used to determine PCP concentrations in water samples.

Results for the separation of all other phenols from PCP using the HPLC technique are presented in Table 4.8.

It is worth noting from Figure 4.9 that each phenol can be separated very well from the other phenols using this HPLC procedure. Calibration standard graphs for each phenol were drawn of peak area *vs* quantity injected for each of the eluent mixture. The slope and regression coefficient R for each calibration $Y = aX + c$ are given. Various dilutions of each phenol could be

Table 4.8: Detection Limits at 280 nm for Each of the Phenols (based on peak area)

| **Compound** | **LDL (ppm)** | **R** | **a** | **R.T.** | **R.S.D. (%) at 10 ppm** |
|---|---|---|---|---|---|
| CHB | 0.8 | 0.9958 | 3.490 | 2.936 | 5.59 |
| CHQ | 0.8 | 0.9979 | 3.999 | 4.047 | 12.787 |
| 2-chlorophenol | 0.8 | 0.9936 | 1.928 | 5.579 | 4.826 |
| TeCHQ | 0.8 | 0.9963 | 4.415 | 8.012 | 19.544 |
| 2,4-dichlorophenol | 0.8 | 0.9966 | 2.138 | 12.66 | 4.057 |
| 1,4,6-trichlorophenol | 0.8 | 0.9978 | 1.696 | 15.34 | 4.576 |
| PCP | 0.6 | 0.9960 | 0.913 | 18.275 | 6.180 |

LDL = Lowest Detecting Limit in ppm;
R = Regression Correlation Coefficient;
R.T.= Retention Time;
R.S.D. = Relative Standard Deviation;
CHB = 2-chloro-1,4-dihadrozybenzene;
CHQ = 2,5-dichlorohydroquinone;
TeCHQ = 2,3,5,6-tetrachloro-1.4-benzenediol;
PCP = Pentachlorophenol.

Table 4.9: The Variability of HPLC Analysis of PCP and Phenols Standards (all concentrations in ppm)

| **Phenols** | **Mean Concentration (ppm)** | **Standard Dev.** | ***CV%*** |
|---|---|---|---|
| PCP | 20.94 | 0.60 | 2.87 |
| CHB | 20.12 | 0.38 | 1.87 |
| CHQ | 20.94 | 0.62 | 3.01 |
| 2-chlorophenol | 20.65 | 0.63 | 3.06 |
| TeCHQ | 20.23 | 0.37 | 1.85 |
| 2,4-dichlorophenol | 19.10 | 0.36 | 1.88 |
| Trichlorophenol | 19.91 | 0.61 | 3.03 |

run under identical conditions. The lowest detectable limit was determined by the integrator's capacity to integrate the peak arising from the injected sample.

From the results in Table 4.9 the coefficient of variation for HPLC analysis of PCP and the other phenols is very low. Thus it can be recognized as a reliable method for determining PCP and phenols concentration. Numerous other analysis of standards containing PCP and PCP-containing water samples carried out with this HPLC have shown similar good reproducibility. Obviously the HPLC method developed is very reliable for the analysis of PCP in water samples.

### 4.1.2 Conclusions

HPLC is a very convenient method for accurately, directly and rapidly determining PCP concentrations in wastewater samples. Although analysis time for one sample run was about 50 minutes, the water sample could be directly injected to HPLC system for analysis without any sample pre-preparation. If an autosampler was installed and connected to the HPLC system, the analysis could be done on a 24 hours basis, which would give very convenient and useful

analysis of PCP in wastewater.

The method developed above was used for determining PCP and possible intermediates in water samples for all the experiments necessary for this thesis. External standards were used to calculate the PCP concentration in samples by comparison of the measured peak area of samples with the standard.

## 4.2 Decomposition of PCP in Fed-batch Reactor by Free Cells

The feasibility of biological degradation of PCP-containing wastes was examined by using an aerated fed-batch system. Basically, this series of experiments contained two parts. One was studying the PCP degradability of cells under aerobic conditions. The other was investigating the volatilization of PCP from the batch reactors.

**Degradability** The initial experiment in this series was a comparison of the degradability of PCP by *Flavobacterium sp.* only, activated sludge only, and *Flavobacterium sp.* with activated sludge together, under batch conditions as has been noted.

Concerning PCP disappearance from batch reactors it is necessary to determine the importance of abiotic removal mechanisms. Two potentially important ones are volatilization (stripping) and sorption (e.g. physical adsorption, chemisorption, partitioning, bioadsorption, etc.). Moos *et al.* (1983) used activated sludge in batch reactors to conduct analysis of the rate constant for PCP sorption isotherms onto biosolids. Their results suggested that the sorptive characteristics of the activated sludge biomass for PCP were insignificant. They revealed that the sorptive losses were only 0.31, 0.15 0.10 and 0.08% with SRT of 3.2, 7.8, 12.8 and 18.3 days, respectively, which showed sorption was a minor mechanism for PCP removal. Kirsch (1981) also found that sorption was responsible for less than 0.1% of the observed PCP removal in

acclimated activated sludge. Consequently sorption was not considered in the test of PCP removal in fed-batch reactors in this thesis. However, the contribution of volatilization to the PCP removal rate was determined.

**Volatilization** Regarding the contribution of volatilization to total PCP removal, another type of test was conducted to determine the rate of PCP volatilization (stripping) from the batch reactors. The protocol of these tests and results were described as following.

### 4.2.1 Volatilization of PCP

Although the physical and chemical characteristics (PCP's boiling point is 309°C) of PCP suggest that it would not be susceptible to volatilization at room temperature, a long term air stripping test was performed to determine if any volatilization of PCP from the fed-batch reactor contents occurred. A stripping test was done by spiking PCP-containing authentic wastewater with sufficient nutrients into a sterile Erlenmeyer flask reactor. The flask was aerated. Any water lost by evaporation was replaced daily with deionized water. Over a two-week period samples were periodically removed from the flask and an HPLC analysis for PCP was done. Some volatilization did occur. See Figure 4.12.

Assuming that the equilibrium concentration of PCP in the incoming air was zero, the first-order rate constant for the volatilization (K) was determined by plotting the natural log of the PCP concentration versus time and measuring the slope. The result is shown in Figure 4.12.

The value of K was -0.0031 $day^{-1}$, calculated from the slope of the graph. According to the description of Metcalf & Eddy (p. 1207), an estimate of the contribution by volatilization to the removal of PCP from the batch reactor can be expressed by equation below:

$$R_v = KSV$$

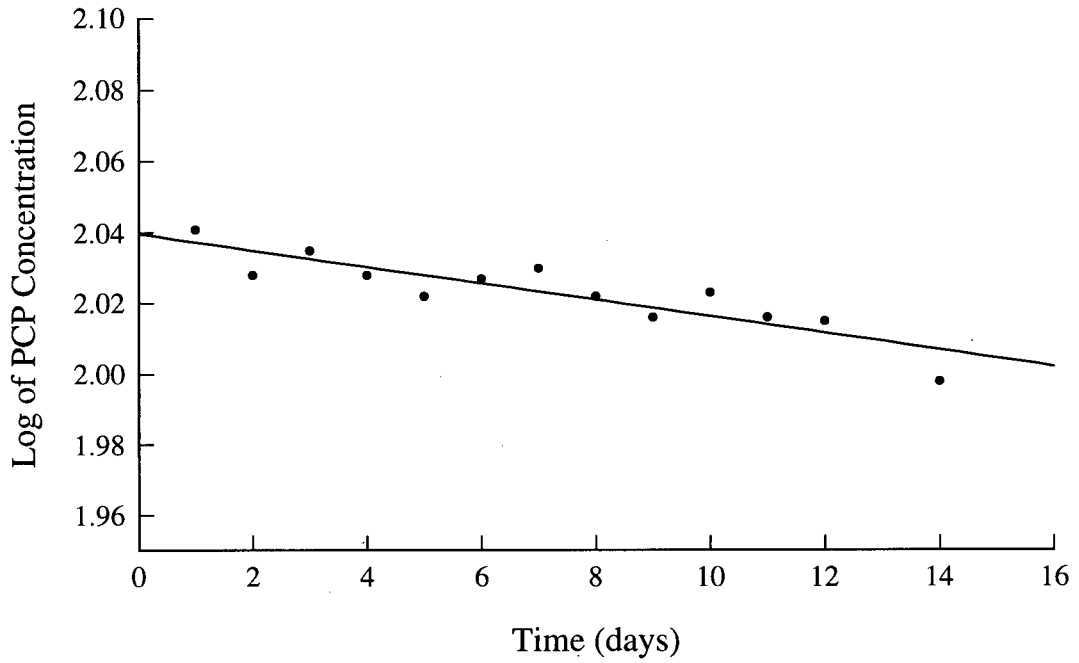



Figure 4.12: First-order Plot for Physical Removal of PCP in Batch Reactor

Where $R_v$ is the mass removal rate of PCP by volatilization (mg/day); K is the first-order rate constant ($day^{-1}$); S is the mean PCP concentration in the reactor (mg/L); and V is the reactor volume (L). Calculations of the stripping losses were made for each reactor, based upon the assumption that the value of K is the same in all reactors and equal to the value measured experimentally in the stripping test, i.e. -0.0031 $(day)^{-1}$. Calculation results showed that the maximum volatilization loss was only 0.87% of the PCP.

From the results of this experiment the conclusion can be drawn that the volatilization contributes of insignificant amount to the removal rate of PCP from the batch reactor contents.

The contribution of volatilization to the removal of PCP in the continuous reactors would be even lower because the hydraulic retention times in those systems were much smaller than the values employed in the volatilization test. The shorter the hydraulic retention times, the less the importance of volatilization. Thus for a given influent loading rate, the choice of reactor

volume will have an impact upon the importance of volatilization.

Even though there is quite a number of papers published about the biological removal of PCP, not very many papers were found in the literature search regarding the contribution of PCP volatilization to the PCP mineralization by microorganisms. Only two papers were found. These had totally conflicting results.

Moos *et al.* (1983) conducted an analysis of the rate constant for PCP volatilization in batch reactors. The concentration of PCP used for volatilization test was 0.1 mg/L. The test of PCP volatilization from the reactor showed that the maximum volatilization loss was only 0.037%. Volatilization of PCP was considered to be insignificant for PCP removal. The test was done using activated sludge cells in batch mode. Lamar *et al.* (1990) tested the mineralization and volatilization of PCP by *Phanerochaete chrysosporium* and *Phanerochaete sordida* cultures. A significant amount of volatilization from all cultures was found in their study, ranging from 1.05% volatilization to 9%. HRT values were not given in their paper.

According to the results of air stripping tests done in our laboratory, the volatilization of PCP from the reactor contents was very small. It was neglected in the following study of PCP degradation in either fed-batch reactors or continuous reactors.

### 4.2.2 Degradability of PCP by Free Cells in Fed-batch Reactor

Fed-batch experiments were conducted in 2-liter Erlenmeyer flasks with working volume of 1 liter. As has been noted in Chapter 4, fed-batch reactors were inoculated with free cells, either activated sludge only; *Flavobacterium sp.* only; or *Flavobacterium sp.* with activated sludge together. PCP was added as the only carbon and energy source, no supplementary carbon source was added at the same time. Samples from the supernatant of the cultures were removed periodically and filtered through a 0.4 $\mu$m polycarbonated membrane filter, and then analyzed for

pentachlorophenol concentration using the HPLC method as described previously.

The initial PCP concentration in the reactors was 10 mg/L. Whenever PCP was found to have disappeared from the supernatant, a new PCP degradability test, with a higher initial PCP concentration, was started. Thus three others were done with PCP concentrations of 30 mg/L, 50 mg/L and 65 mg/L respectively.

**Fed-batch Reactors with Activated Sludge only**

Fed-batch reactors #1 and #2 were inoculated with activated sludge only. After 10 mg/L PCP were added, aeration was provided continuously, and the change of PCP concentration in the reactors was monitored. It was expected that acclimation of the activated sludge to growth on PCP would be accomplished through this process. Cell growth was monitored by measuring the turbidity of the cell suspension (at 600 nm). The results are shown in Figure 4.13.

The results indicate that the growth of activated sludge cells, which could degrade PCP, was not found. From Figure 4.13, it can be seen that the turbidity of the cultures was very small (very low absorption of light at $A_{600}$) after several days. This suggests that all cells in the reactors were dead, because a decrease in bacterial numbers would result in a decreased absorption of light (or an decrease in the turbidity of the suspension). Figure 4.13 illustrates the change of absorption $A_{600}$ in the reactors, which dropped as the time went by. Further checks using a microscope verified that no live bacteria could be seen in the suspension after 6 days.

Meanwhile, analysis of chlorine concentration in the reactors showed that there was not an increase in chlorine ion concentration generated as a result of PCP decomposition, which led to the conclusion that no biological degradation of PCP had happened.

The study of fed-batch reactor seeding with activated sludge resulted in the conclusion that activated sludge was unable to acclimate to PCP degradability when PCP at 10 mg/L was used

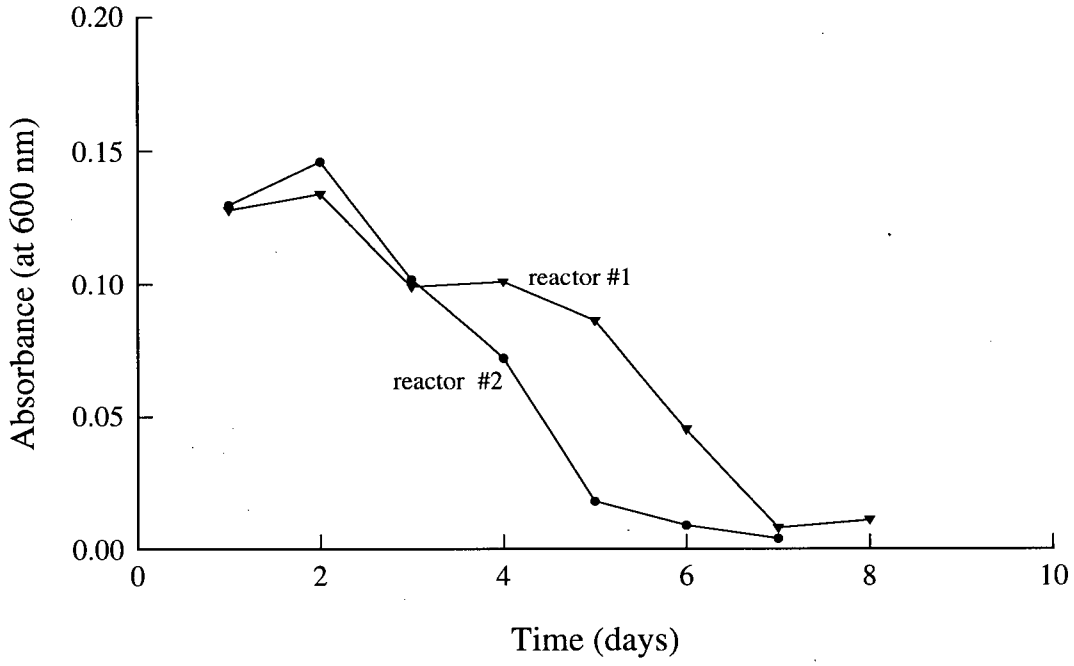



Figure 4.13: Changes of Supernatant Turbidity ($A_{600}$ in Reactors Seeded with Activated Sludge only)

initially. Kincannon and Gaudy (1967), Watanabe (1977) observed the same result as I did, i.e. that PCP at a concentration of 10 mg/L significantly inhibited the growth of PCP-degrading organisms in the activated sludge. Some published reports seem contradictory to this. Kirsch *et al.* (1981) found that organisms existed in activated sludge with the genetic capability for degrading PCP and that the acclimation procedure was satisfactory. The explanation of these contradictions need further experimentation.

### Fed-batch Reactors with *Flavobacterium sp.* only

#3 and #4 flasks were seeded with *Flavobacterium sp.* to test the cell ability to degrade PCP in water. The result, showing the changes of parameters such as PCP concentration, pH, and chlorine ion concentration in each fed-batch reactor seeded with *Flavobacterium sp.* are illustrated in Figures 4.14 to 4.16.

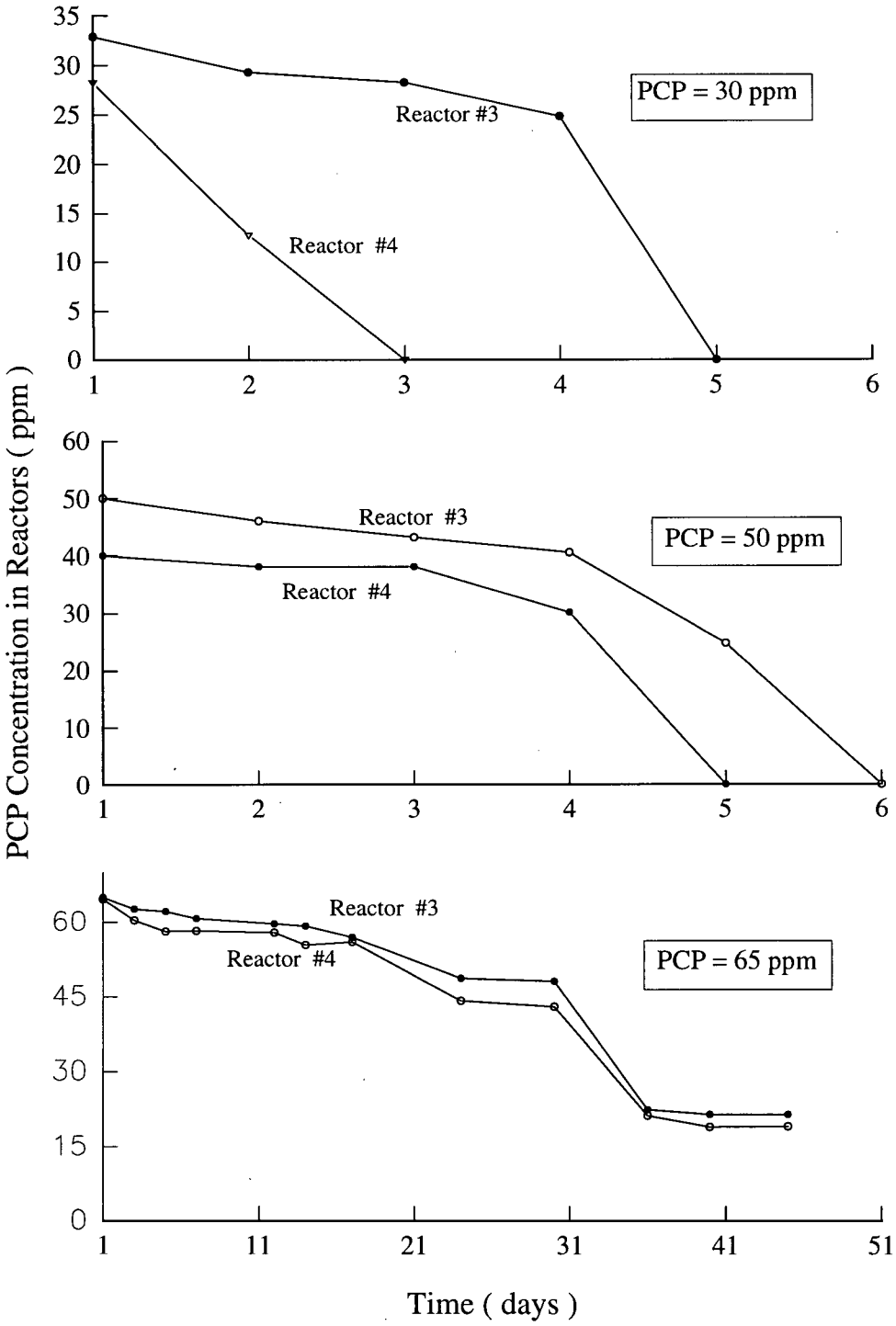



Figure 4.14: PCP Changes in Reactors Seeded with *Flavobacterium sp.* only

Figure 4.14 shows PCP disappearance from the reactors seeded with *Flavobacterium sp.* only. Figure 4.16 shows the increase in chlorine ion concentration in the reactors as PCP concentration decreased. These results demonstrate that *Flavobacterium sp.* is able to cause extensive degradation of PCP. Biodegradation was demonstrated by the disappearance of PCP, and the increase of chlorine ions in the fed-batch reactors. *Flavobacterium sp.* was able to grow very well when using PCP as the only source of carbon and energy.

*Flavobacterium sp.* demonstrated rapid degradation of PCP at initial concentrations of 30 mg/L and 50 mg/L. It is of interest to notice that even at an initial PCP concentration of 65 mg/L the cells retained their degradation ability. From Figure 4.14, it can be seen that *Flavobacterium sp.* cells could only partially degrade PCP even after cells were left in the reactors for more than 30 days. PCP concentration remained almost unchanged after 35 days at around 20 ppm level. These results are not in agreement with the results of others (Saber and Crawford, 1985; Xun and Orser, 1992; Steiert and Crawford, 1986; Matinson *et al.*, 1986). According to Saber and Crawford's study (1985) *Flavobacterium sp.* isolated from PCP-contaminated soils could completely degrade a suspension containing 200 ppm of PCP into $CO_2$ in the rather short time of 2.5 days. Xun and Orser's report showed *Flavobacterium sp.* ATCC 39723 could decompose PCP from 70 ppm to 0 ppm in 4 days. Steiert and Crawford (1986) reported *Flavobacterium sp.* could completely degrade 50 ppm PCP in 3 hours. Martinson *et al.* (1986) also reported that *Flavobacterium sp.* could completely decompose 100 ppm PCP to undetectable levels, usually within 48 hours.

Figure 4.15 demonstrates a slight change in pH in the reactors as time passed. As the PCP degradation proceeded, chlorine ion concentration increased, and pH dropped in the fed-batch reactors. Adjustment of the pH back up to 7.5 was made using 0.2 NaOH because the pH should be maintained at as constant a level as possible commensurate with adequate bacteria growth. Decomposition of PCP by *Flavobacterium sp.* in water is most effective between pH 7.5 and

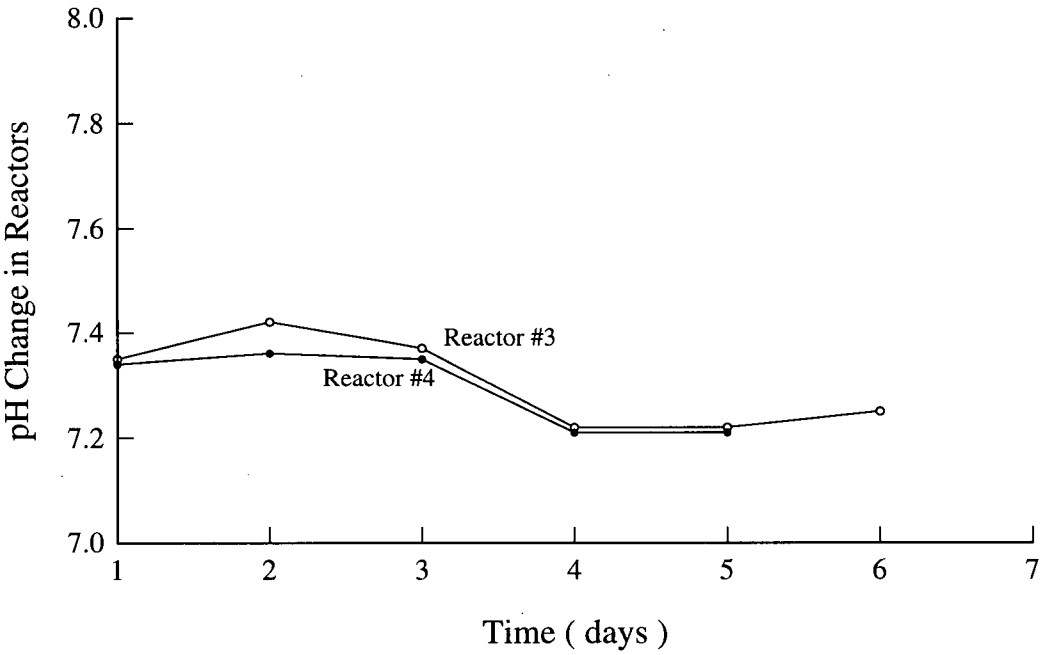



Figure 4.15: pH Changes in Reactors Seeded with *Flavobacterium sp.* only

pH 9.0 as has been noted.

If PCP is degraded biologically, a dechlorination reaction should happen. As a result the concentration of chlorine ions ($Cl^-$) would increase in the reactors. Moreover if PCP were completely degraded the total measured concentration of chlorine ions in the reactor should correspond to the concentration of chlorine ions calculated from the initial known amount of PCP.

Figure 4.16 demonstrates the change in chlorine ion concentration and shows that the concentration of chlorine ions did increase in the reactors, and that dechlorination had happened. In short, stoichiometric amounts of free chloride were detected which confirmed the degradation of PCP in the fed-batch reactors seeded with *Flavobacterium sp.* However the measured concentrations of chlorine ions (solid lines in Figure 4.16) in the reactors did not correspond to the calculated concentration of chlorine ions (dotted lines in Figure 4.16). This leads to the hypothesis that the total disappearance of PCP from the fed-batch reactors to some extent results from

other removal mechanisms which do not contribute to free chloride generation, rather only to biodegradation of PCP. Further study is needed to figure out what else (sorption, or photolysis, or probably other mechanisms) contributes to the PCP disappearing from the batch reactor, and how much such mechanisms would contribute to the total PCP removal.

Even though an attempt was made to try to find intermediates resulting from PCP degradation, no positive results were obtained from the fed-batch study. Why this came about needs further research work.

**Fed-batch Reactors with both *Flavobacterium sp.* and activated sludge**

The degradability of PCP was also studied in fed-batch reactors seeded with both activated sludge and *Flavobacterium sp.* cells (ATCC 39723). Figures 4.17 to 4.20 illustrate the profiles of the degradation parameters: PCP concentration, chloride ion concentration, pH and optical density at 600 nm, in each fed-batch reactor seeded with both activated sludge and *Flavobacterium sp.*.

Figure 4.17 illustrates that PCP disappeared from the reactors seeded with both *Flavobacterium sp.* and activated sludge. Figure 4.18 illustrates that chlorine ion concentration increased significantly in those reactors as PCP was disappearing. It is noteworthy that biodegradation in the reactors seeded with *Flavobacterium sp.* and activated sludge definitely happened. This result is very valuable because it suggests that inoculation of PCP-degrading *Flavobacterium sp.* to activated sludge system, or to natural waters containing PCP is an effective decontamination technique for removing PCP from a contaminated environment. Stated in another way, if *Flavobacterium sp.* were inoculated into an activated sludge system, PCP contained in the influent could be removed biologically without acclimating activated sludge to the toxic chemical. Martinson *et al.* (1986) tried to inoculate *Flavobacterium sp.* into natural water polluted

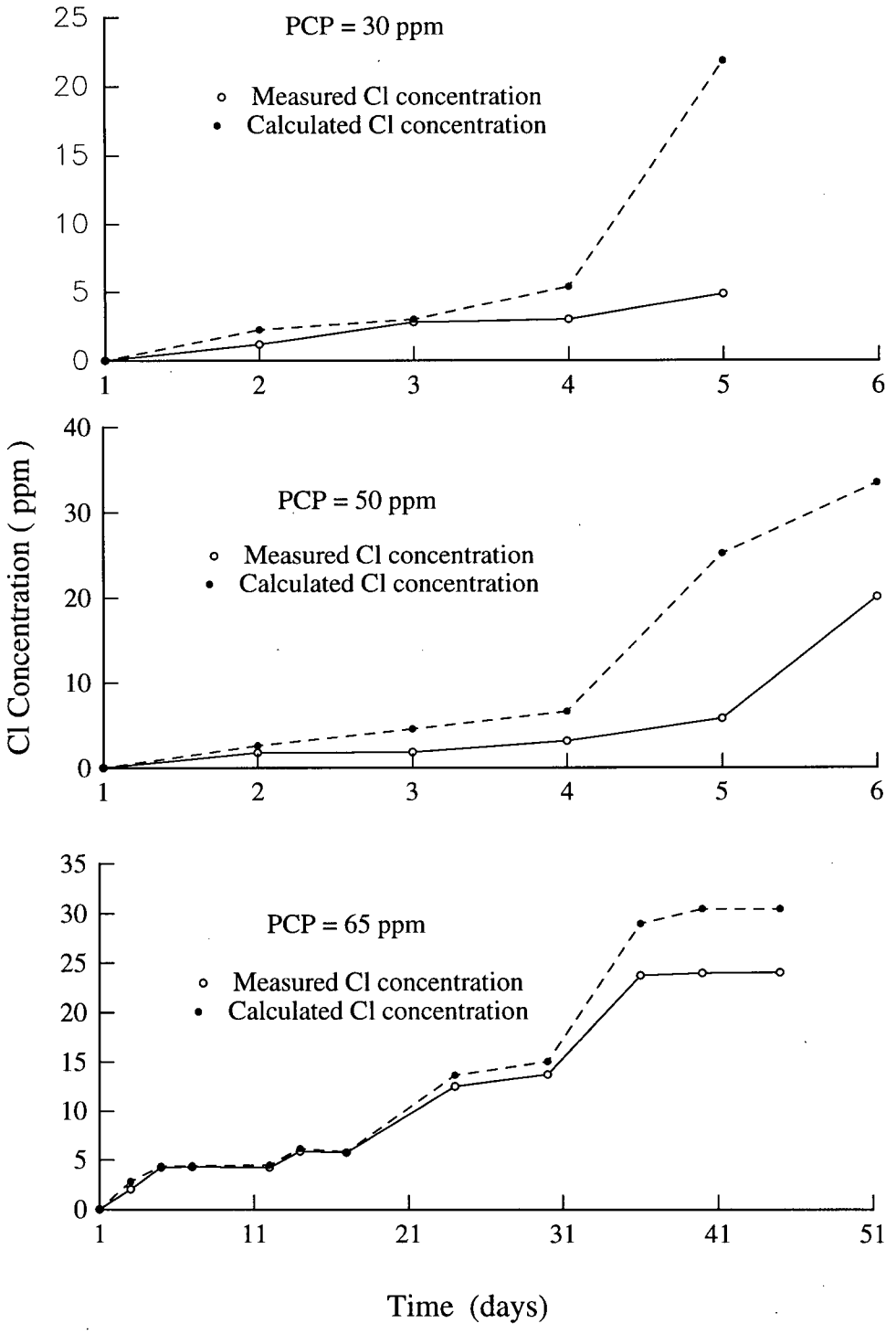



Figure 4.16: Changes of the Concentrations of Free Chloride Ions in Reactors Seeded with *Flavobacterium sp.* only

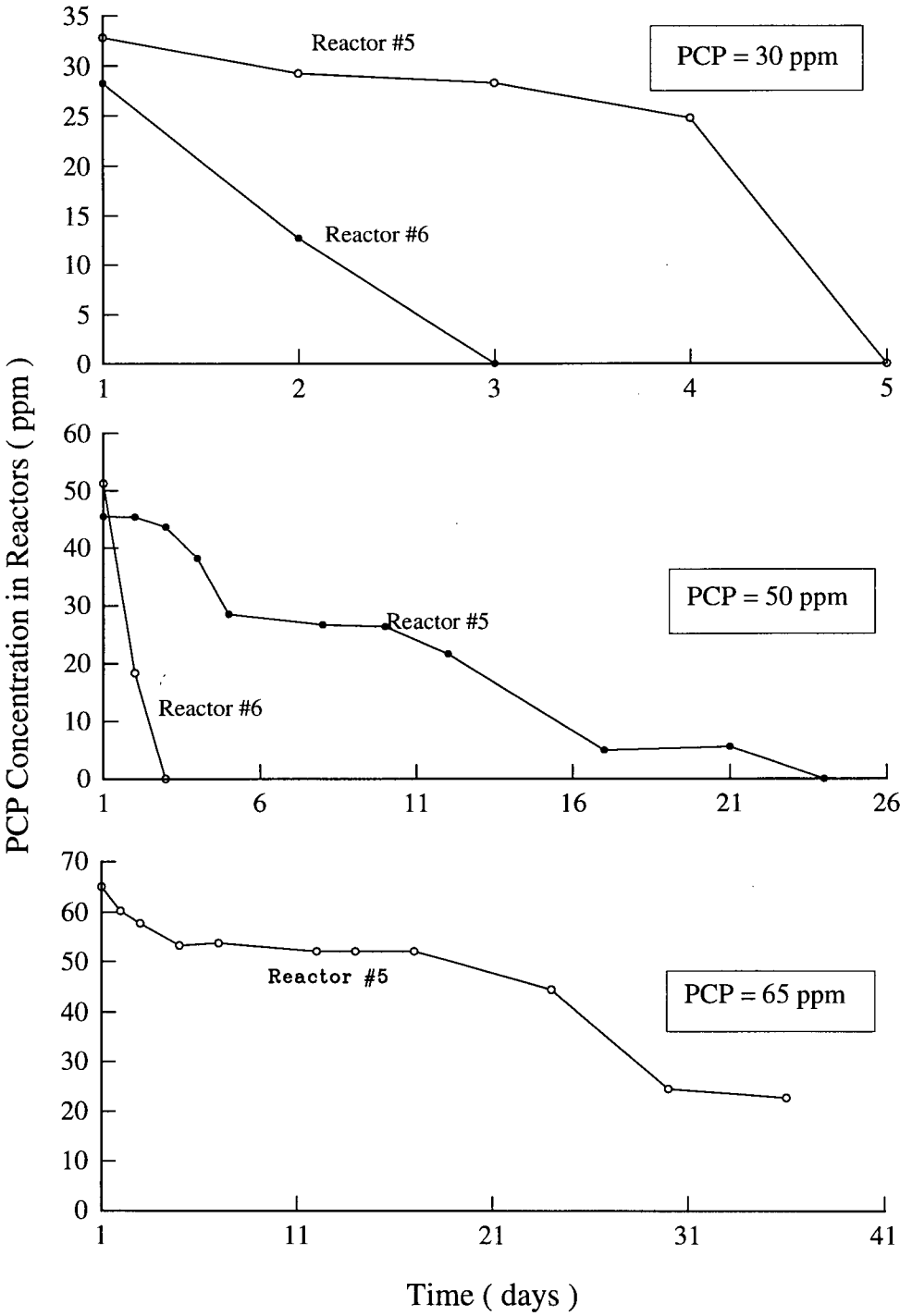



Figure 4.17: PCP Changes in Reactors Seeded with both *Flavobacterium sp.* and Activated Sludge

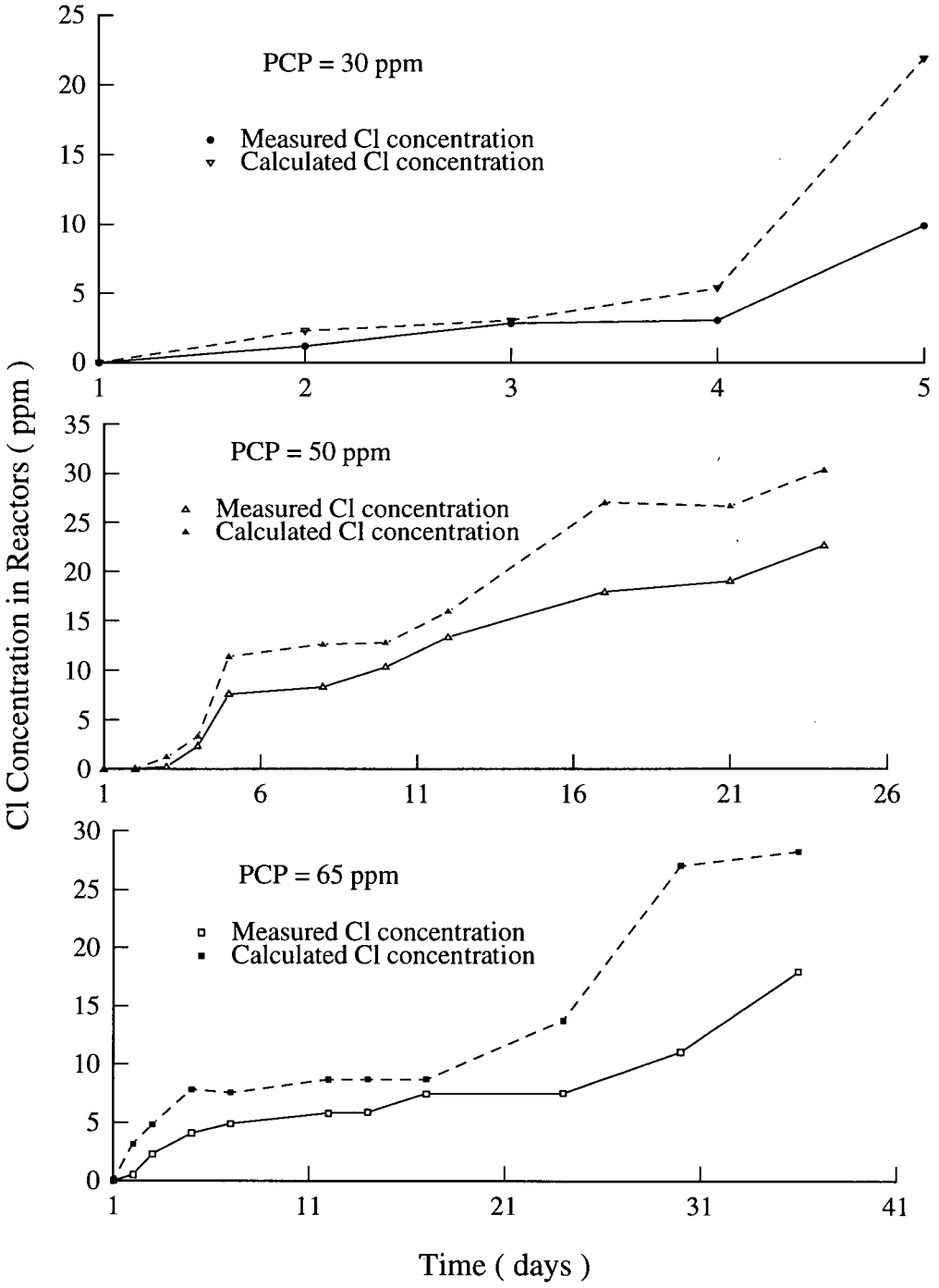



Figure 4.18: Changes of the Concentrations of Free Chloride Ions in Reactors Seeded with *Flavobacterium sp.* and Activated Sludge

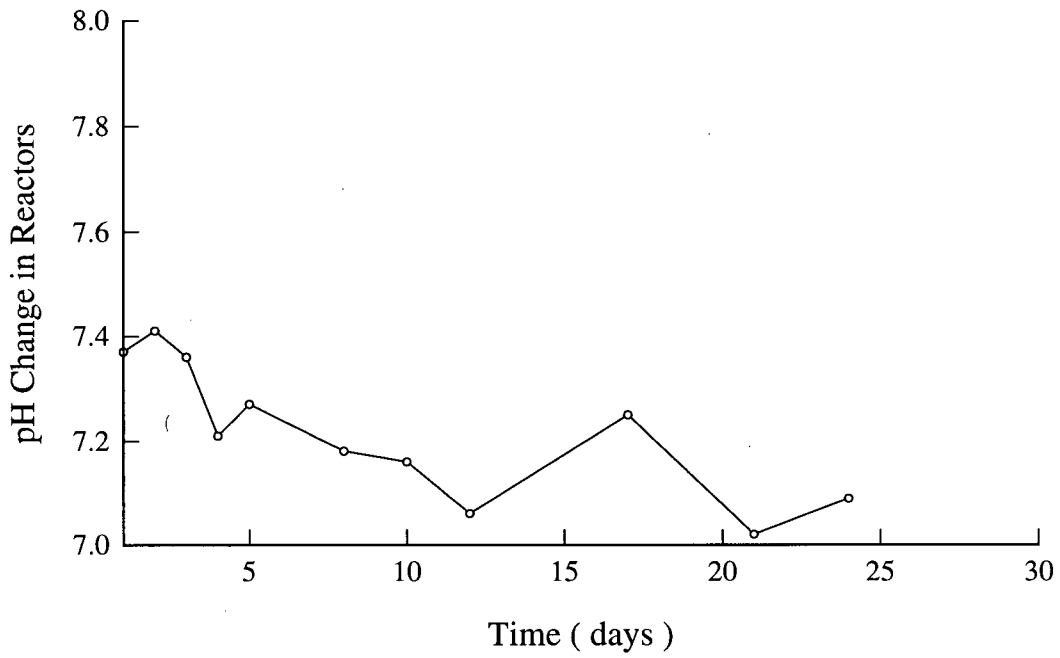



Figure 4.19: pH Changes in Reactors Seeded with *Flavobacterium sp.* and Activated Sludge

with PCP, and examined the removal efficiency. Their results suggested that PCP was removed from natural water by *Flavobacterium sp.* PCP concentrations ranging between 10 ppb and 100 ppm were decontaminated equally well, and usually reduced to levels below the detection limit of about 0.1 ppb. It is a feasible method to inoculate PCP-degrading *Flavobacterium sp.* into some biological waste treatment systems or natural waters for biological removing PCP or other phenol from such environment.

The changes of pH (Figure 4.19) in the reactors with *Flavobacterium sp.* and activated sludge were found to be very similar to those of the reactors with *Flavobacterium sp.* only (Figure 4.15). PCP degradation occurred, the cell mass increased, chlorine ion concentration increased, and pH dropped. In the same way, the measured concentration of chlorine ions increased with time but was always less than the calculated concentration of chlorine ions.

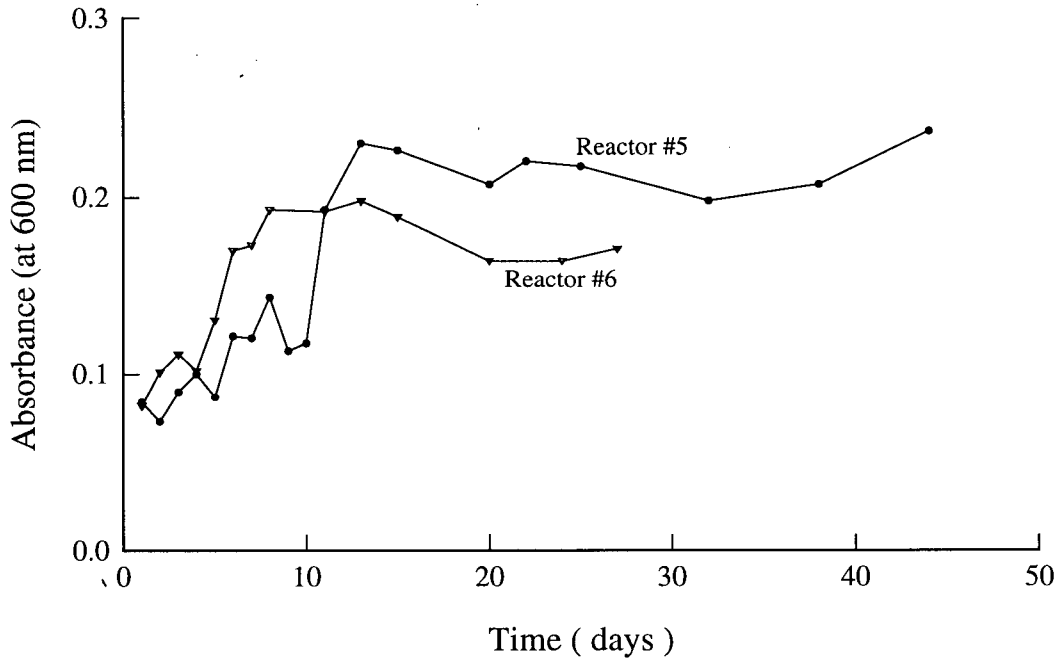



Figure 4.20: $A_{600}$ Changes in Reactors Seeded with *Flavobacterium sp.* and Activated Sludge

However, comparing the changes in PCP, chloride ions ($Cl^-$), and pH in batch reactors under those two conditions, seeding with *Flavobacterium sp.* only (mentioned as Condition-1), and with *Flavobacterium sp.* and activated sludge together (Condition-2) , it is worth noting that better replicate results were achieved under Condition-1, whereas PCP degradations were quite variable under Condition-2. Under Condition-2, reactor #6 finished degrading 50 ppm of PCP in 3 days, but reactor #5 did not finish degrading 50 ppm of PCP until 24 days had passed. Obviously activated sludge affects the degradability of PCP in some unknown ways.

### 4.2.3 Filtered Samples and Non-filtered Samples

Because an HPLC column is very expensive and needs very special care, samples with any particles in them would destroy the column and make analysis unreliable and unreproducible.

Table 4.10: The Effects of Filtering on PCP Concentration When PCP=107 ppm

| | **PCP Concentration(ppm)** | | |
|---|---|---|---|
| **Replicate** | **Filtered** | **Non-filtered** | **Difference (%)** |
| 1 | 100.72 | 106.63 | 5.54 |
| 2 | 103.69 | 107.44 | 3.49 |
| 3 | 103.91 | 107.32 | 3.18 |
| 4 | 100.16 | 105.42 | 4.99 |
| 5 | 102.71 | 107.16 | 4.15 |
| 6 | 101.06 | 105.19 | 3.92 |
| 7 | 100.57 | 105.56 | 4.72 |
| | | $\mu$ | 4.07 |
| | | Standard Dev. | 0.64 |
| | | *CV%* | 15.69 |

Therefore water samples were initially filtered through a 0.4 $\mu$m polycarbonate membrane filter before going to HPLC analysis. The filtrate was collected in a sample vial and injected into the HPLC system for analyzing PCP concentration as the method described before. It was not expected that would be any difference between the original samples and the filtrate. But the question was raised before starting the continuous reactor experiments: whether or not some of PCP in original samples would stick in the polycarbonated membrane filter instead of going completely through to the filtrate? Some preliminary experiments were done to try to figure this out.

A series of standard PCP solutions was run through the HPLC analysis respectively under conditions of either filtered or non-filtered. Results are demonstrated in Table 4.10 and Table 4.11 for PCP = 107 ppm and PCP = 20 ppm respectively.

From the results in Table 4.10 and Table 4.11, it can be seen that the filter used could retain on average 4.07% PCP from passing the filter at PCP concentration of 107 ppm, however, an

Table 4.11: The Effects of Filtering on PCP Concentration When PCP=20 ppm

| | **PCP Concentration(ppm)** | | |
|---|---|---|---|
| **Replicate** | **Filtered** | **Non-filtered** | **Difference (%)** |
| 1 | 14.96 | 20.08 | 25.49 |
| 2 | 16.79 | 20.79 | 19.24 |
| 3 | 15.87 | 19.52 | 18.66 |
| 4 | 17.14 | 18.91 | 9.36 |
| 5 | 18.93 | 19.82 | 4.49 |
| 6 | 15.79 | 18.11 | 12.81 |
| 7 | 14.82 | 20.19 | 26.59 |
| | | $\mu$ | 15.19 |
| | | Standard Dev. | 7.22 |
| | | *CV%* | 47.52 |

average 15.19% of PCP would remain on the filter, instead going to the filtrate, at a PCP concentration of 20 ppm. Moreover, the filter could cause a very big variance in the HPLC analysis results with a coefficient of variation of 15.69% at PCP=107 ppm and 47.52% at PCP=20 ppm, which means that it was not reliable method to use for filtering PCP-containing wastewater. Maximum retention of PCP was 26.59% at PCP=20 ppm, and 5.54% at PCP=107 ppm.

Therefore it can be concluded that filter with a 0.4$\mu$m pore size is not very reliable for filtering PCP-containing wastewater for HPLC analysis. The PCP concentration used in my study was around 60 ppm. The 0.4$\mu$m pore filter was then abandoned. Instead, a precolumn was connected to the HPLC system which works like a filter but will not retain any PCP according to the column manufacturer.

Literature research indicated that Klecka and Maier (1985) had done similar experiments on the filter problem. They reported that $> 90\%$ of the PCP present in a standard solution was recovered in the filtrate, The filter they used was a pore size of 0.2 $\mu$m which was smaller than the ones I was using with a pore size of 0.4 $\mu$m.

## 4.3 Decomposition of PCP in Continuous Reactor by Immobilized *Flavobacterium* Cells

The utilization of immobilized *Flavobacterium* cells to remove PCP from water was studied. Under various conditions, immobilized cells have advantages over free cells, such as, immobilization allows a high cell density be maintained in a reactor at any flow rate, and some immobilized microorganisms are able to tolerate higher concentrations of toxic compounds than their free counterparts, while degrading toxic compounds at faster rates.

Although the technique of immobilization of microorganisms is a widely applied in drug manufacturing, food processing, and biological waste treatment, few studies have been reported regarding the utilization of immobilized *Flavobacterium sp.* cells to degrade PCP. PCP degrading *Flavobacterium sp.* were effectively immobilized in alginate, and then used in the continuous reactors to study the PCP degradability efficiency of such immobilized cells.

### 4.3.1 Continuous Reactors with Immobilized *Flavobacterium* Cells

The experimental setup of the continuous reactor is shown in Figure 3.5. In the first place, two types of reactors with different configurations were studied to determine if there was any unaccounted for scale-up factor (such as a wall effect) on the performance of the packed-bed reactor. Column A was glass tube with working volume of 180 ml (about 4.8 cm i.d. x 60 cm long). Column B had a working volume of 1500 ml (approximately 100 $cm^2$ cross sectional area and 15 cm bed height).

For the glass column A, however, the beads containing *Flavobacterium sp.* did not fluidize well in the reactors, instead they clumped together at the top of the column. This resulted in a pressure build up in the column. The alginate beads did not remain intact. Their structure began to deteriorate within 12 to 24 hours. This column A was not considered to be practical, and so was not used in the following study.

Table 4.12: Operating Conditions of Continuous Reactors for PCP Degradation

| **Run** | **Influent Flowrate** (ml/min) | **HRT** (hours) | **PCP in influent** (ppm) |
|---|---|---|---|
| 1 | 0.3 | 75.75 | 59.11 |
| 2 | 0.66 | 37.88 | 60.99 |
| 3 | 1.38 | 18.11 | 60.88 |
| 4 | 1.58 | 15.82 | 60.03 |
| 5 | 3.15 | 7.93 | 60.01 |

For the column B reactors, the reactor was prepared by adding 67 g of beads and operating at an air flow rate of approximately 0.50 L/min, which was sufficient to suspend the beads in the reactor. A feed stream (influent) of synthetic wastewater containing PCP and nutrients was introduced from the bottom of reactor at different rates. Three reactors were set up with one working as control, other two working as replicates. The reactors were run at a series of flow rates with about 60 ppm of PCP. Five different loading rates of influent were utilized to test the treatabilities of PCP by immobilized *Flavobacterium sp.* cells in the continuous reactor. The operating conditions of continuous reactors for PCP degradation are described in Table 4.12 (hydraulic retention time is based on empty bed volume). Whenever a new flow rate was started, newly-made beads replaced the old ones. This kept each run at identical starting conditions except for flowrate. After steady state had been reached for a given flow rate, the effluent PCP concentration and PCP degradation rate were determined. Results are presented in Figure 4.22 to Figure 4.27.

**Beads stability** Several factors will affect the stability of the alginate beads. Alginate is a natural resin and is relatively, physically weak being easily biodegraded within a short time. High flow rates can damage the bead structure. The stability of the alginate beads with immobilized *Flavobacterium sp.* was tested when the influent with PCP at 60 ppm was at a flowrate

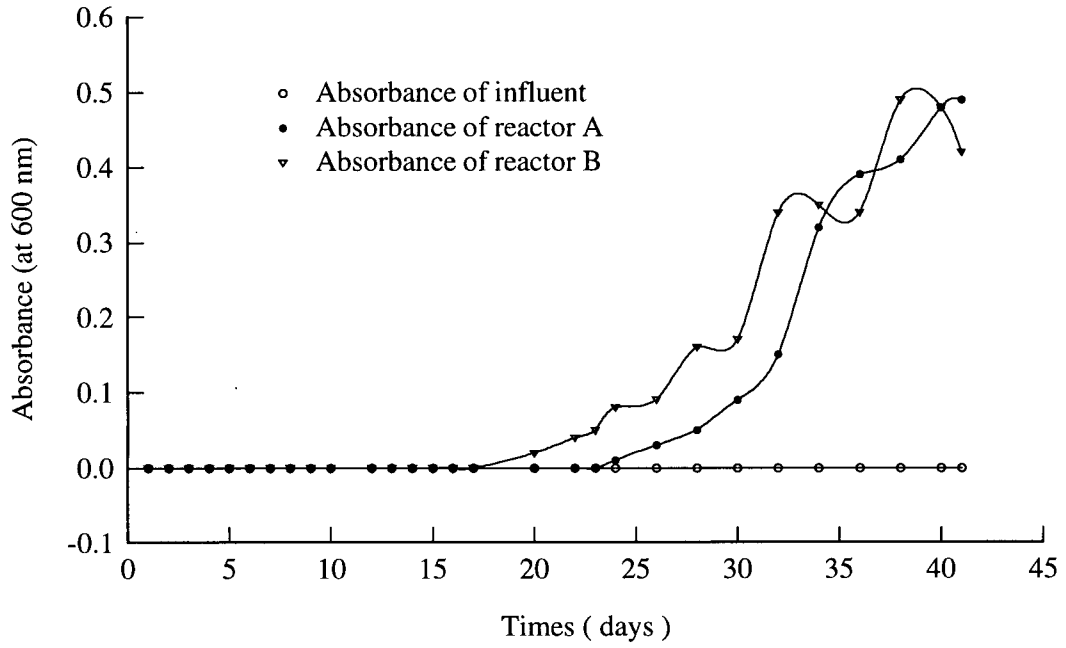



Figure 4.21: Turbidity Changes in Continuous Reactors

of 0.66 ml/min. It was observed that visible bead breakage was found on Day 29. No more alginate beads were found existing in the reactors after day 38. However, From Figure 4.21 (Absorption change in the reactors), we can see that the turbidity ($A_{600}$) of the solution in the continuous reactors was starting to increase from day 20, which demonstrated that free *Flavobacterium* cells were being released from the alginate beads. Further confirmation was done by septically streaking small amounts of solution onto plates which contained medium and substrate. *Flavobacterium sp.* were observed to be growing. This result confirmed that free *Flavobacterium* cells really existed in the reactors which were released from the alginate beads or from breakage of the beads.

From Figure 4.22, which displays curves describing the PCP concentration in the effluent at a flow rate of 0.66 ml/min, it can be seen that alginate beads with immobilized-*Flavobacterium sp.* worked very efficiently to remove PCP before day 30. After this some beads started to break releasing a large amount of free cells. These free cells could be washed out of the continuous

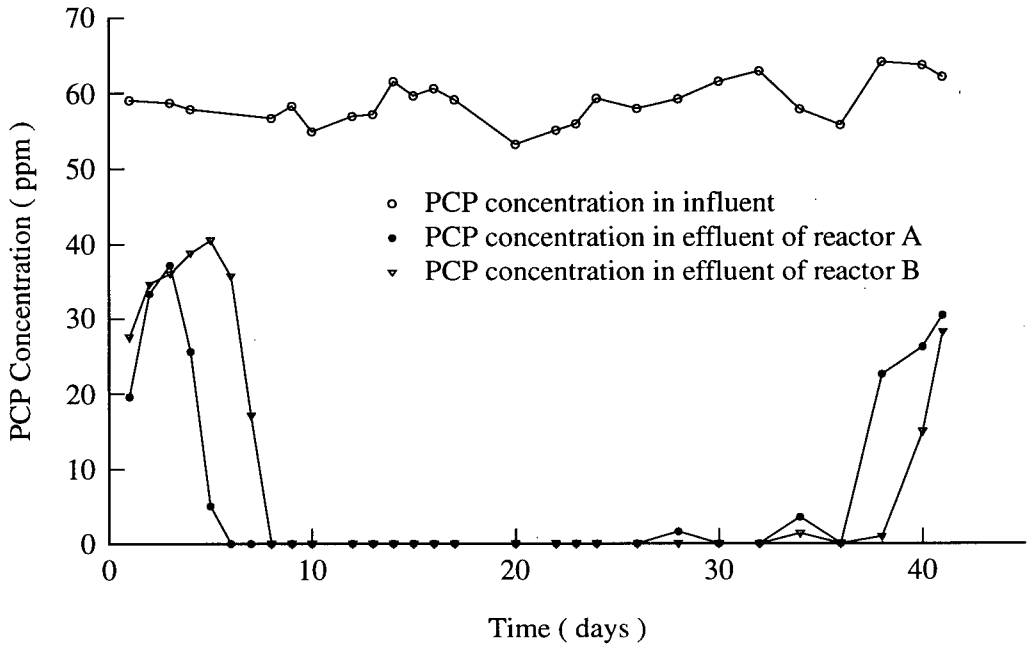



Figure 4.22: Changes of PCP Concentrations in Effluents

reactors, resulting in a decrease in cell numbers in the reactors and a decrease in PCP degradation.

Obviously, at this modest condition, alginate beads could not last longer than 38 days. Therefore it is believed that alginate beads are not a preferred immobilization matrix for the immobilization of cells in these kind of reactors because they lack the necessary mechanical strength. However, work done with alginate beads containing immobilized *Flavobacterium sp.* still has been very valuable in two respects: it can test how the reactor works, and allow prediction of the reactor efficiency and it can predict the PCP degradation efficiency by immobilized cells. This is very valuable information for future design of full-scale reactors using immobilized *Flavobacterium sp.* to degrade PCP.

**The concentration of chloride ions** The change of concentration of chloride ion was not

monitored in the immobilized-*Flavobacterium sp.* continuous reactors. The reason was that alginate beads were made from alginate-cells suspension mixed with calcium chloride. So chloride ions might be trapped in the alginate, which could then be released later from the beads during PCP degradation. It would be impossible to tell which part of the chloride ions came from the original calcium chloride trapped by the beads, and which came from the release by PCP degradation. The control continuous reactor with cell-free alginate beads confirmed this hypothesis. High concentrations of free chloride ions existed in the solution in the control reactor, where no PCP degradation happened. Hence the concentrations of chloride ions were not monitored in the test of PCP degradation by immobilized-*Flavobacterium* cells.

**Food-to-microorganism** Figure 4.23 describes the effect of food-to-microorganism ratio on the efficiencies of PCP degradation by immobilized-*Flavobacterium sp.* According to Metcalf & Eddy (1990), the food-to-microorganism ratio is a commonly used parameter in biological wastewater treatment systems. The relationship of food-to-microorganism ratio to the process efficiency (%) is described as:

$$F/M = \frac{U*100}{E}$$

Where F/M is food-to-microorganism ratio, and E is process efficiency. We can see from the relationship that the bigger the food-to-microorganism ratio, the smaller the process efficiency. Stated in another way, at big food-to-microorganism ratios relatively less microorganisms work on food (PCP in this case) conversion, thus resulting in a smaller degradation rate of substrate. In order to test the effects of the ratio of loading PCP (food) to immobilized-*Flavobacterium sp.* on the PCP degradation, initially four different ratios of food-to-microorganism were applied at 27.91, 57.44, 63.12, 130.85 mg PCP applied/$10^{10}$cells·day, as shown in Figure 4.23. The result of the effects of the food-microorganism ratio on the PCP degradation rate are shown in Figure 4.23.

Table 4.13: The Applied Food-to-Microorganism Ratio

| **Flowrate** (L/day) | **Cells** ($10^8/cm^3$-bead) | **F/M Ratio** (mg PCP applied/$10^{10}$cells.day) |
|---|---|---|
| 0.95 | 5.04 | 27.91 |
| 1.99 | 5.10 | 57.44 |
| 2.28 | 5.19 | 63.72 |
| 4.54 | 5.37 | 130.85 |

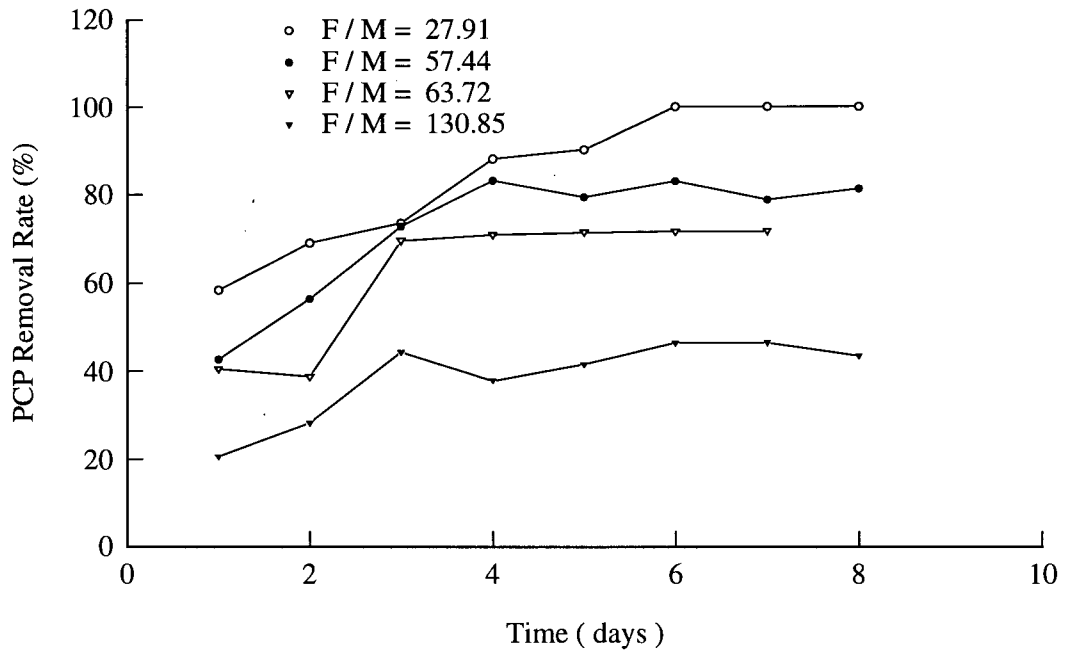



Figure 4.23: The Effects of Food-to-Microorganism Ratio on PCP Degradation

As was expected, the result indicated that the smaller the food-to-microorganisms ratio, the faster the start-up time, and the larger the removal of PCP. Thus we can conclude that if the amount of *Flavobacterium sp.* is increased by immobilization, a high percent removal of PCP is expected to be achieved even with an increasing the loading rate of substrate (PCP).

**Bead protection of immobilized-*Flavobacterium* cells from PCP toxicity** The ability of alginate-immobilized *Flavobacterium sp.* to degrade 60 ppm of PCP was tested. From the study of PCP degradability by free *Flavobacterium sp.* in fed-batch reactors done in our laboratory, the concentration of 65 ppm PCP is the concentration known to start inhibiting the growth of free *Flavobacterium* cells, and PCP could only be partially degraded at this concentration. The results from continuous reactors with immobilized *Flavobacterium sp.* are shown in Figure 4.26 for hydraulic retention times (HRT) of 7.93, 15.82, 18.11, 37.88, 75.75 hours respectively. It can be seen that 100% degradation of 60 ppm PCP could be accomplished in the reactors at $HRT = 37.88$ hours. Obviously, alginate appears to protect *Flavobacterium* cells from inactivation by high concentrations of PCP.

**Physical adsorption of PCP by alginate beads** The physical removal of PCP by alginate beads was investigated. This was done by running the control reactor under identical conditions of temperature and air flow rate (without biomass) to account for any physical removal, mainly by physical adsorption by the beads. The cell-free beads in the control reactor were made as has been noted above. The isotherm of PCP adsorption by cell-free beads generated at PCP influent flowrate of 3.15 ml/min is shown in Figure 4.24, indicating that PCP was physically adsorbed by alginate beads to the extent of 10% of the added amount. However, eventually alginate beads became saturated with PCP; for this particular case saturation happened starting from day 15. The conclusion is that physical removal of PCP by alginate beads plays an significant role in aerobic biodegradation of PCP by immobilized *Flavobacterium sp.* in a continuous reactor.

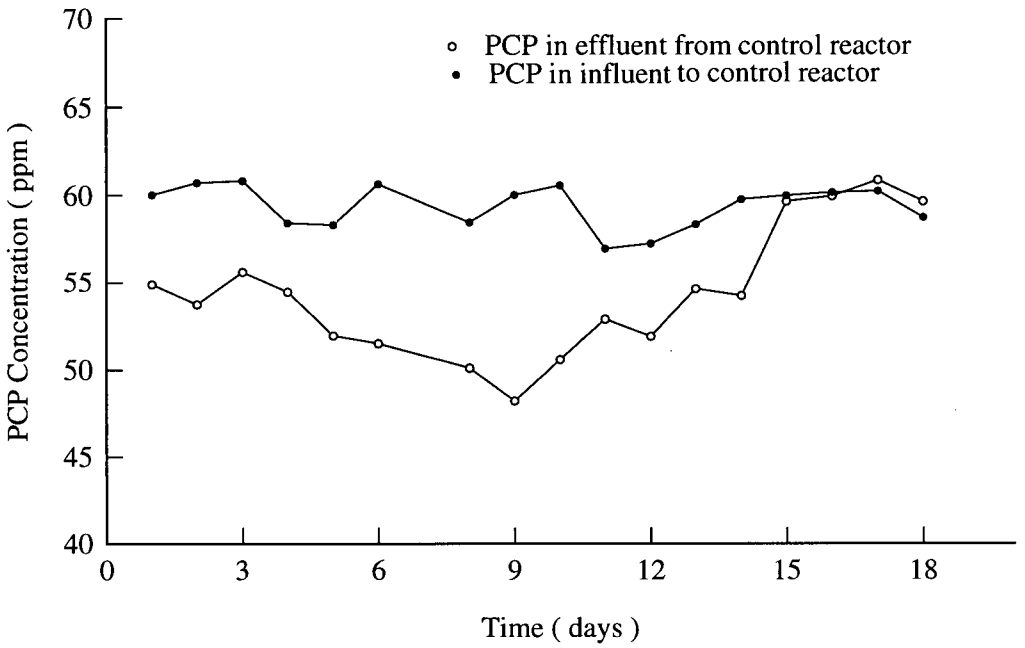



Figure 4.24: The Effects of Physical Adsorption of Alginate Beads on PCP Degradation

**Effect of PCP loading rate** The flow rate of influent may affect the performance of a biological treatment system. The effects of different loading rates of PCP influent on PCP removal rates by immobilized *Flavobacterium sp.* in continuous reactors were studied. Results are presented in Figure 4.25.

From these results we can see that PCP concentrations remaining in the effluent were zero for an influent PCP loading at 0.38-0.68 ml/min, 100% removal of PCP was achieved. As the influent PCP loading rate increased, the PCP concentrations remaining in the effluent increased. The higher the influent loading rate, the higher PCP concentrations in the effluent. This suggests that the continuous reactor with immobilized *Flavobacterium sp.* is strongly affected by the loading rate of PCP influent during PCP degradation treatment. Thus PCP loading rate is a very important parameter to be considered in reactor design.

**Effect of HRT** Hydraulic Retention Time (HRT) should be checked to make sure it provides efficient treatment in the treatment system. As the HRT of the system is increased, the time that

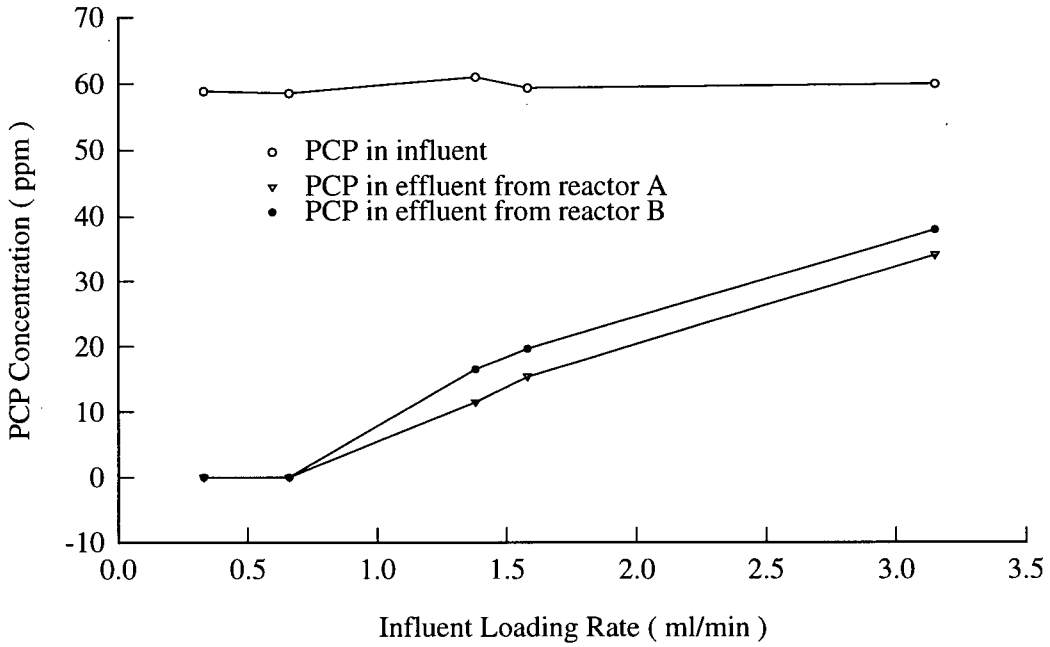



Figure 4.25: The Effects of Influent Loading Rate on PCP Degradation

cells have to work on the substrate is increased, which should result in increasing the treatment efficiency. A relationship was established for percent removal of PCP versus HRT in continuous reactors with immobilized *Flavobacterium* cells as shown in Figure 4.26.

It is obvious from Figure 4.26 that PCP removal rate decreased when the HRT decreased. For an HRT between 37.88 to 75.75 hours 100% removal could be achieved. As HRT decreased from 18.11, 15.82, to 7.93 hours, removals of PCP decreased from 81.19%, 71.61%, to 43.39% respectively. Thus, HRT is believed to be a very important design parameter for a PCP treatment system, enough HRT has to be provided so that the cells can work under optimum conditions. The dotted lines on Figure 4.26 show the PCP degradation removal considering physical adsorption of PCP by alginate beads. The solid lines are the total PCP removal rate in the continuous reactors.

**Changes in concentration of phosphate ions** Sofer *et al.* (1990) used alginate to immobilize activated sludge for studying the biodegradation of 2-chlorophenol. They reported that

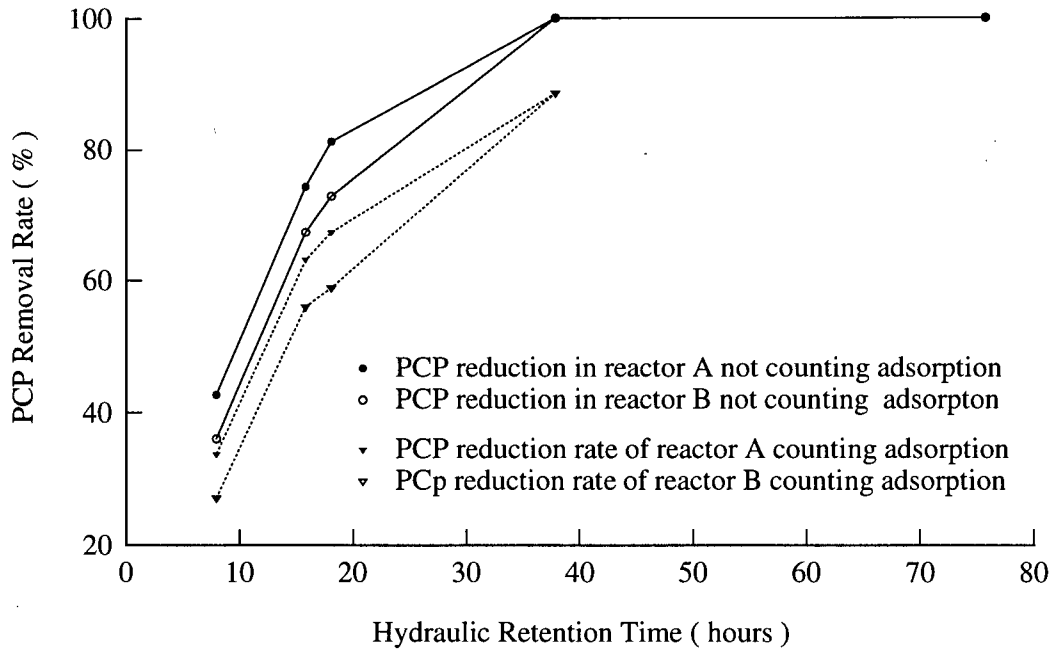



Figure 4.26: The Effects of HRT on PCP Removal

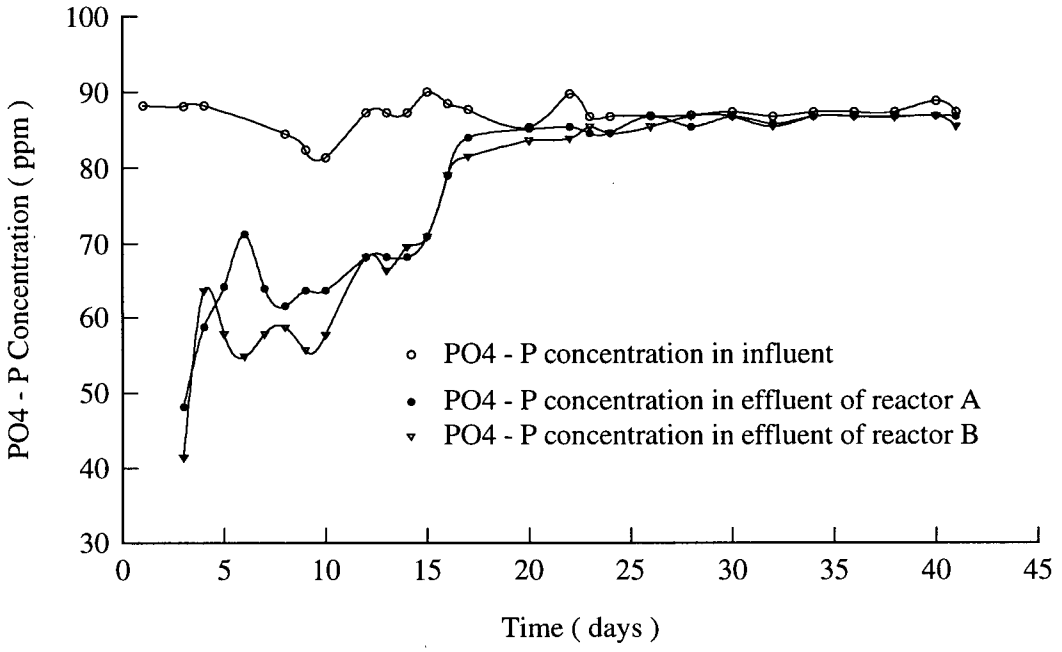



Figure 4.27: $PO_4$ Change in the Continuous Reactors

the concentration of potassium phosphate in wastewater up to 1 g/L would dissolve alginate beads within 24 hours, subsequent decreasing the concentration of potassium phosphate to 10 mg/L would allow alginate beads to work up to 1 month without any physical damage. The ones used in the batch study were at $K_2HPO_4$ concentration of 500 mg/L, and $KH_2PO_4$ concentration of 650 mg/L, which were quite high concentrations. The tests using these concentrations of $K_2HPO_4$ and $KH_2PO_4$ in wastewater were also done to check the stability of alginate beads in continuous reactors. The results confirmed that this high concentration of potassium phosphate would damage alginate beads within 48 hours. Therefore, considering this adverse effect of potassium phosphate on alginate beads, the potassium phosphate concentration used in synthetic wastewater for the continuous reactor study was reduced to $K_2HPO_4$=4.5 mg/L, and $KH_2PO_4$=5 mg/L, respectively.

The change of the concentration of phosphate ions in effluent was monitored at a PCP influent loading rate at 0.66 ml/min. Figure 4.27 shows the result.

From Figure 4.27, it can be seen that the concentration of phosphate in the reactor effluent decreased a lot compared to the influent phosphate concentration before day 20, which implies that phosphate removal was happening when PCP was degraded by immobilized *Flavobacterium sp.* However, after day 20, the concentration of phosphate became almost the same as the influent. This may have occurred because after day 20 some *Flavobacterium* cells were dead and released phosphate from their cell structures. This may have provided enough phosphate for the needs of the live *Flavobacterium* cells. Therefore there was no further change in the concentration of phosphate in the influent and effluent. Further research is needed to confirm this hypothesis.

This unexpected result suggests that PCP degradation may enhance nutrient phosphate removal in biological treatment. An interesting future research topic would be the effect of PCP on biological phosphate removal in biological treatment systems if the proper strategy is used.

In the study of both batch reactors and continuous reactors, no intermediates were found by HPLC analysis. One reason is probably that the intermediates from such a biodegradation were at very low concentrations, below the HPLC detecting limits. Another reason could be that the intermediates generated are easy to degrade thus not enough can accumulate in the effluent to be detected. According to Suzuki's (1977) research report, who used a KC-3 culture to degrade PCP, TeCHQ and TCC from PCP degradation could be degraded rapidly as soon as they were produced. Moreover the intermediates were present in extremely small concentrations, only 0.005 to 0.02% TCC and 0.2 to 0.4 % TeCHQ of the original PCP could be produced. That means that 60 ppm PCP would only produce maximum concentrations of 0.012 ppm TCC and 0.24 ppm TeCHQ, which are far below the HPLC detecting limits of these chemicals. Xun and Orser (1991) used *Flavobacterium sp.* to study degradation of PCP, and reported that the intermediate TeCHQ was detected in their experiment, however in the same way, TeCHQ was unstable and at a very low concentration of 0.009 ppm. This is below the detecting limit of

TeCHQ by the HPLC used in my study. Further study regarding intermediates released from PCP degradation is worth doing by using a better HPLC detector.

In summary, this study of PCP degradation in continuous reactors demonstrates the feasibility of biological removal of PCP in wastewater by using immobilized-*Flavobacterium* cells. Moreover, immobilized-*Flavobacterium* cells can use PCP as the sole carbon and energy source.

# Chapter 5

# Conclusions and Recommendation

## 5.1 Conclusions

1) *Flavobacterium sp.* ATCC 39723 can effectively degrade PCP in batch reactors. The rates of degradation are dependent on the PCP concentrations used. Complete removal of PCP can be achieved at PCP concentrations of 30, and 50 ppm. Only partial degradation happens at PCP concentration of 65 ppm, implying the degradation of PCP by *Flavobacterium sp.* is inhibited by PCP toxicity at higher PCP concentrations. The degradation is confirmed by the increasing concentration of free chloride ions in the reactor.

2) There are not big differences of degradability between reactors seeded with only *Flavobacterium sp.* and ones seeded with activated sludge and *Flavobacterium sp.* together, which suggests that activated sludge systems, or other aerobic biological treatment systems, or natural streams can treat PCP-containing waste provided that an appropriate organism such as *Flavobacterium sp.* capable of degrading PCP is present and is maintained in the system.

3) The generated concentration of chloride ions from PCP degradation does not correspond to the calculated concentration of chloride ions. Thus other mechanisms for PCP removal exist which do not contribute to the generation of free chloride ions. Other mechanisms could be photolysis, bioadsorption and so on.

4) Immobilized cells of *Flavobacterium sp.* ATCC 39723 can effectively degrade up to 60 ppm of PCP in a continuous reactor. The alginate film appears to protect the *Flavobacterium*

*sp.* cells from inhibition by high concentrations of PCP. Alginate beads without immobilized *Flavobacterium sp.* demonstrated some physical removal of PCP from influent by adsorption. Eventually these beads saturated with PCP.

5) In the continuous reactors with immobilized *Flavobacterium sp.* cells, the level of PCP degradation decreased as the influent loading rate increased, or as the hydraulic retention time (HRT) decreased. This is important in scale-up and design of reactors for treating PCP-containing waste by using immobilized *Flavobacterium sp.* cells.

6) Alginate is not a perfect matrix for immobilizing cells for this biological treatment system because the alginate beads are subject to be damaged by mechanical agitation, and are biodegradable by toxic chemical. Besides, high concentrations of potassium phosphate can destroy alginate beads within 48 hours.

7) PCP-degradable *Flavobacterium sp.* immobilized in alginate are able to utilize phosphate efficiently. As a consequence, PCP might enhance phosphate removal in a biological treatment system if a proper control strategy was used.

## 5.2 Recommendations

1) Investigation of the fate of PCP should be directed towards understanding what other mechanisms, such as bioadsorption, photolysis, and so on, are responsible for the PCP total removal in a reactor with either free *Flavobacterium* cells or immobilized *Flavobacterium* cells, and how much they contribute respectively to the total PCP removal.

2) Whether or not activated sludge cells are still alive when PCP is degraded by *Flavobacterium sp.* should be investigated in a reactor seeded with both activated sludge and *Flavobacterium sp.* The existing information about how PCP affects activated sludge with PCP-degrading *Flavobacterium sp.* is very useful in determining the feasibility of biological treatment of PCP

in other wastewater treatment systems when inoculating with *Flavobacterium sp.*.

3) Supplementary substrates, such as glucose, glutamate, etc. are believed to enhance PCP degradation when added to a PCP treatment system. Studies should be made to investigate what kind of and how much supplementary substrate will work best in enhancing PCP degradation in immobilized *Flavobacterium sp.* treatment systems.

4) Studies should be undertaken to quantify the range of PCP which could enhance phosphate removal in biological system with immobilized *Flavobacterium* cells.

## Bibliography


[1] Agriculture Canada. 1987. "Discussion document. pentachlorophenol - wood preservative" Pesticide Directorate, Food Production and Inspection Branch, Ottawa K1A OL6.

[2] Andrew, M. D., Canas, B., Ixquierdo, R. C. and Polo, L. M. 1990. "Transient changes of mobile phase in the high-performance liquid chromatographic separation of priority pollutant phenols" J. of Chromatography V.507, p.399.

[3] Apajalahti, J. H. A. and Salkinoja-Salonen, M. S. 1987. "Dechlorination and *para*-hydroxylation of polychlorinated phenols by *Rhodococcus chlorophenolicus*" J. Bacteriol. V.169(2), p.675.

[4] Arvin, E., Jensen, B. K. and Gundersen, A. T. 1991. "Biodegradation kinetics of phenols in an aerobic biofilm at low concentrations" Bat. Sci. Tech. V.23, p.1375.

[5] Bettmann, H. and Rehm, H. J. 1985. "Continuous degradation of phenols by *Pseudomonas putida* P8 entrapped in polyacrylamide-hydrazide" Appl. Microbiol. Biotech. V.22, p.389.

[6] Bevenue, A. and Beckman, H. 1967. "Pentachlorophenol: a discussion of its properties and its occurrence as a residue in human and animal tissues" Residue Rev. V.19, p.83.

[7] Bigley, F. P. 1980. "Determination of phenols in water and wastewater by post-column reaction detection high-performance liquid chromatography" J. of Chromatography, V.350, p.407.

[8] Bollag. J. M., Helling, C. S. and Alexander, M. 1967. "Metabolism of 4-chloro-2-methylphenolxyacetic acid by soil bacteria" Appl. Microbiol. V.15, p.1393.

[9] Borsetti, A. P. and Grob, R. L. 1985. "Determination of pentachlorophenol in milk and blood of dairy cattle" J. Agric. Food Chem. V.28, p710.

[10] Breed, R. S. et al. 1957. "Bergey's of determinative bacteriology".

[11] Brown, E. J., Pignatello, J. J., Martinson, M. M. and Crawford, R. L. 1986. "Pentachlorophenol degradation: a pure bacterial culture and an epilithic microbial consortium" Applied and Environmental Microbiology V.52, p.82.

[12] Buckman, N. G., Hill, J. O. and Magee, R. J. 1984. "Separation of substituted phenols, including eleven priority pollutants using high-performance liquid chromatography" J. of Chromatography V.284, p.441.

[13] Burger, K., Macrae, I. C. and Alexander, M. 1962. "Decomposition of phenoxyalkyl carboxylic acids" Soil Sci. Soc. Am. Proc. V.26, p.243.

[14] Carey, J.H. and Hart, J. H. 1988. "Sources of chlorophenolic compounds to the Fraser River esturary" Water Poll. Research J. Canada, V.23(1), p.55.

[15] Chu, J. P. and Kirsch, E. J. 1972. "Metabolism of pentachlorophenol by an axenic bacterial culture" Applied Microbiology V.23, p.1033.

[16] Chu, J. P. and Kirsch, E. J. 1973. "Utilization of halophenols by a pentachlorophenol metabolizing bacterium" Development in Industrial Microbiology V.14, p.264.

[17] Crosby, M. D. G., Beynon, K. T., Greve, P. A., Lorte, R., Still, G .G. and Vouk, J. W. 1981. "Environmental chemistry of pentachlorophenol" Pure Appl. Chem. V.53, p.1951.

[18] Cserjest, A. J. 1967. "The adaptation of fungi to pentachlorophenol and its biodegradation" Canadian Journal of Microbiology, V.13, p.1243.

[19] Dust, J. V. and Thompson, E. J. 1973. "Pollution control in the wood preserving industry, part 4, bilogical methods of treating wastewater" Forest Prod. J. V.23, p.59.

[20] Dust, J. V.; Thompson, E. S.; Shindala, A. and Francingues, N. R. 1980. "Chemical and biological treatment of wastewater from the wood-preserving industry" The Proceeding Of 35th Industrial Waste Conference, Purdue University.

[21] Dwyer, D. F., Krumme, M. L., Boyd, S. A. and Tiedje, J. M. 1986. "Kinetics of phenol biodegradation by an immobilized methanogenic consortium" Appl. Environ. Microbiol. V.52, p.217.

[22] Edgehill, R. U. and Finn, R. K. 1982. "Isolation, characterization and growth kinetics of bacteria metabolizing pentachlorophenol" Eur. J. Appl. Microbiol. Tiotechnol. V.16, p.179.

[23] Etzel, J. E. and Kirsch, E. J. 1975. "Biological treatment of contrived and industrial wastewater containing pentachlorophenol" Dev. Ind. Microbiol. V.16, p.287.

[24] Faghani-Shoja, S., DeVantier, B. A., Ray, B. T. and Cook, E. E. 1988. "Removal of phenol in mixed substrate form by a fixed film process" The Processing of 43rd Industrial Wastes Conference. Purdue University.

[25] Fisher, B. 1991 "Pentachlorophenol: toxicology and environmental fate" J. Of Pesticide Reform V.11 (1), p.15.

[26] Fountaine, J. E., Joshipura, P. B. and Keliher, P. N. 1976. "Some observations regarding pentachlorophenol levels in Haerford Township, Pennsylvania" Water Res., V.10, p.185.

[27] Haggblom, M. M., Nohynek, L. J. and Salkinoja-Salonen, M. S. 1988. "Degradation and P-methylation of chlorinated phenolic compounds by *Rhodococcus* and *Mycobacterium* strains" Appl. Environ. Microbiol. V.54(12), p.3043.

[28] Health and Welfare Canada 1989. "Chlorophenols and their impurities: a health hazard evaluation"

[29] Heidman, J. A., Kincannon, D. F. and Gaudy, A. F. 1967. "Metabolic response of activated sludge to sodium pentachlorophenol" Proceedings of 22nd Purdue Industrial Waste Conference. p.661.

[30] Hickman, G. T. and Novak, J. T. 1984. "Acclimation of activated sludge to pentachlorophenol" Journal WPCF, V.56(4), p.364.

[31] Ide, A., Niki, Y., Sakamoto, F., Watanabe, I. and Watanabe, H. 1972. "Decomposition of pentachlorophenol in paddy soil" Agr. Biol. Chem. V.36, p.1937.

[32] Kincannon, J. and Gaudy, M. 1967. "Metabolic response of activated sludge to sodium pentachlorophenol" Purdue Univ. Eng. Bull. Ext., V129, p.661.

[33] Kirsch, E. J. and Etzel, J. E. 1973. "Microbial decomposition of pentachlorophenol" J. of WPCF V.45(2), p.359.

[34] Kirsch, E. J., Grady, C., Guthrie, M. A., Moos, L. P. and Wukasch, R. F. 1981. "Experimental strategy for evaluating the fate of priority pollutants in wastewater treatment systems" In G.C. Llewellyn and C.E. O'Rear (ed.), Biodeterioration 5. C.A.B. International, Slough, United Kingdom. p.212.

[35] Klecka, G. M. and Maier, W. J. 1985. "Kinetics of microbial growth on pentachlorophenol" Appl. and Environ. Microbiol. V.49, p.46.

[36] Kloppfer, W., Kaufman, E., Rippen, E. and Poremski, H.J. 1982. "A laboratory method for testing the volatility from aqueous solution: first results and comparison with theory" Ecotoxicol. Environ. Saf. V.6(6), p.545.

[37] Kotzias, D., Klein, W. and Korte, F. 1975. "Occurrence of xenobiotica in seeping waters of landfills" Chemosphere V.24, p.257.

[38] Kutney, J. P., Choi, L., Hewitt, G. M., Salisbury, P. J. and Singh, M. 1985. "Biotransformation of dehydrobietic acid with resting cell suspensions and calcium alginate immobilized cells of *Mortierella isabellia*" Appl. Environ. Microbiol. V.49(1), p.96.

[39] Lamar, R. T., Larsen, M. J. and Kirk, T. K. 1990. "Sensitivity to and degradation of pentachlorophenol by *Phanerochaete* spp." Appl. Environ. Microbiol. V.56(11), p.3519.

[40] Lin, J., Wang, H. Y. and Hickey, R. F. 1990. "Degradation kinetics of pentachlorophenol by *Phanerochaete chrysosporium* " Biotech. Bioeng. V.35, p.1125.

[41] Lewandowski, G. A., Armenante, P. M. and Pak, D. 1990. "Reactor design for hazardous waste treatment using a white rot fungus" Wat. Res. V.24(1), p.75.

[42] MacRae, J. C. and Alexander, M. 1963. "Metabolism of phenoxyalkyl carboxylic acids by a *Flavobacterium species*" J. Bacteriol. V.86, p.1231.

[43] Markowski, M., Dzido, T. H. and Soczewinski, E. 1990. "Computer simulation for optimization of high-performance liquid chromatography of some phenolic pollutants" J. of Chromatography V.523, p.81.

[44] Martinson, G. L., Steiert, J. G., Saber, D. L., Mohn, W. W. and Crawford, R. L. 1986. "Microbiological decontamination of pentachlorophenol-contaminated natural waters" p.529. In G.C. Llewellyn and C.E. O'Rear (ed.), Biodeterioration 6. C.A.B. International, Slough, United Kingdom.

[45] Mercier, M. P. 1988. "Pentachlorophenol" Rev. of Env. Contents and Toxicology. V.104, p.184.

[46] Mileski, G. J., Bumpus, J. A., Jurek, M. A. and Aust, S. D. 1988. "Biodegradation of pentachlorophenol by the white rot fungus *Phanerochaete Chrysosporium*" Appl. Environ. Microbiol. V.54(12), p.2885.

[47] Moos, L. P., Kirsch, E. J., Wukasch R. F. and Grady, C. P. L. 1983. "Pentachlorophenol biodegradation-I" Water Res. V.17(11), p. 1575.

[48] Morgade, C., Barquet, A. and Praffenberger, C. D. 1980. " Determination of polyhalogenated phenolic compounds in drinking-water, human blood-serum, and adipose tissue" Bull. Environ. Contam. Toxicol. V.24, p.257.

[49] Mueller, J. G., Lantx, S. E., Blattmann, B. O. and Chapman, P. J. 1991. "Bench-scale evaluation of alternative biological treatment processes for the remediation of pentachlorophenol- and creosote-contaminated materials: solid-phase bioremediation" Environ. Sci. Technol. V.25, p.1045.

[50] Murthy, N. B. K., Kaufman, D. D. and Fries, G. F. 1977. "Aerobic and anaerobic soil degradation of pentachlorophenol" J. Environ. Sci. Hlth. V.43, p.138.

[51] O'Reilly, K. T., Kadakia, R., Korus, R. A. and Crawford, R. L. 1988. "Utilization of immobilized bacteria to degrade aromatic compounds common to wood-treatment wastewaters" Wat. Sci. Tech. V.20(11/12), p.95.

[52] Pignatello, J. J., Martinson, M. M., Steiert, J. G., Carlson, R. E. and Crawford, R. L. 1983. "Biodegradation and photolysis of pentachlorophenol in artificial freshwater streams" Appl. Environ. Microbiol. V.46, p.1024.

[53] Pignatello, J. J., Johnson, L. K., Martinson, M. M., Carlson, R. E. and Crawford, R. L. 1985. "Response of the microflora in outdoor experimental streams to pentachlorophenol: compartmental contributions" Appl. Environ. Microbiol. V.50(6), p.137.

[54] Prasad, D. Y. and Joyce, T. W. 1992. "Removal of chlorinated organics from kraft soft-wood bleach plant effluent by sequential biological treatment using white rot fungus and an anaerobic reactor" The Processing of 46th Purdue Industrial Waste Conference, Purdue University.

[55] Radehaus, P. M. and Schmidt, S. K. 1992. "Characterization of a novel *Pseudomonas* sp. that mineralizes high concentrations of pentachlorophenol" Appl. Environ. Microbiol. V.58(9), p.2879.

[56] Rao, K. R. 1978. "In Pentachlorophenol: chemistry, pharmacology and environmental toxicology" (Ranga Rao, K.), New York, USA: Plenum Press.

[57] Reiner, E. A., Chu, J. and Kirsch, E. 1978. "Microbial metabolism of pentachlorophenol" In Pentachlorophenol: Chemistry, Pharmacology and Environmental Toxicology. (Ranga Rao), New York, USA: Plenum Press, p.67.

[58] Richardson, N. G. 1978. "Wood preserving effluent and their treatment" Report EPS 3-WP-78-1, p.40.

[59] Saber, D. L. and Crawford, R. L. 1985. "Isolation and characterization of *Flavobacterium* strains that degrade pentachlorophenol" Appl. Environ. Microbiol. V.50, p.1512.

[60] Seech, A. G., Trevors, J. T. and Bulman, T. L. 1991. "Biodegradation of pentachlorophenol in soil: the response to physical, chemical, and biological treatments" Can. J. Microbiol. V.37, p.440.

[61] Sethunathan, N. and Yoshida, T. 1973." A *Flavobacterium sp.* that degrades diazinon and parathion" J. Can. Microbiol. V.19, p.873.

[62] Sofer, S. S., Lewandowski, G. A., Lodaya, M. P., Lakhawala, F. S., Yang, K. C. and Singh, M. 1990. "Biodegradation of 2-chlorophenol using immobilized activated sludge" Research Journal WPCF. V.62(1), p.74.

[63] Stanlake, G. J. and Finn, R. K. 1982. "Isolation and Characterization of a pentachlorophenol-degrading bacterium" Appl. Environ. Microbiol. V.44, p.1421.

[64] Steenson, T. I. and Walker, N. 1957. "The pathway of breakdown of 2,4-dichloro- and 4-chloro-2-methyl-phenoxyacetic acid by bacteria" J. Gen. Microbiol. V.16, p.146.

[65] Steiert, J. G. and Crawford, R. L. 1986. "Catabolism of pentachlorophenol by a *flavobacterium* sp." Biochem. and Biophys. Res. Comm. V.141(2), p.825.

[66] Stretz, L. A. and Vavruska, J. S. 1984. "Controlled air incineration of pentachlorophenol-treated wood" U.S. EPA Report No. EPA-600/54-84-089.

[67] Suzuki, T. 1977. "Metabolism of pentachlorophenol by a soil microbe" J. Environ. Sci. Health, B12(2), p.113.

[68] Thompson, G. E., Husain, H., Parry, J., Wardrop, W. L. and Gilbride, P. J. 1978. "Hydrogeological control and clean-up of soil and groundwater contaminants at Northern Wood Presevers Ltd." Proceedings of the Ontario Industrial Waste Conference, Toronto, p.18.

[69] Topp, E. and Hanson, R. S. 1990. "Degradation of pentachlorophenol by a *flavobacterium* species grown in continuous culture under various nutrient limitations" Appl. and Environ. Microbiol. V.56(2), p.541.

[70] Trevors, J. T. 1982. "Effect of temperature on the degradation of pentachlorophenol by *Pseudomonas* species" Chemosphere. V.11, p.471.

[71] Tokuz, R. Y. 1989. "biodegradation and removal of phenols in rotating biological contactors" Wat. Sci. Tech. V.21, p.1751.

[72] Watanabe, I. 1977."Pentachlorophenol decomposing and PCP-tolerant bacteria in field soil treated with PCP" Soil Biol. Biochem., V.9, p.99.

[73] Westmeier, F. and Rehm, H. J. 1985. "Biodegradation of 4-chlorophenol by entrapped *Alcaligenes sp.* A 7-2" Appl. Microbiol. Biotech. V.22, p.301.

[74] Wong, A. S. and Crosby, D. G. 1981. "Pentachlorophenol: chemistry, pharmacology and environmental toxicology"(Ranga Rao, K.), New York, USA: Plenum Press, p.19.

[75] World Health Organization, Geneva, 1987. "Pentachlorophenol: environmental health criteria 71".

[76] Xun, L. and Orser, C. S. 1991. "Purification and properties of pentachlorophenol hydroxylase, a flavoprotein from *Flavobacterium sp.* strain ATCC 39723" J. Bacteriol. V.173(14), p.4447.

[77] Xun, L., Topp, E. and Orser, C. S. 1992. "Confirmation of oxidative dehalogenation of pentachlorophenol by a *Flavobacterium* pentachlorophenol hydroxylase" J. Bacteriol. V.174(17), p.5745.