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ABSTRACT

Inability of Phanerochaete chrysporium to Degrade Polychlorinated Biphenyls in Sand

by

Pankaj Pankaj

The white rot fungus *Phanerochaete chrysosporium* has been previously found to successfully degrade a wide spectrum of chlorinated aromatic hydrocarbons (some of them in soil or sand matrices). In this research, it was tested if the fungus could also degrade a very recalcitrant class of pollutant, i.e. Polychlorinated Biphenyls (PCBs) in sand at a concentration of 100 ppm (w/w PCB to sand). For this purpose, a number of experiments were performed, in which several operating parameters, such as nutrient concentrations (nitrogen and glucose), amount of initial biomass, aeration, and degree of chlorination of the PCBs were varied. The results obtained indicate that the fungus is unable to degrade the PCBs under the experimental conditions tested in this work. In order to further validate this conclusion a new set of experiments was carried out, in which the activity of the fungus was tested against PCBs as well as 2,4,6-trichlorophenol (TCP). The experiments with 2,4,6-trichlorophenol were run in parallel with those with PCBs, and under similar conditions. The fungus used to inoculate both the compounds came from the same batch. It was evident from these experiments, that the fungus was able to mineralize the TCP completely, but could not degrade the PCBs. From all the results obtained during this research, it can be concluded that the fungus is ineffective to degrade the PCBs.

INABILITY OF PHANEROCHAETE CHRYSOSPORIUM TO DEGRADE POLYCHLORINATED BIPHENYLS IN SAND

by

Pankaj Pankaj

A Thesis
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APPROVAL PAGE

Inability of Phanerochaete chrysporium to Degrade Polychlorinated Biphenyls in Sand

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CHAPTER 1
INTRODUCTION

The rapid growth of industries in the first half of the twentieth century resulted in the large scale production of a number of commodities. Along with useful products, some unwanted items were also produced, quite a few of which could be termed as hazardous waste. Due to the absence of proper environmental regulatory bodies and proper laws, unhealthy waste disposal activities were being practised, and these hazardous wastes were released into the environment. This posed a severe health hazard not only to human beings, but also to the whole ecological system. In the second half of the twentieth century, global environmental consciousness arose, and this resulted in much tighter environmental laws and practices. With the establishment of the regulatory bodies like the U.S. Environmental Protection Agency (U.S.E.P.A.), these laws became stricter, and the environment became cleaner.

There are several compounds which, up to a certain time in the past, were believed to be less toxic but were later proven to be hazardous. Polychlorinated Biphenyls (PCBs) are a classic example of such compounds. PCBs were first synthesized in 1881 but their mass production began only in 1929. Because of their remarkable insulating capacity and their nonflammable nature, they gained widespread use as coolants, lubricants and insulators in transformers and other electrical equipments. Between 1929 and 1977, it is estimated that about 1.2 billion pounds of PCBs were produced in the United States alone (Cairns et al.,1986).

Concerns about the presence of PCBs in our environment began around 1966, when the results of research studies in Sweden revealed some buildups of PCBs in the environment. These studies confirmed that the natural breakdown of some of the PCB compounds was a very slow process. In 1968, a widespread human poisoning occurred in Japan, which is referred to as the Yusho case. This mass food poisoning was caused by consumption of a rice-bran oil contaminated with a commercial PCB mixture (Kanechlor 400, containing 48% chlorine, produced by Kanegafuchi Industrial Co. Ltd., Japan), which had been used as a heat exchanger fluid to deodorize the oil. More than 1600 people were affected. The clinical symptoms showed dermal, ocular, respiratory, neurological and reproductive abnormalities. The level of PCBs in the blood of these patients declined rapidly, once the oil ingestion was terminated. However, the above symptoms persisted even after 10 years of the incidence. In certain other studies, PCBs have been found to cause liver cancer in rats, when they were fed with PCB-containing food.

PCBs were released into the environment, primarily through two main practices: 1) Under legal permits, industries involved in the manufacture or use of PCBs in their processes discharged the PCB-containing wastes into rivers and other water streams; 2) Certain other PCB-containing wastes were often disposed off in open landfills. These practices were then permitted by law, but were inappropriate and potentially hazardous. In addition, there was another way of releasing PCBs in the environment i.e. as a result of accidents occurring in these industries which used PCBs. When used in transformers and capacitors, PCB compartments were sealed. Occasionally, these seals leaked or the PCB containers were damaged, resulting in PCB leakages. These factors lead to the increase in amount of the PCBs in the environment.

When a PCB release occured a river or water stream, the PCBs migrated to the river sediment. Due to their persistent nature, they remained in the sediments and were slowly taken up by the local fauna, including the fish. Due to bioaccumulation (larger fishes eating smaller ones), PCB concentration in them started increasing. Consumption of these fishes by humans, posed serious threat to human health.

The present situation in the USA is that there are about 2500 sites that are highly contaminated with PCBs. Since PCBs are very resistant to natural degradation, it cannot be expected that their removal from the environment will be a natural process. The existing technologies to remove PCBs are :

1) Nonthermal Methods for PCB Disposal:
   a) the Sun Ohio PCBX Process
   b) the Acuren Waste Technologies Inc. (AWT) PCB Destruction Process.
2) High Temperature PCB Incineration
3) Burning PCB wastes in Cement Kilns

Some of the above processes can be useful only at a small scale, and if PCB concentration is quite high. Some processes are used only when PCBs are in the oil phase. For the cases in which PCBs have contaminated landfills or have been spilled in a large area, the application of the above technologies is not only a labor intensive process, but is also very expensive. One of the alternatives at this stage would be to investigate into the possibility of biodegradation of the PCBs using microorganisms. Many hazardous wastes have been found to be biodegradable, and microorganisms have been used to treat sites contaminated with such wastes.

The white rot fungus, *Phanerochaete chrysosporium*, has been found to degrade a lot of hazardous wastes, including chlorinated ring-structured compounds. This fungus decomposes wood by breaking down lignin, a complex O-methylated polymer of phenolic hydroxyl groups, that is otherwise very resistant to decay. The fungus produces an extracellular enzyme (ligninase) system, which is capable of degrading lignin. It is observed that near complete degradation of lignin by the fungus is possible (Hammel et al., 1987).

Lignin is an irregular polymer. Therefore, the ligninase system must be nonspecific in its activity. The ligninolytic enzyme system produced by the fungus has the unusual property of being able to degrade a wide variety of compounds. Indeed, the fungus has been shown to degrade a wide spectrum of chlorocarbons and polycyclic aromatics (Bumpus et al., 1985; Huynh et al., 1985; Bumpus et al., 1987; Sangalard et al., 1986; Lewandowski et al., 1990). There is a great hope of degrading many hazardous wastes using this fungus.

There is very little information available about the activity of microorganisms in degrading PCBs. Also, little is known about the activity of the fungus in sand or soil. The aim of this research was to determine if the PCBs in sand could be degraded by the fungus. In this work, sand was artificially spiked with PCBs, and then exposed to the fungus. The concentration of PCBs was monitored as a function of time. During the later stages of the research, another compound, 2,4,6-trichlorophenol (TCP) was introduced. The TCP was used in order to determine the activity of the fungus against it, in sand. It has already been shown that the fungus degrades TCP in water (e.g. by N. Pal, PhD student, Department of Chemical Engineering, Chemistry and Environmental Science, NJIT, Newark, NJ). In the present work, sand artificially spiked with TCP was also exposed to the fungus, and its concentration was also monitored as a function of time. In the later

stages of the experiments, separate sand samples (one spiked with PCB and the other with TCP) were exposed to the fungus which came out from the same batch of fermenter. All the subsequent experiments were conducted under similar conditions. Also, this compound (TCP) was used as a control for the experiments with PCBs. This provided a comparative study on the activity of the fungus against the two hazardous wastes, that is the PCBs and TCP, under similar conditions.

CHAPTER 2
LITERATURE REVIEW

2.1 POLYCHLORINATED BIPHENYLS

2.1.1 Introduction

After being synthesized in 1881, the large scale industrial applications of the PCBs were realized only decades later. Since they first went into industrial production in 1929, PCBs have played an important role as industrial chemicals to be used as a nonflammable oils in a variety of products. PCBs were marketed under various trade names like Aroclor, Clophen, Phenochlor, Kanechlor, Pyralene, Sovol and others.

The PCBs quickly became popular for their use in industrial products, where nonflammability and heat-resistant properties were highly desired. Apart from this, they have remarkable insulating properties. For these reasons, they were used as coolants and lubricants in transformers, capacitors and other electrical equipments, where these properties are much desired. PCBs replaced combustible insulating fluids. They were used in many other industrial applications too, such as constituent of heat transfer systems, hydraulic/lubricant, plasticizer applications, adhesives, paints and varnishes, carbonless copying paper, newsprint, fluorescent light ballasts, petroleum additives etc. (Cairns et al., 1986; Whelan, 1985)

2.1.2 Chemical Structure of PCBs

PCBs are a class of synthetic chlorinated organic compounds, with a biphenyl as their basic structural unit, and a varying number of chlorine atoms attached to the carbon atoms. As shown in Figure 2.1, there can be a maximum of ten chlorine atoms attached to a single nucleus of the molecule. In Figure 2.1, chlorine atoms can take any of the places marked with numbers (primed or unprimed). Therefore, theoretically, the total number of possible compounds resulting from the chlorination of the biphenyl nucleus is 209, as is evident from the calculations in Table 2.1 .

Table 2.1 Number of different possible isomers of the chlorinated biphenyl structure

Chlorine Substitution	Number of Possible Isomers
Mono -	3
Di -	12
Tri -	24
Tetra-	42
Penta-	46
Hexa -	42
Hepta-	24
Octa -	12
Nona -	3
Deca -	1
Total	209

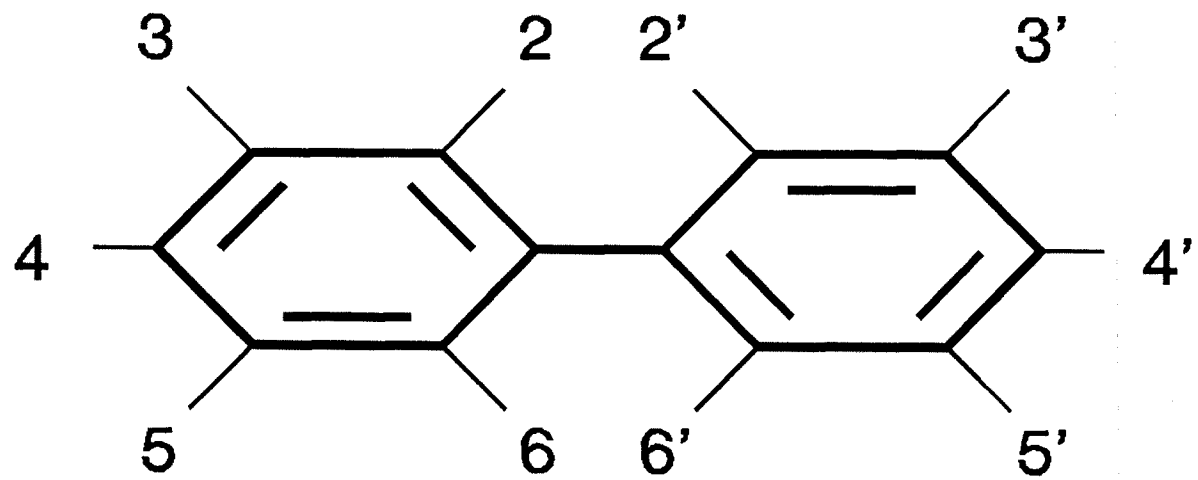


Figure 2.1. Structure of a PCB Molecule
(number represent Cl or H atoms)

2.1.3 PCB Nomenclature

According to the IUPAC definitive rules for nomenclature of organic chemistry, one ring system in the biphenyl ring assembly is assigned unprimed numbers and the other, primed numbers, as shown in Figure 2.1. The order for assigning priorities to the substituents in the ring assembly, as Cl in PCBs is (1) an unprimed number is assigned lower order than the corresponding primed number, as 2 vs 2'; (2) a lower number is assigned to a point of attachment in equivalent position, as 2 vs 6, for a substituent in one of the ortho positions; (3) when the number of substituents in the two ring systems is the same, unprimed numbers are assigned to the ring system with smaller numbered substituents (Sawhney, B.L., 1986).

2.1.4 Commercial PCBs

Chlorination of biphenyl in the presence of a catalyst, such as iron filings or iron chloride was used for the industrial preparations of PCBs. The chlorination process produced a mixture of chloro-biphenyls, whose composition was influenced by the ratio of chlorine to biphenyl. This means that the PCBs which were commercially available, were basically a mixture of chloro-biphenyls. In the U.S., PCBs have been manufactured by Monsanto, under the trade name 'Aroclor'. The most common Aroclor preparations include Aroclor 1242, 1248, 1254 and 1260. In the commercial names like these, the first two digits (that is 12, here) are the number of carbon atoms in the biphenyl group, and the last two digits give approximate percentage of chlorine content (Cairns et al., 1986). Therefore, Aroclor 1254 means that there are 12 carbon atoms in the nucleus of the molecule, and the PCB mixture contains approximately 54 % chlorine in it. GC analysis of the Aroclors show that they are complex mixtures of different chlorobiphenyls.

2.1.5 Physical Properties of PCBs

PCBs are among the most stable organic compounds known. They have a low dielectric constant and a high heat capacity, which render them ideal for use in electrical capacitors and transformers. While most individual chlorobiphenyls are solid at room temperature, commercial preparations are generally resins or viscous liquids of density greater than water.

Solubility of PCBs in water is very low. The solubility in water generally decreases with an increase in the degree of chlorination. Individual chlorobiphenyls vary in their solubility from about 6 ppm for monochlorobiphenyl to as low as 0.007 ppm for octachlorobiphenyl (Sawhney, B.L., 1986). They are more soluble in fats, than in water. Hence, upon consumption, by human beings, they tend to get absorbed in the fat tissues. The solubility of PCBs is greatly influenced by the environment. For example, aqueous phases in the environment generally contain dissolved organic substances, which probably enhance the concentration of PCB in the solution.

PCBs have very low vapor pressure which, like their solubility in water, decrease with increased chlorination. The vaporization rates decrease about 200 folds from Aroclor 1221 to Aroclor 1260.

2.1.6 Toxicity of PCBs

In the Yusho mass poisoning case, in which people were exposed PCBs, the symptoms shown by the affected persons were: persistent chlorance (acute skin rash), discoloration of the gums and nailbeds, swelling of the joints, swelling of arms and legs, liver disorders and waxy secretions of the glands in the eyelids. Some more general manifestations, like lethargy, fatigue and nausea, were also reported. Some

babies born to exposed mothers were smaller than usual (Cairns et al., 1986; Whelan, 1985).

In other studies conducted at Center for Disease Control in Atlanta, rats fed with 100 ppm of PCBs in their diet for twenty-one months, were reported to have developed liver cancer. In human beings, conclusive evidence has not been found, to classify PCBs as a carcinogen (Whelan, 1985).

Some of the important properties of commercially available PCBs are listed in Table 2.2 (Cairns et al., 1986).

Table 2.2 Some important properties of PCBs

PCB, Aroclor Number	Density (g/cc)	Distillation Range °C	Rats Oral LD_{50} (mg/kg)	Vaporization Rate at 100 °C (g/cm^2 /hr)
1242	1.38	325 - 366	8,650	0.000338
1248	1.44	340 - 375	11,000	0.000152
1254	1.53	365 - 390	11,900	0.000053
1260	1.62	385 - 390	10,000	0.000009

2.1.7 PCB Releases into the Environment

Two practices believed to be acceptable and hazard-free in the past, have led to the release of PCBs into the environment. (1) Usually under permit, industries using PCBs in their processes and products, discharged the PCB-laden wastes into rivers and streams. (2) Other PCB-containing wastes were often dispersed in open landfills. Thus an ever increasing amount of PCBs became part of environment. These practices, though permitted by law then, were inappropriate and potentially hazardous procedures.

In transformers and electrical capacitors, PCBs were placed in closed compartments so that the chemical remains in place for the life of the equipment. Sometimes, these containers leaked or they were damaged, resulting in PCB leakage.

2.1.8 PCB Regulations

As seen earlier, the toxic effects of PCBs became evident during 1970s. The Food and Drug Administration (FDA) became concerned about the tolerance limit of PCBs in various food products. Through biological chain, PCBs were entering into the human bodies, for example, through consumption of fish from the rivers in which PCB wastes were released. After various studies, FDA changed the PCB tolerance limits in various food items in 1979. A comparative data of these tolerance limits has been provided in Table 2.3, which shows these limits in 1973 and in 1979 (Cairns et al., 1986; Sawhney, 1986).

As a result, EPA issued rules governing the continued deployment of PCBs in certain industrial applications. The regulatory control acts were made under the Toxic Substances Control Act (TSCA) of 1976, and proposed the termination of the use of PCBs in heat transfer systems, in plants manufacturing or processing food, drugs and cosmetics. By October 1977, no one was allowed to manufacture, process, distribute, or use any PCBs except in a "totally enclosed manner", defined as any manner that ensures no exposure to human beings or the environment.

Table 2.3 FDA Tolerances for PCBs in Several Classes of Food

Product	Concentration (ppm)	
	1973	1979
Milk & dairy products (fat basis)	2.5	1.5
Poultry (fat basis)	5.0	3.0
Eggs	0.5	0.3
Fish & shellfish (edible portion)	5.0	2.0*
Infant & junior food	0.2	0.2
Feed for food producing animals	0.5	0.2
Animal feed components including fishmeal & other byproducts of marine origin	5.0	2.0
Paper food packaging materials intended for or used with human food	10.0	10.0

* This value was proposed in 1979, but came into effect only in 1984.

2.1.9 Analysis of PCBs

Gas chromatography has been widely used to detect the PCBs in environmental and biological samples (Cairns et al., 1986; Sawhney, 1986). Halogen sensitive chromatographic detectors such as Hall electrolytic conductivity detectors (HECD) and the electron capture detectors (ECD) are capable of detecting PCBs in the subnanogram range and offer the chemist a convenient analytical approach when dealing with trace levels of PCBs (even below 1 ppm). These detectors are sensitive to chlorine, and are used for trace analysis. Flame ionization detector (FID) is also used to detect PCBs, but these can be succesfully used only when the concentration of PCB is high. In terms of relative sensitivity, both HECD and ECD demonstrate a

level usually three orders of magnitude greater than that observed with FID (Cairns et al., 1986).

GC/MS technique is used to identify the particular specie of the PCB in the mixture. Other analytical techniques such as nuclear magnetic resonance (NMR) and liquid chromatography with UV detection are also used as alternative methods for analysis of PCBs, but can be successfully used when the concentration of PCB is over 1000 ppm.

2.1.10 Destruction of PCBs

Various methods have been developed to destroy PCBs. Some of them are (Lauber, J.D., 1986) -

I) Non thermal methods for PCB disposal:

- The Sun Ohio PCBX Process. By this process, PCBs are broken down into two primary components, biphenyl derivatives and chloride.
- Accurex Waste Technologies Inc. (AWT) PCB destruction process: Its a mobile unit, that chemically detoxifies PCBs in oil using sodium reagent.

II) High temperature PCB incineration.

III) Burning low level PCB/mineral oil wastes in high efficiency boilers.

IV) Burning PCB wastes in cement kilns.

Most of the technologies currently used are based on thermal destruction of PCBs. PCB incineration requirements are-

1) Maintenance of the introduced liquids/solids for 2 seconds at 1200 °C ($\pm$100 °C) at 3% excess oxygen in the stack gas, or

2) Maintenance of the introduced substances for 1/2 second at 1600 °C ($\pm$100 °C) at 2% excess oxygen.

3) The mass air emissions from such an incinerator burning such wastes shall be no greater than 0.001 kg PCB per kg of PCB introduced in the incinerator. (99.999% destruction and removal efficiency).

The stack emissions must be monitored (when incinerator is being used) for the following emissions- Oxygen, CO, NOx, HCl, Total Chlorinated Organic Content, PCBs and Total Particulate Matter.

Temperature is an important factor in thermal destruction of PCBs, since at lower temperatures (till about 700 °C), some very toxic materials like - polychlorinated benzofurans (PCDFs), are produced (Sawhney, 1986; Whelan, 1985).

2.1.11 Biological Treatment of PCBs

The thermal destruction techniques of PCBs are very expensive and, in some cases, can be applied only to wastes in the liquid phase (such as oil). When the PCB-contaminated sites are very large in area, application of incineration is a labor intensive and expensive task. Another aspect to look as an alternative to the above methods is the possibility of biodegradation of the PCBs.

Bedard et al. (1989), reported that PCBs are undergoing biodegradation in nature. The analysis of PCBs extracted from the Hudson river sediments showed that the GC fingerprints of these samples were quite different from the commercial PCBs originally discharged.

Other investigations of biodegradation of PCBs in soils, sediments, lakes and rivers showed that both aerobic and anaerobic microorganisms decompose and metabolize PCB (Cairns et al., 1986). The degree of chlorination has been found to have influenced the biodegradability (Furukawa et al., 1976)

Iwata et al. (1973) have reported that biodegradation is affected by the environment. Out of six soil samples they tested, biodegradation was observed in four. Hankin and Sawhney observed that out of eight soil samples, seven of them showed degradation of Aroclor 1248, three showed degradation of Aroclor 1254, while none showed degradation of 1260.

Sample chromatograms showing the evidence of biodegradation of PCBs in soil, are shown in Figure 2.2 (Source- Envirogen Inc., Princeton, NJ). In Figure 2.2, Chromatogram A is for a control experiment, to which no biomass was added. Chromatogram B represents the same PCB sample, after exposure to one unit dose of organisms. From this chromatogram, it can been be clearly seen that some of the peaks have been reduced after exposure to the microorganism. This can be attributed to the biodegradation caused by the microorganisms. Chromatogram C shows the PCB after exposure to six consecutive unit doses of the microorganism. The decrease in height of the peaks is more visible in this chromatogram.

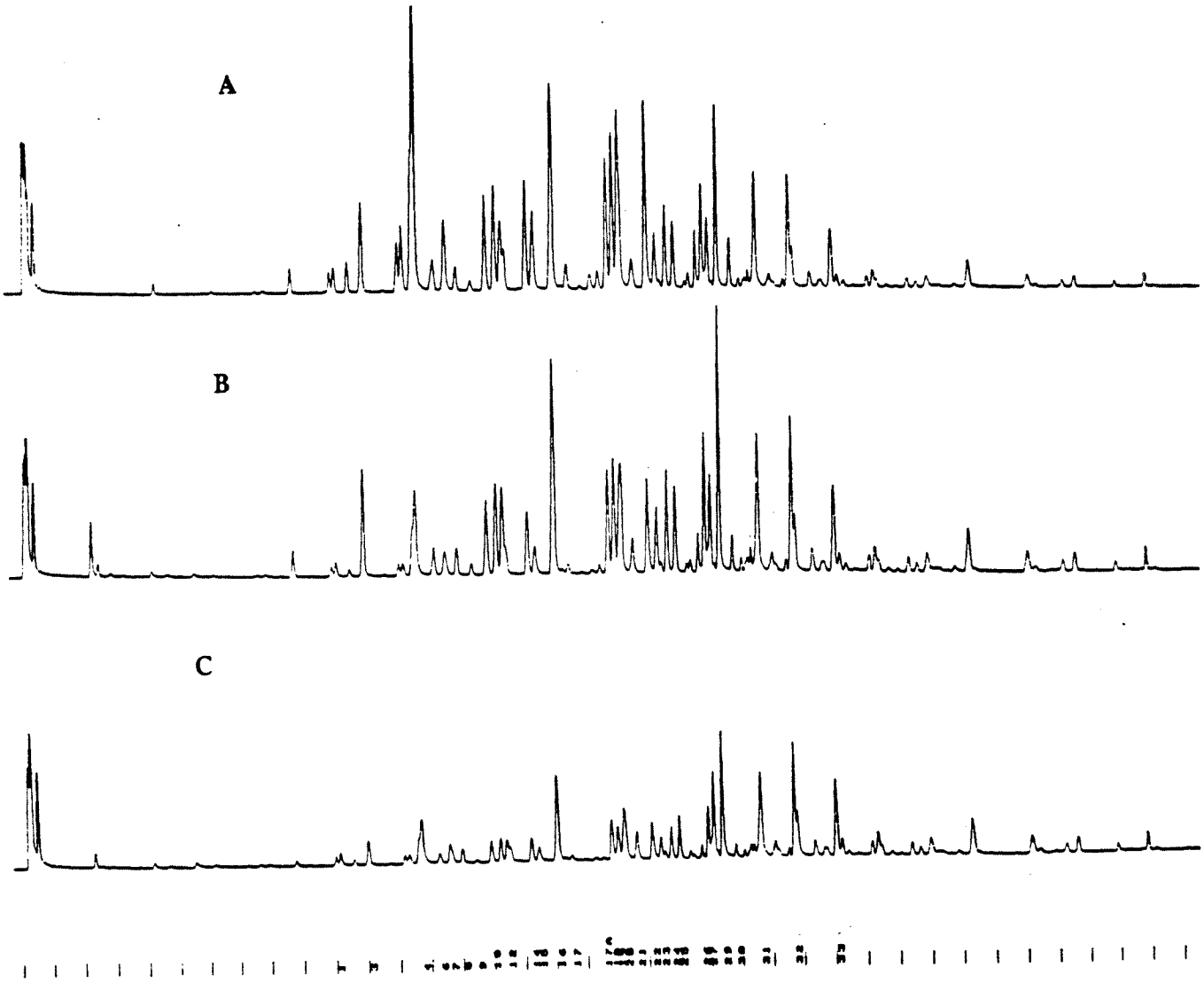
2.2 PHANEROCHAETE CHRYSOSPORIUM

2.2.1 Introduction

Phanerochaete chrysosporium belongs to a family of wood rotting fungi, that are found all over the Northern Hemisphere. It is a white filamentous fungus, which has been classified as a member of Hymenomycetes subclass of Basidiomycetes. Like other fungi, they are eukaryotic, that is, they possess a nuclear membrane, and as microorganisms, are considered as plants without chlorophyll, and having no photosynthetic abilities (Glaser et al., 1989).

Figure 2.2 Chromatograms Showing Evidence of Biodegradtion of PCBs

A - Control (No degradation)
B - PCBs After Exposure to 1 Dose of Microorganisms
C - PCBs Exposed to 6 Consecutive Doses of Microorganisms



As the name suggests (wood rotting fungus), this fungus breaks down the cellulose and complex lignin structure of the wood. These naturally occurring polymers of cellulose and lignin are used by this fungus as their major source of carbon. The carbon in the lignin is not available to the fungus as a primary carbon source. In order to access it, the fungus excretes a highly effective extracellular oxidative enzyme (ligninase) system capable of degrading lignin through a secondary metabolic cycle (Glaser et al., 1989).

2.2.2 Enzymatic Activity

Lignin is a naturally occurring highly complex polymer, very resistant to degradation because of its complex structure and molecular size (these polymer structures have a size, which cannot go in to the cells of microorganisms through known cellular transport mechanisms) (Glaser et al., 1988). Therefore the ligninase system, which was found to be degrading the lignin structure completely, should be nonspecific.

Lignin degradation is believed to occur in two steps. In the first step, the extracellular enzymes break down the lignin structure into smaller fragments, and in the second step, the intracellular enzymes break these fragments into carbon dioxide. During lignin breakdown, the main steps involved are the breaking of the alkyl sidechains, ring demethylation and ring cleavage. Hydrogen peroxide based enzymes take active part in these oxidative steps (Glaser et al., 1988).

This non-specificity of the fungus has been successfully used to degrade many hazardous wastes, which are otherwise recalcitrant to degradation because of their complex structure. Several researchers have reported successful degradation of hazardous wastes by this fungus. Many of the chlorinated and aromatic compounds, in this category of wastes, like DDT, Polychlorinated dibenz(p)dioxins, chlorinated alkanes, and chlorophenols (Tien et al., 1984; Bumpus et al., 1985;

Faison et al., 1985; Lewandowski et al., 1990) have been degraded using this fungus. This clearly shows that the fungus holds a lot of hope in future to economically degrade hazardous wastes, and treat the sites contaminated with such wastes.

2.2.3 Parameters Affecting the Enzymatic Activity and Growth of the Fungus

2.2.3.1 Effect of Nitrogen Concentration

The ability of the fungus to degrade the lignin structure is greatly influenced by the concentration of nitrogen (Eaton, 1985,1986,; Reid et al., 1983; Bumpus et al., 1990). Reid et al. (1983) studied the effect of nitrogen sources and supplements on degradation of aspen wood lignin, and found out that addition of nitrogen greatly inhibits the ligninolytic activity of the fungus. They also reported that the addition of complex nitrogen sources such as peptone, albumin, casein-hydrolysate and yeast extract can stimulate the rate of degradation. Kirk et al. (1986) showed that the source of nitrogen has little effect on lignin degradation, but the concentration of nitrogen is critical. The rate of degradation of lignin was 25-30 % in a medium containing 24 mM of nitrogen than in the one containing 2.4 mM of nitrogen.

2.2.3.2 Effect of Oxygen Concentration

P. chrysosporium grows aerobically, that it needs oxygen for its growth. Oxygen concentration has been found to be an important parameter for the growth and the degradation activity of the fungus. It was reported that the rate of lignin degradation was about two to three folds greater under 100 % oxygen than in air (21% oxygen concentration). Further, no degradation was observed in a 5 % oxygen environment (Kirk et al., 1986).

2.2.3.3 Effect of pH

The optimum pH for the growth of the fungus has been found to be 5.5 (Kirk et al., 1986). For lignin degradation, the optimum pH has been observed to be about 4.5. Below a pH of 3.5 and above 5.5, the decrease in degradation activities were substantial.

2.2.3.4 Effect of Carbon Source

Several carbohydrates can serve as the carbon source for the growth of the fungus and also to induce its ligninolytic activity (Kirk et al., 1986). When simple carbohydrates are used as the carbon source, the degradation activities against the xenobiotics ceases after about 20-25 days (Bumpus et al., 1990), whereas this activity period of the fungus can be prolonged to about 90 days, when complex carbohydrates such as cellulose and starch are used as the carbon source. The degradation of pollutants is reported to stop after the carbon source has depleted (Reid et al., 1983).

Different kind of soils contain different types of nutrients for the fungus. During the study of degradation of pentachloro phenol (PCP) in soil, Lamar et al. (1990), observed that the highest rate of degradation was observed in masham soil. It was suggested that differences in degradation rates could be attributed to the differences in the nature and quantity of the various nutrients in different soils.

Since sand as such does not contain appreciable amount of nutrients for the growth of fungus, it becomes essential to add an additional carbon source, such as glucose, in order to sustain the growth and ligninolytic activity of the fungus.

2.2.3.5 Effect of Temperature

In soil, the fungus has been reported to grow in a wide range of temperatures between 10 - 39 °C (Glaser et al., 1988). No growth was observed at 10 ° C, whereas it considerably increases with temperatures from 15 to 39 °C. No significant difference in growth was observed between 30 and 39 °C. The optimum temperature for growth in the culture medium has been found to be 39 °C (Kirk et al., 1978).

2.2.3.5 Effect of Agitation

Studies have shown that increase in agitation reduces the ligninolytic activity of the fungus (Faison et al., 1985; Jager et al., 1985, Leisola et al., 1985; Reid et al., 1983).

2.3 ACTIVITY OF P. CHRYSOSPORIUM AGAINST PCBs

The fungus has been reported to degrade PCBs by Bumpus et al. (1985) and Eaton et al. (1985). Eaton et al. carried out experiments with [U-^{14}C] Aroclor 1254, and observed that $^{14}CO_2$ was released after exposing the PCBs (at a concentration of 250 ppb) to the fungus. They suggested that this was due to the ligninolytic activity of the enzymes produced by the fungus. Biodegradation was confirmed by extracting the PCBs and running it on the GC equipped with Hall electrolytic conductivity detector (HECD). The peaks corresponding to various congeners in the mixture (Aroclor 1254) were almost completely reduced, indicating almost complete biodegradation of the PCBs. Bumpus et al. (1985) also reported biodegradation of PCB using this fungus. They carried out experiments with a particular congener of PCB (2,4,6,2',4',6' Hexachloro biphenyl). They reported

about 1 % conversion of HCB to CO_2. The maximal rate of biodegradation was observed between day 3 and 18, after which the rate began to decrease. They carried out some other experiments with Aroclor 1254 in silt loam soil and reported about 11.5% mineralization of the PCB, when exposed to the fungus.

Both researches were carried out at a very low concentration of PCBs, that is below 1 ppm. For effective use of fungus as an alternative to treat sites contaminated with the PCBs, it is important for the fungus to be able to degrade the PCBs at a reasonable concentration of about 100 ppm (w/w PCB to sand).

CHAPTER 3
MATERIALS AND METHOD

3.1 MATERIALS

3.1.1 Sand

The sand used in these experiments was the same as that commonly used for building construction, and was purchased from a local retailer. The sand was sieved using U.S.A. Standard Testing Sieve No. 25. The fraction below 710 um was (Tyler equivalent 24 mesh) was used for the experiments. The sand was cleaned of its inorganic chemicals, present as impurities, by first washing it with ordinary tap water (about 10 washes) and then with distilled water (about 5 washes).

3.1.2 Soil

Soil was provided by the USEPA (Risk Reduction Engineering Laboratory Release Control Branch, Edison, NJ). The composition of the soil is given in Table 3.1.

Table 3.1- Composition of EPA Soil

Component	Weight %
Sand	31
Gravel No.9	6
Silt	28
Topsoil	20
Clay	15
Montmorillonite(5%)	
Kaolinite(10%)	
	100

The experiments with soil were performed by Mr. T. Poncet and more information about the soil and the results of the experiments with soil can be obtained from his thesis submitted for the degree of Master of Science in Environmental Sciences, at New Jersey Institute of Technology, Newark NJ, (Jan. 1992).

3.1.3 Organism

Phanerochaete chrysosporium BKM-1767 (ATCC 24728) was obtained from the American Type Culture Collection (ATCC), Maryland. The culture was maintained on yeast malt extract agar medium, which was prepared using the following constituents given in table 3.2.

Table 3.2 Composition of the Yeast Malt Extract Agar Medium

Yeast extract	0.3 g
Malt extract	0.3 g
Peptone	0.5 g
Agar	2.0 g
Glucose	1.0 g
Water	100 ml

The mixture was mixed and autoclaved at 121 °C for 20 minutes, following which it was cooled down to about 50-60 °C and transferred to the petri dishes.

3.1.4 Culture Media

Different culture media were prepared depending upon their application for either growing the fungus (growth medium) , or stimulating the production of the enzyme system for degrading the contaminants (induction medium). All the media were prepared using distilled water.

3.1.4.1 Growth Medium

The fungus was grown separately from the sand, either in shaker flasks or in a fermenter (as more clearly described in Section 3.2.3). The growth medium used for the growth of fungus had a composition similar to that described by T. K. Kirk (Kirk et al, 1986) as modified by N. Pal, Ph.D. student in Chemical Engineering at New Jersey Institute of Technology, Newark, NJ. The composition of the growth medium used in this work is given in Table 3.3.

Table 3.3 Composition of the Growth Medium for the Fungus

KH_2PO_4	2.00 g
$MgSO_4$	0.50 g
$CaCl_2$	0.10 g
$NaNO_3$	0.18 g
NH_4Cl	0.02 g
Glucose	10.0 g
Thiamine	1.0 mg
Mineral Salt Solution	5.0 ml
Distilled Water	Balance up to 1.0 l

3.1.4.2 Induction Medium

The induction medium was used in the first set of experiments (Set# 1). In this set, the fungus was grown separately using the growth medium, and then transferred to the bottles containing induction medium, in order to establish the ideal conditions to maximize the enzymatic activity. This was accomplished by reducing the concentration of nitrogen and glucose in the medium. The composition of the induction medium is given in Table 3.4

Table 3.4 Composition of the Induction Medium

KH_2PO_4	2.00 g
$MgSO_4$	0.50 g
$CaCl_2$	0.10 g
NH_4Cl	12.0 mg
Glucose	2.0 g
Thiamine	1.0 mg
Mineral Salt Solution	5.0 ml
Distilled Water	Balance up to 1.0 l

3.1.4.3 Mineral Salt Solution

A mineral salt solution was added to both the growth medium and the induction medium in order to provide the fungus with the trace elements required for growth and production of ligninolytic enzymes. The composition of the mineral salt solution (Kirk et al, 1986) is given in Table 3.5.

Table 3.5 Composition of the Mineral Salt Solution

KH_2PO_4	0.20 g
$MgSO_4.7H_2O$	3.0 g
$CaCl_2$	0.10 g
$MnSO_4.2H_2O$	0.50 g
NaCl	1.0 g
$FeSO_4.7H_2O$	0.10 g
$CoSO_4$	0.10 g
$ZnSO_4$	0.10 g
$CuSO_4.5H_2O$	10.0 g
$AlK(SO_4)2.24H_2O$	10.0 mg
H_3BO_3	10.0 mg
$NaMoO_4$	10.0 mg
Nitriotriacetate	1.50 g
Distilled Water	Balance up to 1.0 l

3.1.5 PCBs

Three different kinds of PCBs used were:

1) AROCLOR 1242
2) AROCLOR 1254
3) AROCLOR 1260

Aroclor 1242 was provided by AccuStandard, New Haven, CT (Lot# 001). Aroclor 1254 and 1260 were obtained from Chem Service Incorporation (Westchester, PA), 1254- LOT # 50-126A, and 1260- LOT # 52-89C.

3.1.6 2,4,6-TRICHLOROPHENOL (TCP)

This compound was purchased from SIGMA Inc. (T-1266, LOT # 69F0636).

3.2 APPARATUS

3.2.1 Flasks

250 ml erlenmeyer flasks were used to grow the fungus in the first set of experiments (Set# 1). The flasks were autoclaved before growing the fungus in it. While growing the fungus, the mouth of the flask was covered with cotton, in order to allow the oxygen to diffuse in, but prevent other organisms from entering the flask.

3.2.2 Shakers

Two kind of shakers were used, one to grow the fungus (but only in Experimental Set # 1), the other to extract the PCBs and the TCP from sand.

3.2.2.1 Shaker Used to Grow the Fungus

This shaker was used only in Set# 1. To grow the fungus, Gyratory Water Bath Shaker, (New Brunswick Scientific Co. Inc., NJ, model G76) was used. The temperature was maintained at 37 ^{o}C using a water bath.

3.2.2.2 Shaker Used for Extraction

Another type of shaker was used (Eberbach Corporation, Ann Arbor, Michigan) to extract the PCBs and the TCP from the sand in the bottles, prior to analysis. The shaking was performed unidirectionally at 240 rpm. While extracting, teflon lined

screw caps were used in order to prevent any leakage of the solvent (ether) from the bottles.

3.2.3 Fermenter

In Experimental Sets # 2, 3 and 4, a significant amount of fungus was used to inoculate all the sand. Therefore, the fungus was grown in a fermenter (Bioflo II Batch/Continuous Fermenter, New Brunswick Scientific Co. Incorporation, Edison, NJ). This could contain about 2.5 liters of growth medium. This amount was enough to carry out experiments in any set. Temperature, agitation and airflow could be constantly monitored and controlled. The pH was continuously monitored by the inbuilt pH probe. The agitation was provided by four bladed impellers, which were axially mounted in the fermenter. The vessel was baffled.

3.2.4 Bottles

All the experiments were performed in clear glass, screw capped , medium round bottles. The volume of each bottle was about 250 ml. During the degradation experiments, the bottles were covered with cotton, in order to allow the air to diffuse in and out. During the extraction of PCBs and TCP, teflon lined screw caps were used, in order to prevent any loss of solvent.

3.2.5 Environmental Chambers

All experiments were performed under controlled conditions of temperature and humidity. For this purpose, special chambers were used. Two different types of environmental chambers were used at different stages.

3.2.5.1 Environmental Chamber for Experimental Set #1 and 2

During Experimental Set #1 and 2, a walk-in environmental chamber was used (Environmental Growth Chamber, Chagrin Falls, Ohio). The temperature was maintained at 30 °C, and the humidity was controlled at about 14 %. To prevent the loss of water from the bottles, it was essential for the humidity to be as close to 100% as possible. This was achieved by building a smaller chamber (placed within the larger environmental chamber), which was covered by plastic from all the sides. The bottom of this smaller chamber was filled with water to about 3 cm. The water would evaporate and fill the chamber with water vapor, thus maintaining high humidity levels. Distilled water was used in order to minimize microbial growth in the environmental chamber.

3.2.5.2 Environmental Chamber for Experimental Set #3 and 4

For Experimental Set #3 and 4, the environmental chamber used was provided by Tabai Espec. Corp. (Model EY 101). In this chamber, the temperature and humidity were controlled by a built-in microprocessor at the values of 30 °C and 100 % respectively. An aeration mechanism was built (as described in Section 7.4.5) to provide air to all the bottles.

3.2.6 Aeration Mechanism

In Experimental Set #3 and 4, the air introduced into each bottle came from the compressed air outlet of the lab. The air at this outlet was dry. The air was tapped at this outlet using a plastic tube, and filtered using a biofilter (pore size 0.3 um) to reduce any contamination risks. The air was then bubbled through a 250 ml glass bubbler, in order to humidify it, thus minimizing water evaporation from the bottles. The outlet from the glass bubbler was connected to a tree of plastic Y connectors,

(O.D = 1/4 inches), at the end of which sixty-four air outlets were obtained. Each Y connector was connected to the other using Nalgene plastic tubes. To these sixty-four air outlets (extended with Nalgene plastic tubes) glass tubes were connected. These glass tubes were then inserted into the glass bottles. The mouth of the bottles were then covered with cotton, in order to prevent external contamination.

3.3 GENERAL METHODS

3.3.1 Fungal Growth

The fungus was grown using two different methods. In the first set of experiments (Set# 1), the growth medium was prepared, autoclaved, and then about 100 ml of it was transferred into a sterile 250 ml erlenmeyer flask. The mouth was covered with cotton. The growth medium in the flask was inoculated under sterile conditions with the fungus, taken from the petri dish in which it was originally grown.

The flask containing inoculated growth medium was put in the shaker as described in section 3.2.2.1. The temperature was maintained at 37 °C using the water bath in the shaker. The flask was then shaked birotationally at 150 rpm. These conditions were maintained for about four days, at the end of which the fungus was fully grown and was ready for the experiments.

For the Experimental Set # 2, 3 and 4 the fungus was grown in a separate fermenter (section 3.2.3). The growth medium was autoclaved, and so was the glass fermenter. The growth medium was transferred to the fermenter and then it was inoculated with the fungus, obtained from the petri dish, under sterile conditions. The temperature of the fermenter was maintained at 37 °C and the agitation at 150

rpm. The airflow was maintained at about 100 ml/min. The pH was continuously monitored using a pH probe.

Samples from the fermenter were periodically taken and analyzed for their nitrogen and glucose content. For optimal enzymatic activity, it was seen from past experience, that nitrogen level should be less than 2 ppm and the glucose level should be less than 4 g/l. Nitrogen was measured using a specific ion electrode (described in Section 3.5.3.4) and glucose was measured by method described in section 3.5.3.3.

3.3.2 Sand Preparation

The prewashed and dried sand was weighed accurately and 20 grams of it was transferred to each bottle. These bottles were then autoclaved at 121 °C for twenty minutes in order to sterilize the sand.

To contaminate the sand artificially, stock solutions of the contaminants (either PCBs or TCP) were prepared by weighing accurately and dissolving completely the contaminants in hexane as a solvent. These stock solutions had concentrations of 200 ppm of their specific contaminant (either PCB or TCP). 10 ml of either one of these solutions were transferred to the appropriate bottles. The bottles were kept under the hoods for about twelve hours to let the solvent hexane evaporate, thus leaving the contaminant in the sand. The contaminated sand, thus, contained 100 parts per million of the respective contaminant (ug of contaminant per gram of sand).

3.4 EXPERIMENTAL SET-UP

3.4.1 Set-up for Experimental Set #1

This was the first set of experiments designed with the hope of getting biodegradation of the PCBs. Experiments were performed on two types of PCBs, that is, Aroclor 1254 and 1260. The second aim of these experiments was to optimize the two important parameters in the enzymatic activity of the fungus, that is

* Initial nitrogen concentration; and
* Initial glucose concentration.

Various kind of control experiments were also run under the same conditions as those of the main experiments, but without fungus, in order to see if there was any degradation or removal of contaminants without the fungus. These controls contained plain water under both sterile and non sterile conditions, and growth medium under both sterile and non sterile conditions. The experimental conditions are tabulated in Table 3.6, 3.7, 3.8 and 3.9.

Table 3.6 Conditions for Experimental Set# 1 with Aroclor 1254

Initial NH_4Cl Conc. (ppm)[a]	Initial Glucose Conc (g/kg sand)	Initial Water Content %[b]	No. of Bottles
2	12	50	5
32	22	50	5
2	22	50	5
32	12	50	5

[a] w/w Nitrogen in NH_4Cl/Sand [b] w/w Water/Sand

Table 3.7 Experimental Conditions for Controls in Set # 1 with Aroclor 1254:

Initial NH_4Cl Conc. (ppm)[a]	Initial Glucose Conc (g/kg sand)	Initial Water Content %[b]	No. of Bottles
0 *	0	50	5 Sterile
32	22	50	5 Sterile
0 *	0	50	5 Non sterile
32	22	50	5 Non sterile

Table 3.8 Conditions for Experimental Set # 1 with Aroclor 1260:

Initial NH_4Cl Conc. (ppm)[a]	Initial Glucose Conc (g/kg sand)	Initial Water Content %[b]	No. of Bottles
2	12	50	5
32	22	50	5
2	22	50	5
32	12	50	5

Table 3.9 Experimental Conditions for Controls in Set # 1 with Aroclor 1260:

Initial NH_4Cl Conc. (ppm)[a]	Initial Glucose Conc (g/kg sand)	Initial Water Content %[b]	No. of Bottles
0 *	0	50	5 Sterile
32	22	50	5 Sterile
0 *	0	50	5 Non sterile
32	22	50	5 Non sterile

* Contained distilled water

[a] w/w Nitrogen in NH_4Cl/Sand

[b] w/w Water/Sand

In order to follow the PCB degradation as a function of time, five bottles (all identical in their content) were prepared for each experiment. The first bottle was harvested at time zero. Then every week one bottle of each kind was harvested and stored in the freezer prior to PCB extraction, in order to determine the extent, if any, of biodegradation.

3.4.2 Set-up for Experimental Set #2

Following the results of the first set of experiments (Set# 1), a new set of experiment was designed. The PCB-contaminated sand was prepared in the same way as in Set# 1. Only Aroclor 1254 was being tested this time. The concentration of the PCB in the sand was 100 ppm (ug PCB per gm of sand). In this experimental set (Set# 2), before inoculating the contaminated sand with fungus, the fungal medium in which the fungus was grown, was tested for its nitrogen and glucose contents. It was being investigated if there was any positive effect on biodegradation of PCBs, if the fungus used was at its best enzymatic activity. Any positive effect of the increased amount of initial concentration of the fungal biomass, on biodegradation of PCBs, was also being tested. The conditions for the best enzymatic activity of the fungus are about 4 g/l of glucose and less than 2 ppm of nitrogen content. When the fungus had grown to such an extent in the fermenter that the level of glucose and nitrogen were brought down to the above levels, due to consumption of nutrients by the growing fungus, 15 ml of the fungal slurry was transferred to each bottle. The bottles in this experiment were harvested every three days.

3.4.3 Set-up for Experimental Set #3

This set of experiments were basically an extension of Experimental Set# 2. In this set, the sand contaminated with Aroclor 1254, with a concentration of 100 ppm (ug PCB per gm of sand), was tested. In order to check the activity of the fungus against some other compound, another set of experiment was run in parallel. The compound chosen was 2,4,6-trichlorophenol (TCP). This compound was reported to be degradable by the fungus, by N. Pal. This compound was added to the sand in the same way as the PCBs. The fungus to be used in both the cases was obtained from the same fermenter batch, and experiments were started at the same time and under similar conditions, as those for the PCBs. This time an aeration mechanism (as described in section 3.2.5) was used to provide air/oxygen to the fungus. The flow rate of air was continuously maintained between 125 - 150 ml per minute per bottle.

For each of the two compounds five bottles were prepared (each containing 20 grams of sand with the appropriate contaminant). The contaminant (PCB or TCP) was added to the sand in such a way, so as to give an initial concentration of 100 ppm (ug contaminant per gm of sand), after the solvent had evaporated. Controls were also run along with the experiments. These controls contained DI water instead of fungal slurry, and were run in order to see any degradation or disappearance of contaminants, in the absence of the fungus, but under identical environmental conditions. There were five control bottles for each one of the compounds, and one was harvested every time a corresponding bottle, containing the same compound and the fungus was being harvested. The conditions for this set of experiments are shown in Table 3.10.

Table 3.10 Conditions for Experimental Set #3

Compound	Amount of Sand per Bottle	Inoculating Medium	No. of Bottles	Harvesting Period
PCB (100ppm)	20 g	20 ml Fungus	5	7 days
PCB (100ppm)	20 g	20 ml DI water	5	7 days
TCP (100ppm)	20 g	20 ml Fungus	5	3 days
TCP (100ppm)	20 g	20 ml DI water	5	3 days

The PCB used was Aroclor 1254, and the TCP was 2,4,6 Trichlorophenol.

3.4.4 Set-up for Experimental Set #4

From the results of the experiments in Set #3, it was evident that the fungus was unable to degrade the PCBs. The Experimental Set # 4 was repetition of Set # 3, except that the PCB was changed from Aroclor 1254 to Aroclor 1242. This was done in order to see if a less chlorinated PCB could be degraded by the fungus or not. The experimental procedure was exactly the same as in Set # 3, except for the number of bottles prepared, which were 7 (for the contaminants with fungus) or 6 (for controls) instead of 5. The experimental conditions for this set are described in the Table 3.11.

Table 3.11 Conditions for Experimental Set #4

Compound	Amount of sand per Bottle	Medium of Contact	No. of Bottles	Samples Harvested
PCB (100ppm)	20 g	20 ml Fungus	7	7 days
PCB (100ppm)	20 g	20 ml DI water	6	7 days
TCP (100ppm)	20 g	20 ml Fungus	7	3 days
TCP (100ppm)	20 g	20 ml DI water	6	3 days

3.5 EXTRACTION AND ANALYSIS OF PCBs AND TCP

3.5.1 Extraction of PCBs

The bottles which were harvested at various times were stored in the freezer at -5°C. For the extraction, the temperature of the bottles and the contents were brought down to room temperature by simply taking the bottles out of the refrigerator and allowing them to equilibrate for about an hour. To all the bottles, DI water was added to such an amount so as to bring the final water content of the solutions in each bottle to about 30 ml (assuming no evaporation of water from the bottles). This means that to all the bottles in Set #1, 20 ml of water was added, and to Set #2, 15 ml of water was added. In Set #3 and 4, 10 additional ml of DI water were added, but only to the bottles containing PCBs.

Then, 50 ml of di-ethyl ether was added to each bottle (each containing 20 gm of sand). The mouth of the bottles were then tightly capped with teflon-lined screw caps. These bottles were then kept in the shaker (described in section 3.2.2.2) horizontally, and were shaken unidirectionally at 150 rpm. The motion of movement of the bottles was parallel to the cylindrical axis of the bottles.

3.5.1.1 Extraction Experiments

The results of the analysis of PCBs in the Experimental Set #1 and 2 suggested that the extraction of PCBs was a function of time. Therefore, a separate set of extraction experiments were run in order to find the extraction efficiency of PCBs as function of extraction time.

3.5.1.1.1 Preliminary PCB Extraction Experiments

Some preliminary experiments were carried out to determine the extraction efficiency of PCBs as a function of extraction time. In the preliminary experiments, it was tested if there was an advantage of adding salt (NaCl) to the bottles before extracting the PCBs. 20 ml of dead (autoclaved) fungus was introduced in the bottles, and 10 ml of DI water was added. The experimental conditions were as shown in Table 3.12 .

Table 3.12 Conditions in Preliminary Extraction Experiments

Bottle #	Dead Fungus	DI Water	NaCl	Ether
1	20 ml	10 ml	0 g	50 ml
2	20 ml	10 ml	2 g	50 ml
3	20 ml	10 ml	4 g	50 ml

All the bottles contained 20 g of sand contaminated with 100 ppm of Aroclor 1254.

The results are shown in Section 4.1.1.1.

3.5.1.1.2 Additional PCB Extraction Experiments

Following the preliminary extraction experiments, another set of extraction experiments were performed, in which more data points (7) were taken. The experimental conditions are shown in Table 3.13 .

Table 3.13 Conditions in Extraction efficiency Experiment

Bottle #	Dead Fungus	DI Water	NaCl	Ether
1	20 ml	10 ml	0 g	50 ml
2	20 ml	10 ml	0 g	50 ml
3	20 ml	10 ml	3 g	50 ml

Note: Bottle #1 and 2 were identical.

The results are shown in Section 4.1.1.2. The PCBs, which were extracted in the ether phase, were analyzed using gas chromatography (Section 3.5.3.1).

3.5.2 Extraction of 2,4,6-Trichlorophenol

Various experiments were performed by Mr. T. Poncet on the extraction of TCP from soil, and the final procedure selected for the analysis of TCP in these experiment was a multiple extraction of TCP, using methanol. This procedure was also used to extract TCP from sand. The protocol finally adopted was as follows:

1) To every bottle containing 20 g of sand, initially contaminated with 100 ppm of 2,4,6-trichlorophenol, 30 ml of methanol were added. The bottles were capped with teflon lined screw caps, and shaken on the shaker in the similar way as in the case of PCBs (Section 3.2.2.2), for 30 minutes. Then, the liquid phase was transferred to a 100 ml volumetric flask.

2) 30 ml of methanol was again added to the bottles, followed by shaking for additional 30 minutes. The liquid phase was transferred to the volumetric flask as in Step 1.
3) 20 ml of methanol was added to the bottle and shaking was done for additional 30 minutes. The liquid phase was transferred to the volumetric flask.

Methanol was added to all the volumetric flasks, to fill it up to a volume equal to 100 ml. The contents were filtered using the syringe microfilters, 0.25 um pore size, to remove all the solid particles, and the filtrate was analyzed for TCP using the HPLC.

3.5.3 Analytical Methods

Different analytical techniques were used to determine the concentration of PCBs, TCP, glucose, nitrogen and chloride. The methods are described in this sub-section.

3.5.3.1 Analysis of PCBs

After the PCB extraction the ether samples containing extracted PCBs were run on the gas chromatographs. For Set #1 and 2, the conditions were the following:

Gas Chromatograph Used : Varian, Model 3400.

Detector : Electron Capture Detector.

Carrier Gas : He-UHP 60 psi at regulator.

Flow rate : 0.67 ml/min

Make up Gas : N_2 UHP 60 psi at regulator.

Flow rate : 33 ml/min

Split ratio : 16

Temperature Programming:

Initial Column Temperature : 160 °C

Initial Hold Time : 0 min

Initial Rate : 2 °C per min for 20 min

First Ramp : 8 °C per min for 5 min

Final Temperature : 24 °C

Final Hold Time : 20 min

Injection Temperature : 300 °C

Detector Temperature : 300 °C

Volume Injected : 1 ul

Column Used : DBI, Methylsilicone, 0.25 um film thickness, 30 m, J & W Scientific (Varian CAT No. JW 122103-20)

For Experimental Set #3 and 4,

Gas Chromatograph Used		: Hewlett Packard (HP)
		: HP 5890 gas chromatograph
Gases Used-	Carrier Gas	: Helium, 50 psi
	Makeup Gas	: Nitrogen 30 psi
Flow Rates-	Split	: 10.4 ml/min
	Purge	: 3.2 ml/min
	Carrier Gas (He)	: 0.64 ml/min
	He + N_2	: 35.2 ml/min
Temperature Programming		
	Initial Temperature	: 160 °C
	Initial Hold Time	: 0 min
	Initial Rate	: 8 °C / min till 200 °C
	First Ramp	: 1 °C / min
	Final Temperature	: 240 °C
	Final Hold Time	: 2 min
	Injection Temperature	: 290 °C
	Detector Temperature	: 290 °C
Detector Used		: ^{63}Ni ECD (Electron Capture Detector)
Column Used		: SPB - 608
		Fused Silica Capillary Column
		30 m, 0.25 mm I.D, 0.25 um Film thickness
		SUPELCO, Inc.

3.5.3.2 Analysis of 2,4,6-Trichlorophenol

A Waters, High Performance Liquid Chromatograph System (HPLC) was used to find the concentration of TCP extracted. The HPLC was equipped with Waters 600 E system Controller, Waters 715 Ultra Wisp Sample Processor and Waters Tunable Absorbance Detector.

Column Used	: Altech Econosphere C8, 4.6 mm I.D, 150 mm 9 (Cat # 70090)
Mobile Phase	: Methanol-Water (60:40)
Flow Rate	: 1 ml/min
Condition	: Isocratic
Injection Volume	: 25 ul

An Altech Direct Connect Refillable Guard Column was also used to protect the column.

3.5.3.3 Analysis of Glucose

To determine the concentration of glucose left in the growth medium, while growing the fungus in the fermenter, samples of medium from the fermenter were taken and centrifuged at 3000 rpm in a microcentrifuge (IEC Centra - M Centrifuge) for ten minutes. 0.1 ml of the supernatant were transferred to a test tube. 0.1 ml of a glucose standard (1g/l) was transferred to another test tube. Two blanks were prepared by putting 0.1 ml of distilled water into two separate test tubes. To all these four test tubes, 5 ml O-toludiene was added and the test tubes were kept in boiling water for about ten minutes and then cooled down to room temperature. These solutions were then transferred to different cuvettes, and analyzed using UV-Visible spectrometer (Varian - DMS 200) at a wavelength of 535 nm. One of the

blanks was used as a reference, and the other was used to zero the spectrometer. The absorbance was converted to concentration of glucose (gm/l) using the absorbance values of the glucose standard.

3.5.3.4 Analysis of Nitrogen

Nitrogen was analyzed as nitrate using a specific ion electrode (Orion, model 93-07). The electrode was previously calibrated using nitrate standards. The reference electrode was Orion, model 90-02. The meter used was Orion, model SA 720.

3.5.3.5 Analysis of Chloride

Chloride concentration was measured only in Set #4. The argentometric method for chloride analysis was used. The method is described in the Standard Methods for the Examination of Water and Wastewater (16th edition). The principle behind this is that in neutral or slightly alkaline solutions, potassium chromate can indicate the end point of the silver nitrate titration of chloride. During the extraction of TCP, the chloride had come in the methanol phase. The method is described for chloride in water, but the solubility of silver chloride in methanol is still less than that in water. Therefore chloride could be safely titrated in the methanol phase.

Reagents Used:

a) *Potassium chromate indicator solution:* 50 g of K_2CrO_4 were dissolved in a small amount of distilled water. $AgNO_3$ solution was added until a definite red precipitate was formed. It was let stand for 12 hours and then filtered. The filtrate was diluted to 1L with distilled water.

b) *Standard silver nitrate titrant:* 0.00705 N of $AgNO_3$ solution was prepared by dissolving 1.1975 g of $AgNO_3$ in 1 L distilled water. This is half the concentration

of what is mentioned in the text. This was done in order to increase the burette reading, as the chloride expected in the samples to be titrated was less.

A known volume of the sample for which chloride was analyzed, was taken in an erlenmeyer flask. About two drops of the indicator solution were transferred in this flask. This solution was then titrated against standard silver nitrate solution. The end point was reached when the color of the solution changed from yellow to pinkish red.

CHAPTER 4
RESULTS

4.1 RESULTS OF THE EXTRACTION EXPERIMENTS

4.1.1 Results of the Extraction Experiments for PCBs

As discussed in Section 3.5.1, there were two sets of extraction experiments for PCBs, the preliminary experiments and the additional extraction efficiency experiments. The results for both experiments are discussed in this section.

4.1.1.1 Results of the Preliminary Extraction Experiments for PCB

The results of the preliminary extraction experiments for PCBs are shown in Table 4.1 and Figure 4.1. The aim of this set of experiment was to find the optimal conditions to carry out the extraction and especially to determine if the addition of salt (NaCl) would help in extracting the PCBs by making the water phase more polarized. Figure 4.1 clearly shows that as we added more NaCl to the bottles, the extraction efficiency of PCBs decreased. So it was decided not to add any NaCl to the bottles, for extracting PCBs. Another important point to be noticed in this graph is that as we increased the extraction time, the extraction efficiency initially increased, but after some time, (somewhere between five and ten hours of extraction time) it began to decrease. This was noticed in all the runs, with or without NaCl.

Table 4.1 Preliminary PCB Extraction Efficiency Results:

Extraction Time(hrs)	PCB Concentration in ppm (Aroclor 1254)		
	Bottle# 1 (No NaCl)	Bottle# 2 (+2g NaCl)	Bottle# 3 (+4g NaCl)
2	99.85	90.41	81.92
5	101.82	92.51	84.89
10	93.92	86.31	84.39

Amount of PCBs in each sample : 100 ppm

4.1.1.2 Results of the Additional Extraction Experiments for PCB

For the next set of extraction experiments (Section 3.5.1.1.2), which was performed to find the extraction efficiency curve for the PCBs with respect to time, the results can be seen in Table 4.2 and Figure 4.2. As expected from the preliminary results, the maxima for extraction was obtained at seven hours of extraction time. After seven hours, a decrease in extraction of PCBs with respect to time was observed. The extraction was performed up to twelve hours. More extraction points were not taken because, as the number of extraction points increased, the bottles had to be opened more often. Since samples were taken from the same bottles to monitor the extraction efficiency vs time, two problems could be encountered. First, the amount of ether evaporating due to the opening of bottles could have introduced some error; and second, about 1 ml of the ether sample was being taken for analysis each time, thus reducing the volume of ether as the extraction proceeded (initially, 50 ml ether were used for extraction). In a separate bottle, extraction was performed for twenty-three hours, and the extraction efficiency was only 37 %. This indeed confirmed that the extractions in excess of twelve hours were found to be less

efficient than those conducted for seven hours. In Figure 4.2, the curves corresponding to Bottle# 1 & 2 (refer to Table 3.13) are identical (with 20 ml of dead fungus, 10 ml of DI water and 50 ml of di-ethyl ether). To the third bottle, 3 gm of NaCl were added, and as expected, the efficiency was less than that of the extraction without NaCl.

The extraction efficiency experiments were performed after the experiments in Set #1 and 2 had been conducted. In these sets the samples were extracted for different lengths of times ranging between 1.5 and 10 hours. Due to the variation in extraction times, the results of extraction of PCBs could not be compared with each others.

Table 4.2 Additional PCB Extraction Efficiency Results

Extraction Time(hrs)	PCB Concentration in ppm (Aroclor 1254)		
	Bottle # 1 (No NaCl)	Bottle # 2 (No NaCl)	Bottle # 3 (+3g NaCl)
1	76.92	80.20	57.25
2	83.54	87.68	66.52
3	83.82	89.12	66.36
5	82.25	92.40	71.98
7	87.49	96.59	76.96
9	81.39	91.52	71.79
12	79.86	89.08	71.00

4.1.2 TCP Extraction Efficiency

As mentioned before, these experiments were performed on soil by Mr. T. Poncet, and due to the similarity between the experiments in soil and sand, the extraction

results of soil were applied to sand also. A summary of all the methods tested is given in Table 4.3.

Table 4.3 Extraction Efficiency of TCP

Method ID ->	M1	M2	M3	M4
Volume of First Methanol Addition (ml)	30	30	40	40
First Extraction Time (hours)	1	1/2	1/2	1/2
Volume of Second Methanol Addition (ml)	30	30	40	40
Second Extraction Time (hours)	1	1/2	1/2	1/2
Volume of Third Methanol Addition (ml)	20	20	20	10
Third Extraction Time (hours)	1	1/2	1/2	1/2
Cumulative TCP Recovery (%)	84.5	90.0	83.0	79.5

The extraction procedure M2 was then used to determine the TCP concentration in the degradation experiments. The repetition of the extraction procedure (M2) gave 92%, 95%, 97% and 98% recovery.

Due to vigorous shaking, the soil particles used get mixed with methanol, to form an unclear liquid phase. Therefore, the extraction in soil required waiting for 2 hours after every extraction step, to let the soil sediment, and then the clear methanol phase was removed from the top. All the removed phases were introduced in a 100 ml volumetric flask. The volume was then balanced up to 100 ml

using methanol, and then these solutions were analyzed on HPLC, after filtering it into microfilters (.25um pore size).

In the experiments with sand, the same TCP extraction procedure was used, except that there was no need for the two hour waiting period, as the problem of sand particles mixing with methanol was not as severe as it was with soil, due to larger particle size of sand particles.

4.2 RESULTS OF THE PCB AND TCP DEGRADATION EXPERIMENTS

4.2.1 Results of Experimental Sets #1 and 2

In the Experimental Set #1 and 2, the extraction of PCBs was performed using different extraction times. An attempt was made to extrapolate these results to get the possible concentration of PCB in sand. This was done assuming a first order extraction of the PCBs in to ether phase. The results of these extrapolated data are shown in Figures 4.3 and 4.4. It can be seen that even after thirty-two days, there was no significant change in the concentration of PCBs in sand.

The chromatograms of these experiments are shown in Figures 4.11 and 4.12. The various peaks in the chromatograms represent various congeners of the PCBs. From these figures, it can be clearly seen that there is no relative reduction of the height of any of the peaks in the contaminated sand samples, which were exposed to the fungus even up to thirty-two days. If there was a minor degradation, which was undetected due to incompleteness of the extraction process, we would have expected to see a relative reduction of the height of at least some of the peaks. This relative reduction would have not been expected to be the same for all the congeners. The equal relative reduction of the heights of the peaks would signify

that all the congeners were degraded by the fungus to the same extent and at the same rate. This is highly improbable, because although the fungus has been found to be degrading many chlorinated compounds, not all of them would be degraded at the same rate and to the same extent. These chromatograms had nearly the same fingerprints. Therefore it was evident that there was no biodegradation of the PCBs by the fungus. The experiments in soil also showed similar negative results.

In Experimental Set # 2, as we have seen, 15 ml of the fungal inocculum was used to see if there was a positive effect of a much larger volume of the initial fungal inocculum and the amount of enzymes (which were produced by the fungus during its growth in the fermenter), on degradation of PCBs. The results of this experiment were also negative. There was no evidence of biodegradation, as is evident from the chromatograms in Figure 4.13.

4.2.2 Results of Experimental Set #3

As discussed in Chapter 3, this set was a sequel to Set # 2. From the disappointing results of the earlier experiments, it was decided to check the activity of the same fungus, which was used for the PCBs, against 2,4,6-trichlorophenol. The PCB being tested was Aroclor 1254. The TCP experiments were run in parallel with those of the PCBs, and the fungus used for inoculating both the compounds came from the same batch, and the samples were inoculated at the same time (that is time zero was same for both the experiments).

The TCP bottles were harvested every three days, and the PCBs samples were harvested every seven days. The compounds (PCB or TCP) were extracted and analyzed by the procedure described in Chapter 3. For PCB extraction, the extraction time was kept uniform at seven hours, as this was the maximum found out

from the extraction efficiency experiment. For the TCP, extraction procedure was same as described in Section 3.5.2 .

The results for this set of experiments are shown in Tables 4.4, 4.5 and Figures 4.5 and 4.6. From these results, it was clear that, once again, there was no degradation of PCBs. The chromatograms of these experiments again prove that there was indeed no biodegradation, as no relative height reduction of any of the peaks was found. All the chromatograms looked alike.

However, the results of TCP experiments indeed showed that there was degradation of this compound. As shown in Figure 4.6, we can see a continuous decrease in the concentration of TCP in sand which was inoculated with fungus, whereas the controls (containing no fungal slurry but only an equivalent amount of DI water) did not show any continuous decrease in the concentration of TCP. The decrease in the concentration of TCP was a result of the addition of the fungus, because in the chromatograms of the TCP analysis, there were some compounds eluting before the TCP eluted, and these peaks were absent in the chromatograms of the controls (Figure 4.16). Similar peaks were found by Mr. N. Pal, during his experiments with the TCP in water using the same fungus.

From these results, it was evident that the fungus was active against the TCP, but not against the PCBs.

Table 4.4 Results of the Experiments with PCB (Aroclor 1254) in Sand, in Experimental Set #3

Harvesting Time(days)	PCB Concentration in ppm	
	Experiment	Control
0	80.90	76.78
7	71.60	83.15
14	72.95	84.22
21	74.35	81.55
28	78.70	92.35

Table 4.5 Results of the Experiments with 2,4,6-TCP in sand, in Experimental Set #3

Harvesting Time(days)	TCP Concentration in ppm	
	Experiment	Control
0	56.20	53.70
3	37.60	38.80
6	17.55	52.65
9	7.11	39.70
12	3.33	52.35

4.2.3 Results of Experimental Set #4

These experiments were a repetition of the experiments in Set # 3, and were performed to confirm that the PCBs in sand are not biodegradable by the fungus at the concentration of 100 ppm. In this set of experiments, another factor was tested, namely, the type of PCB. Therefore the PCB used for this set was Aroclor 1242. This has about 42 % chlorine as compared to 54 % of chlorine in Aroclor 1254. An attempt was made to determine the concentration of chloride ion in the experiments with TCP (since Cl^- would be product of the degradation process).

The results of this set of experiments are shown in Tables 4.6, 4.7 and in Figures 4.7 and 4.8. The extraction time for PCBs was seven hours, and the extraction procedure for both the compound was same as described in Chapter 3. From these figures, it can be seen that there is a decrease in amount of PCBs with time, both in the controls as well as in the experiments with the fungus. The concentration of PCBs in the controls was found to be less than that with the fungus. This is a strange result. A possible explanation is poor recovery of PCBs. Even though the recovery seems to be poor, the chromatograms of this experiments still show the same negative results. There was no relative reduction in the peak height, and all the chromatograms looked alike.

The results of the experiments with TCP show a definite biodegradation by the fungus, as shown in the Figure 4.8. The concentration of the TCP gradually decreased to zero ppm after 18 days, whereas in the controls, it remained more or less constant. The chromatograms of the TCP did show some degraded compounds eluting before the TCP. In this experiment, the concentration of chloride ions was also monitored. The results are shown in Figure 4.9. This figure clearly shows a gradual increase in the concentration of chloride ions with time. This means that the fungus had degraded the TCP and some of the chlorine was liberated as chloride

ions. The theoretical amount of chloride which would have been expected if stoichiometric amounts of chloride ions had been released, is shown by Curve 2 in Figure 4.9. The graphs showing molar amounts of TCP degraded and the chloride recovered, are compared in Figure 4.10. The amount of chloride ion recovered was not equal to this theoretical amount. The possible explanation to this fact could be that not all the chlorine atoms attached to the particular TCP molecules, are reduced to chloride atoms. This means that although the TCP is degraded, the degradation is such that the bigger molecule of TCP is broken down into some smaller structure(s) and during this process, some of the chlorine atoms are liberated as chloride ion, but some remain attached to smaller fragments and are not available as free chloride ion.

This set of experiment was the last in the series of the experiments performed in this research. It is confirmed from this set that the fungus, which was active against some compound (2,4,6-trichlorophenol) in sand, was not able to degrade the PCBs in sand at a concentration of 100 ppm.

Table 4.6 Results of the Experiments of PCBs (Aroclor 1242) in Sand, in Experimental Set #4

Harvesting Time(days)	PCB Concentration in ppm	
	Experiment	Control
0	78.25	81.25
7	72.80	75.78
14	70.62	70.05
21	73.18	69.38
28	68.00	57.00
35	72.50	52.40
42	58.75	-

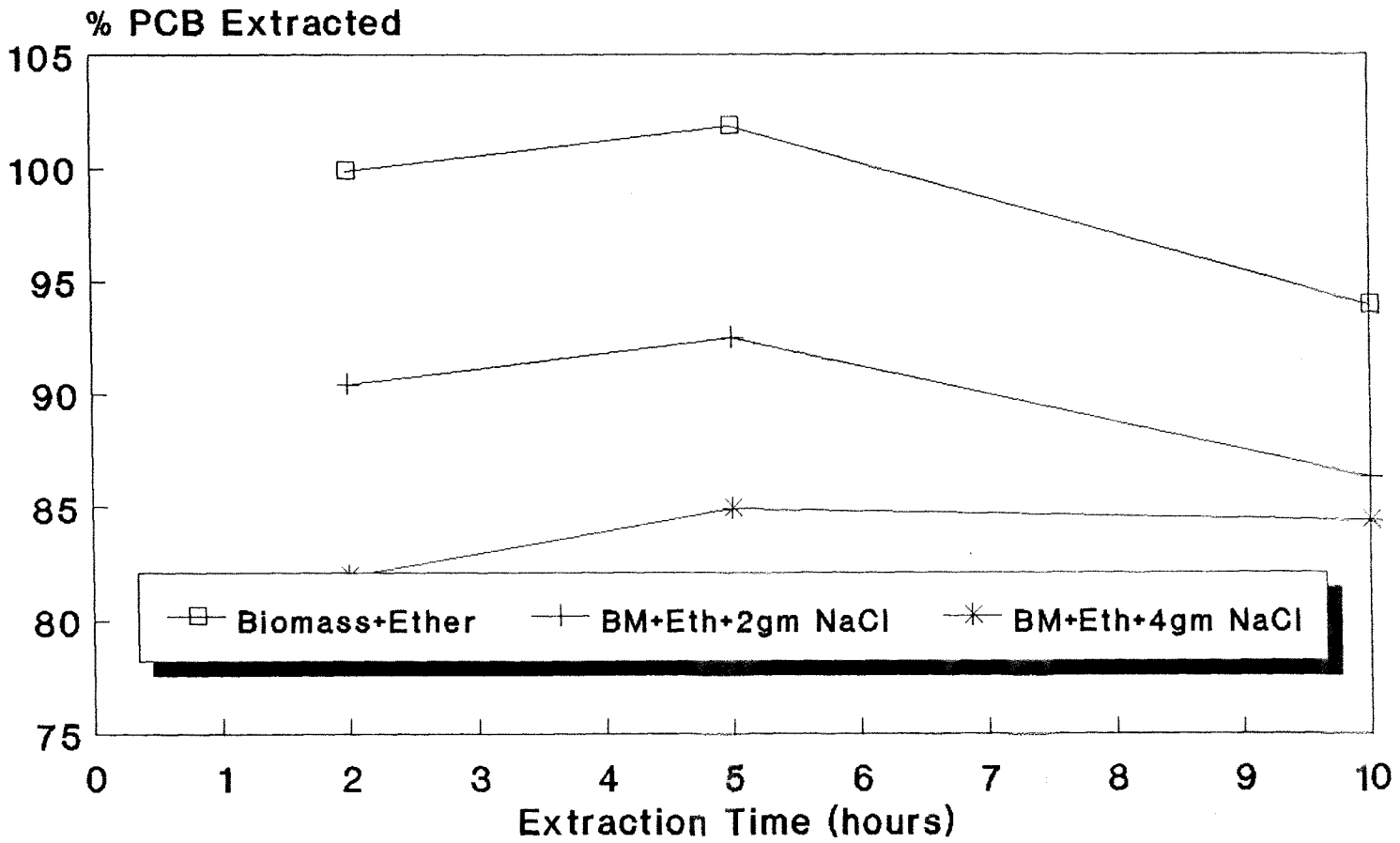
Table 4.7 Results of the Experiments of 2,4,6-Trichlorophenol in Sand in Experimental Set #4

Harvesting Time(days)	TCP Concentration in ppm	
	Experiment	Control
0	40.22	42.46
3	20.74	40.90
6	22.48	34.23
9	6.16	41.72
12	4.01	38.02
15	0.49	38.98
18	0.00	-

Table 4.8 Chloride Content in Degradation of 2,4,6 TCP in Sand in Experimental Set #4

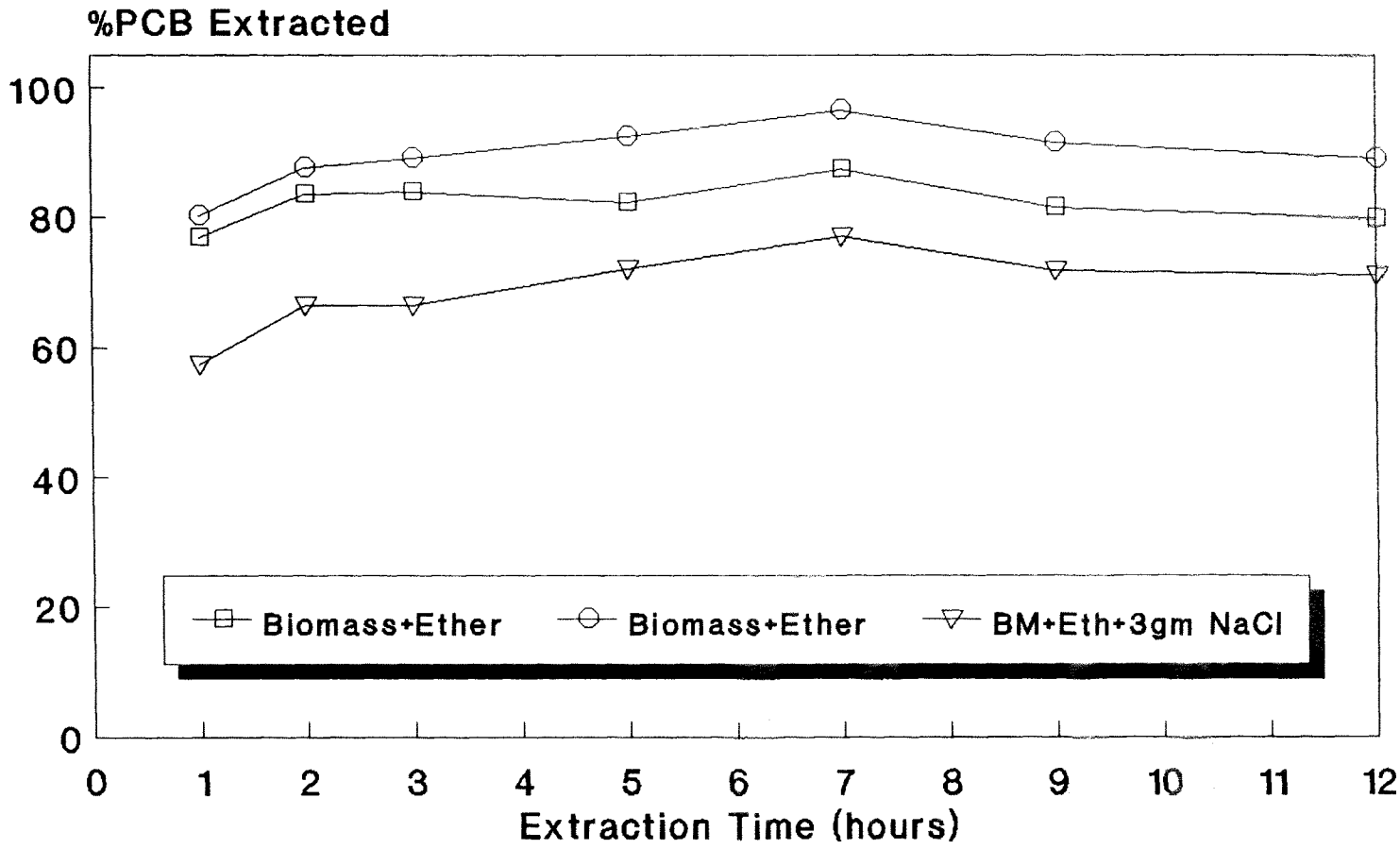
Harvesting	Cl^- Concentration in umol / g of sand		
Time(days)	Actual	As Expected From Degradation of TCP	Control
0	0.072	0.084	0.072
3	0.272	0.387	0.072
6	0.272	0.360	0.072
9	0.362	0.613	0.072
12	0.362	0.646	0.072
15	0.580	0.701	0.072
18	0.507	0.709	-

Figure 4.1. Extraction Efficiency for PCB (Aroclor 1254) in Sand. Preliminary Extraction Results



Initial Concentration of PCB was 100 ppm

Figure 4.2. Extraction Efficiency for PCB (Aroclor1254) in Sand. Additional Extraction Experiments



Initial concentration of PCB was 100 ppm

Figure 4.3. Activity of the Fungus on Aroclor 1260, in Experimental Set #1

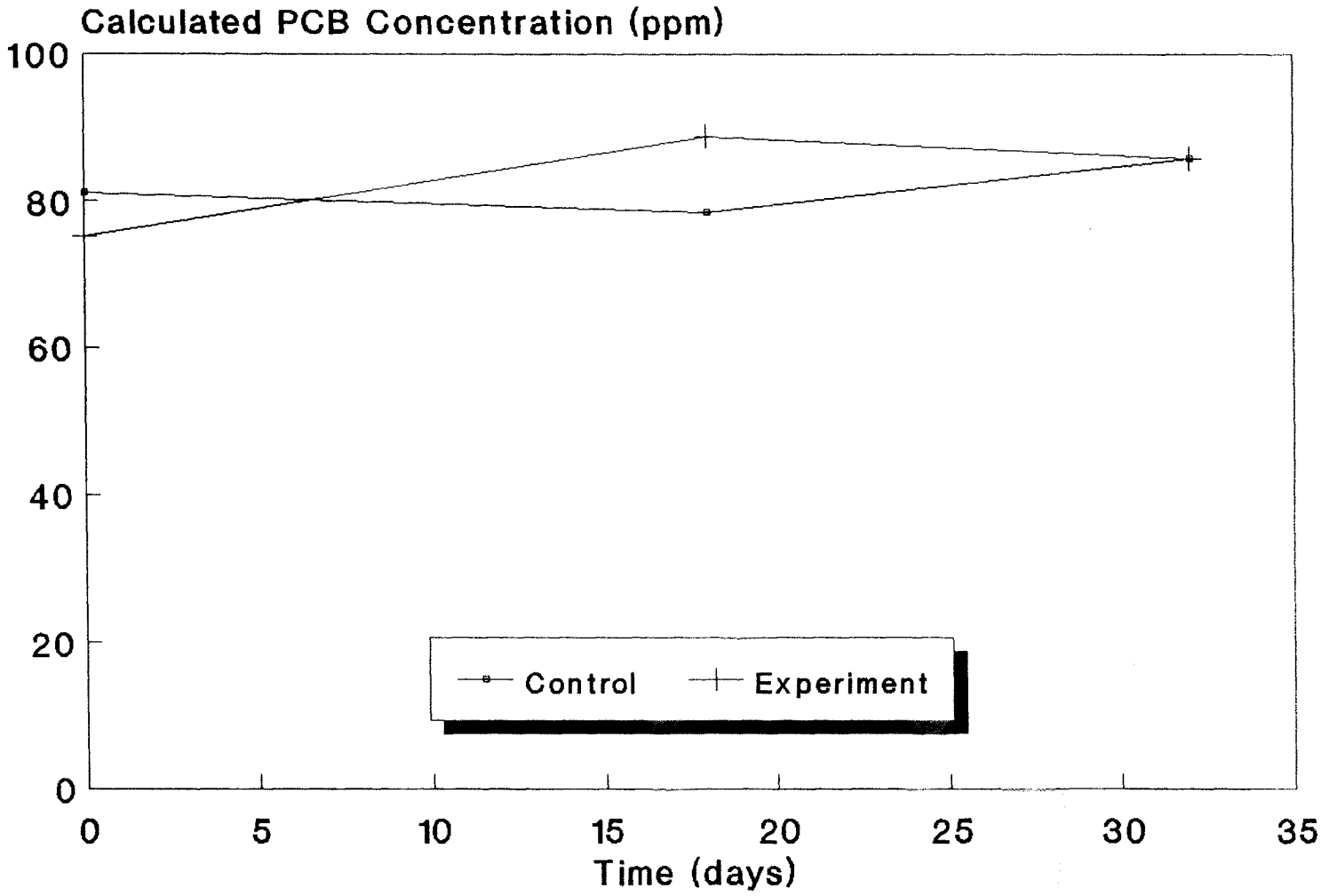


Figure 4.4. Activity of the Fungus on Aroclor 1254 in Experimental Set #1

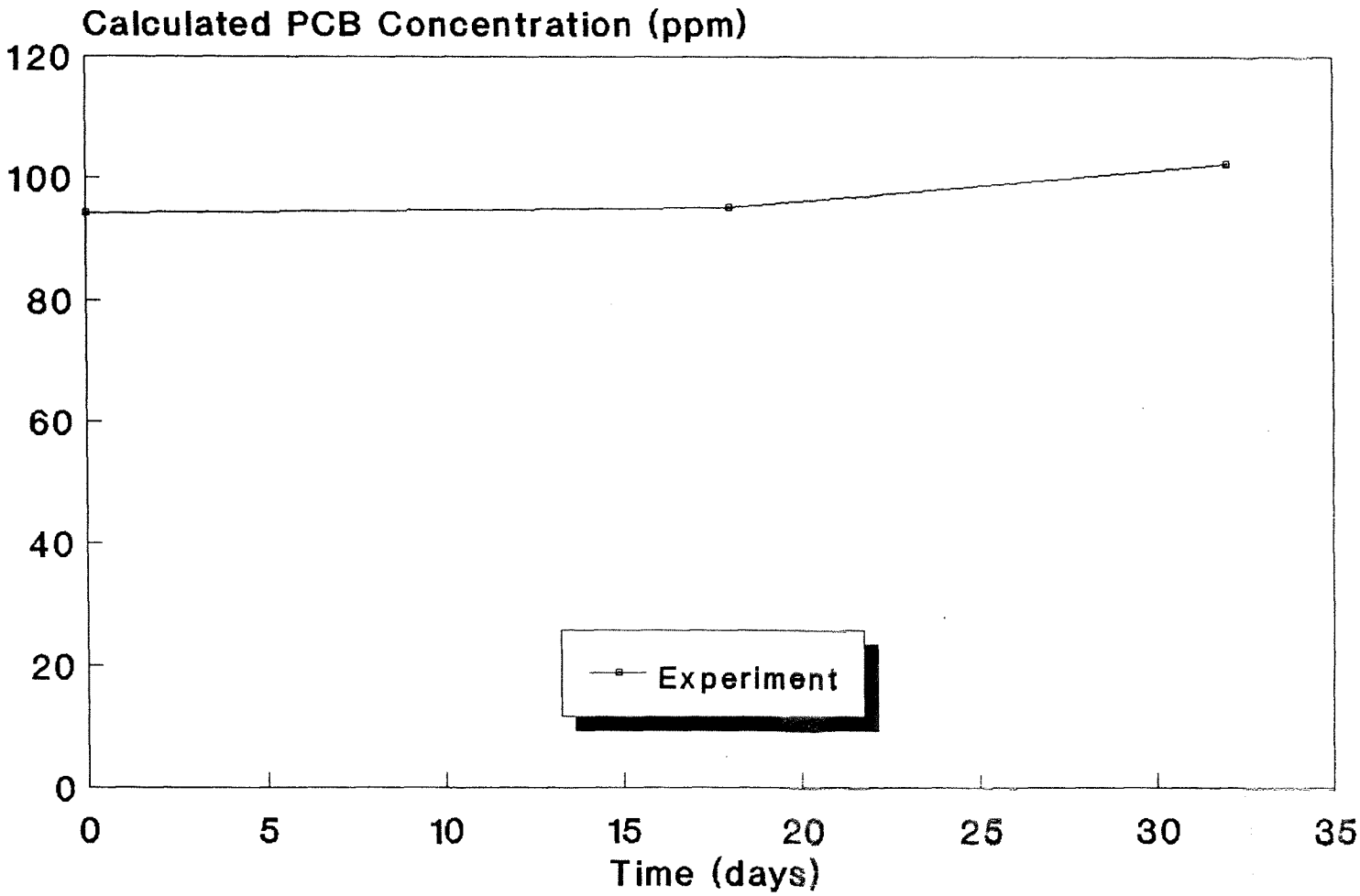


Figure 4.5. Activity of the Fungus on Aroclor 1254 in Experimental Set #3

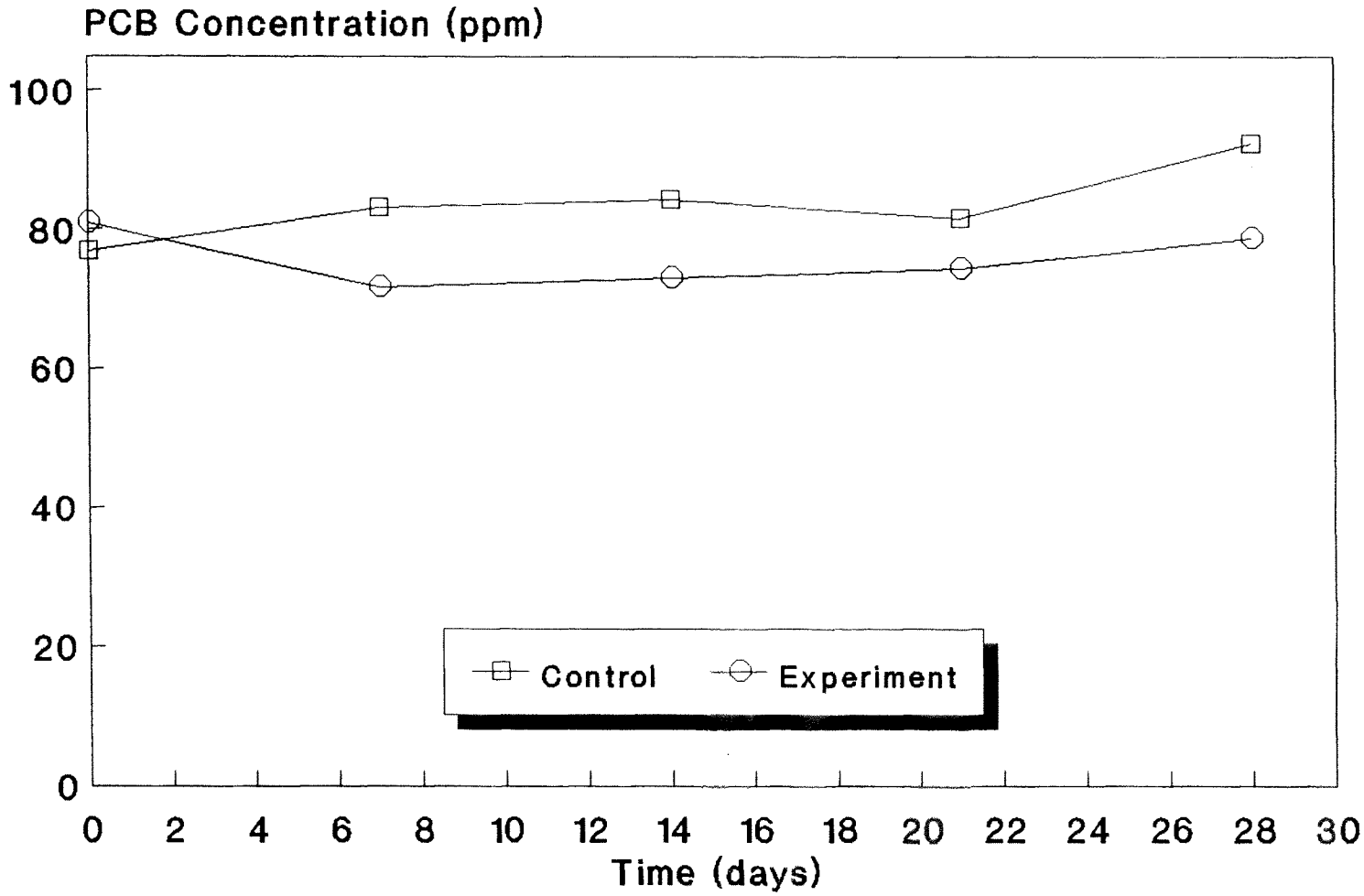


Figure 4.6. Activity of the Fungus on 2,4,6 Trichlorophenol in Experimental Set #3

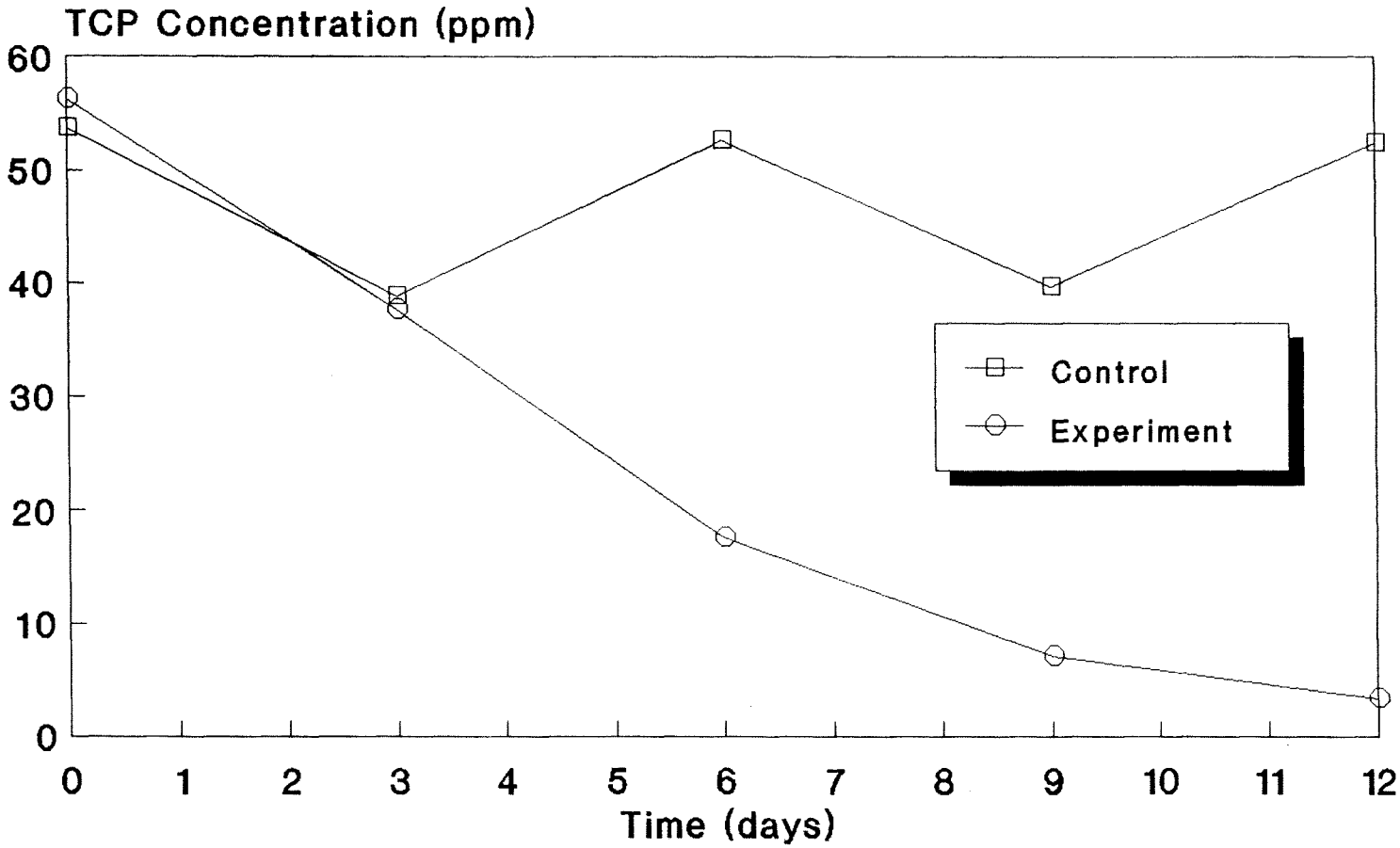


Figure 4.7. Activity of The Fungus on Aroclor 1242 in Experimental Set #4

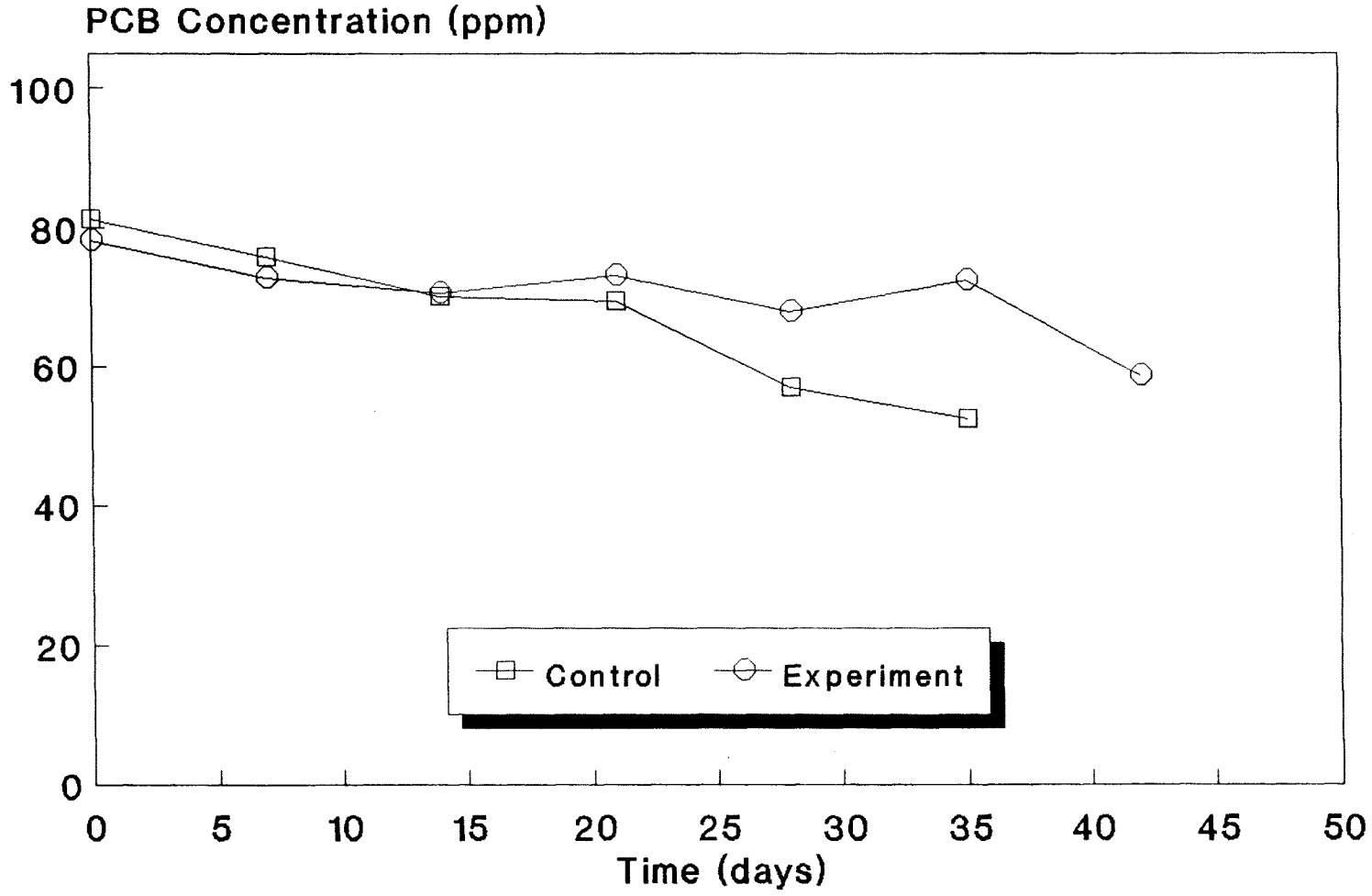


Figure 4.8. Activity of The Fungus on 2,4,6 Trichlorophenol in Experimental Set# 4

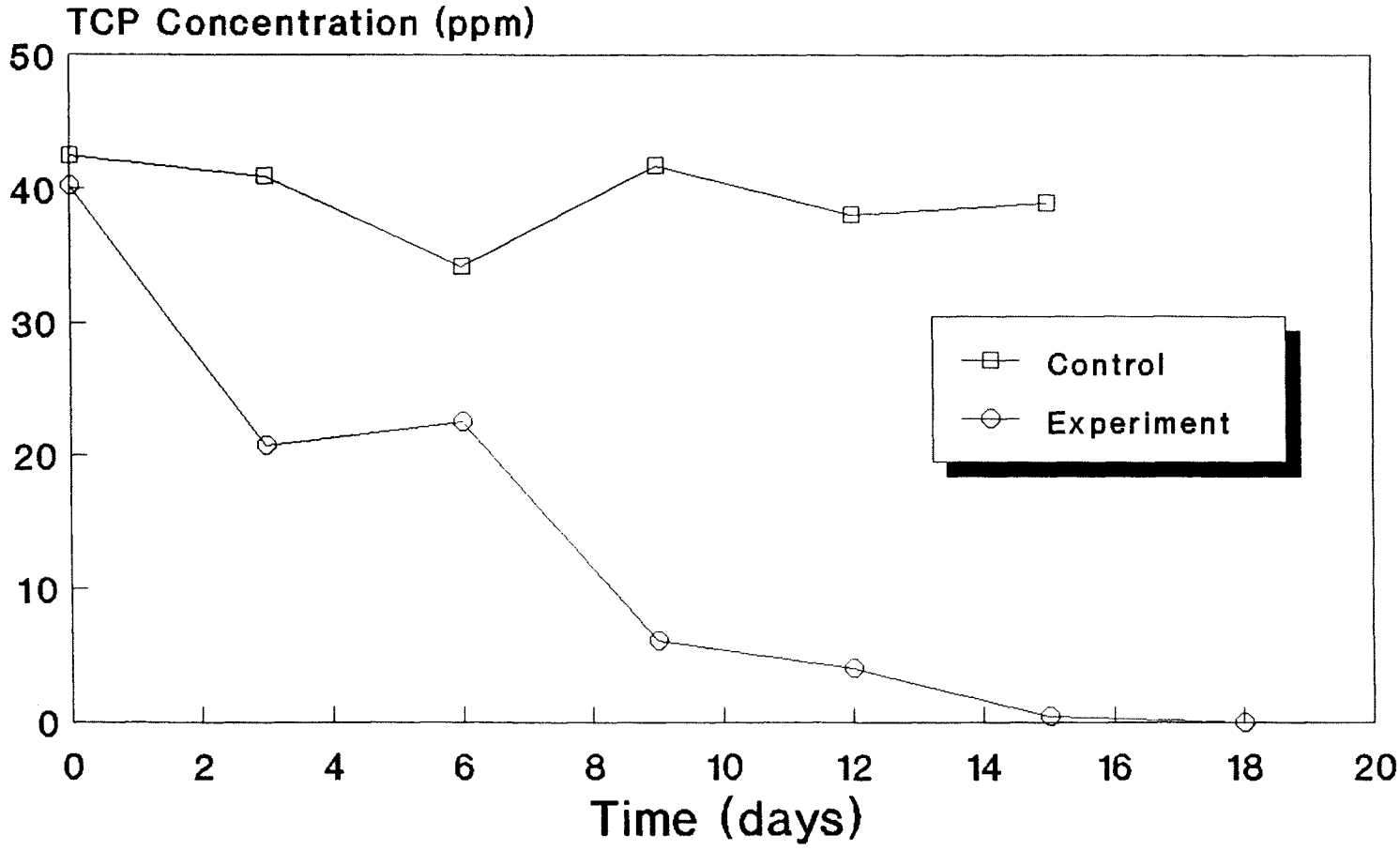
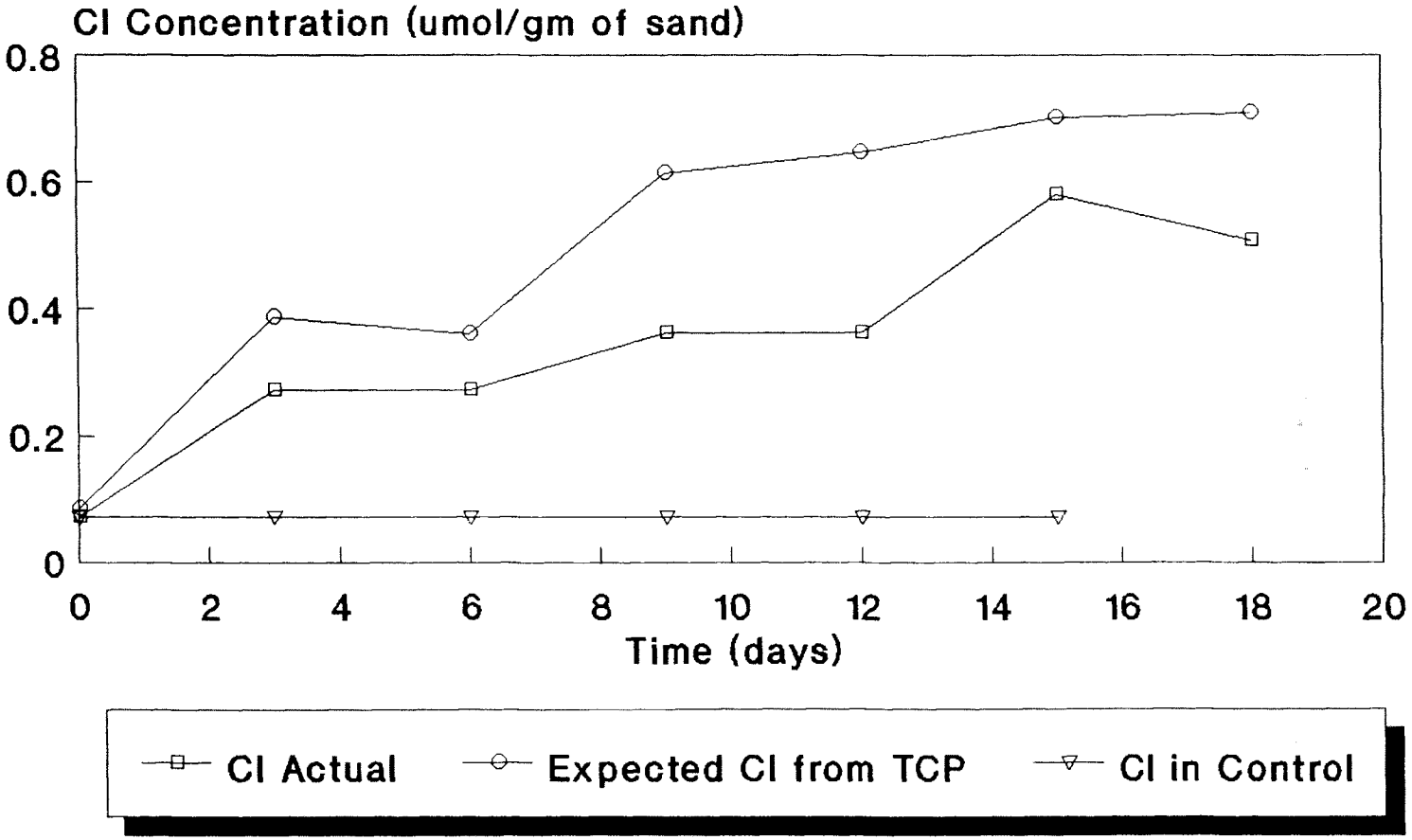


Figure 4.9. Recovery of Cl Ion during Degradation of 2,4,6-Trichlorophenol in Experimental Set #4



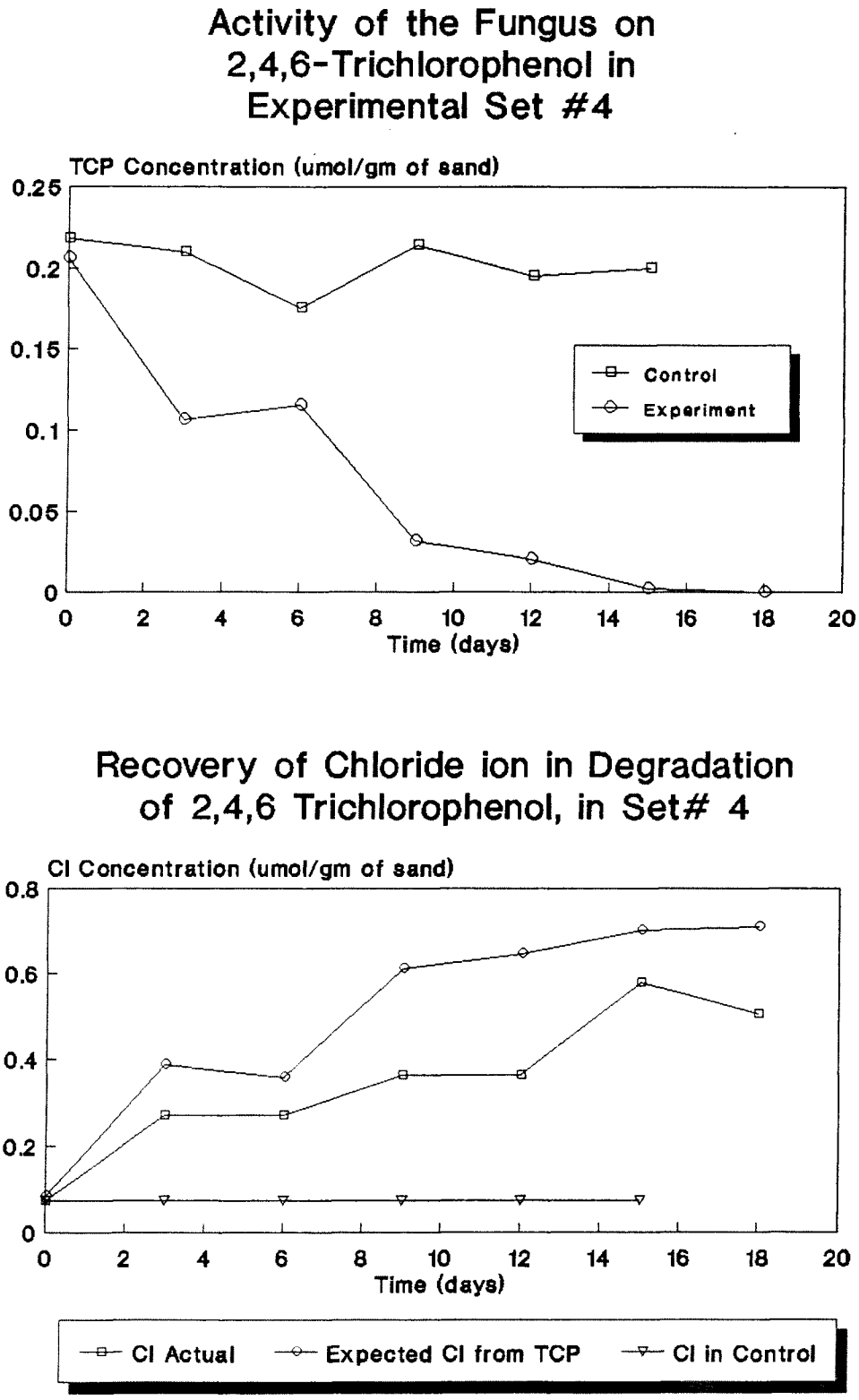


Figure 4.10 A Comparison of TCP and Cl Ion Concentration With Time in Experimental Set #4

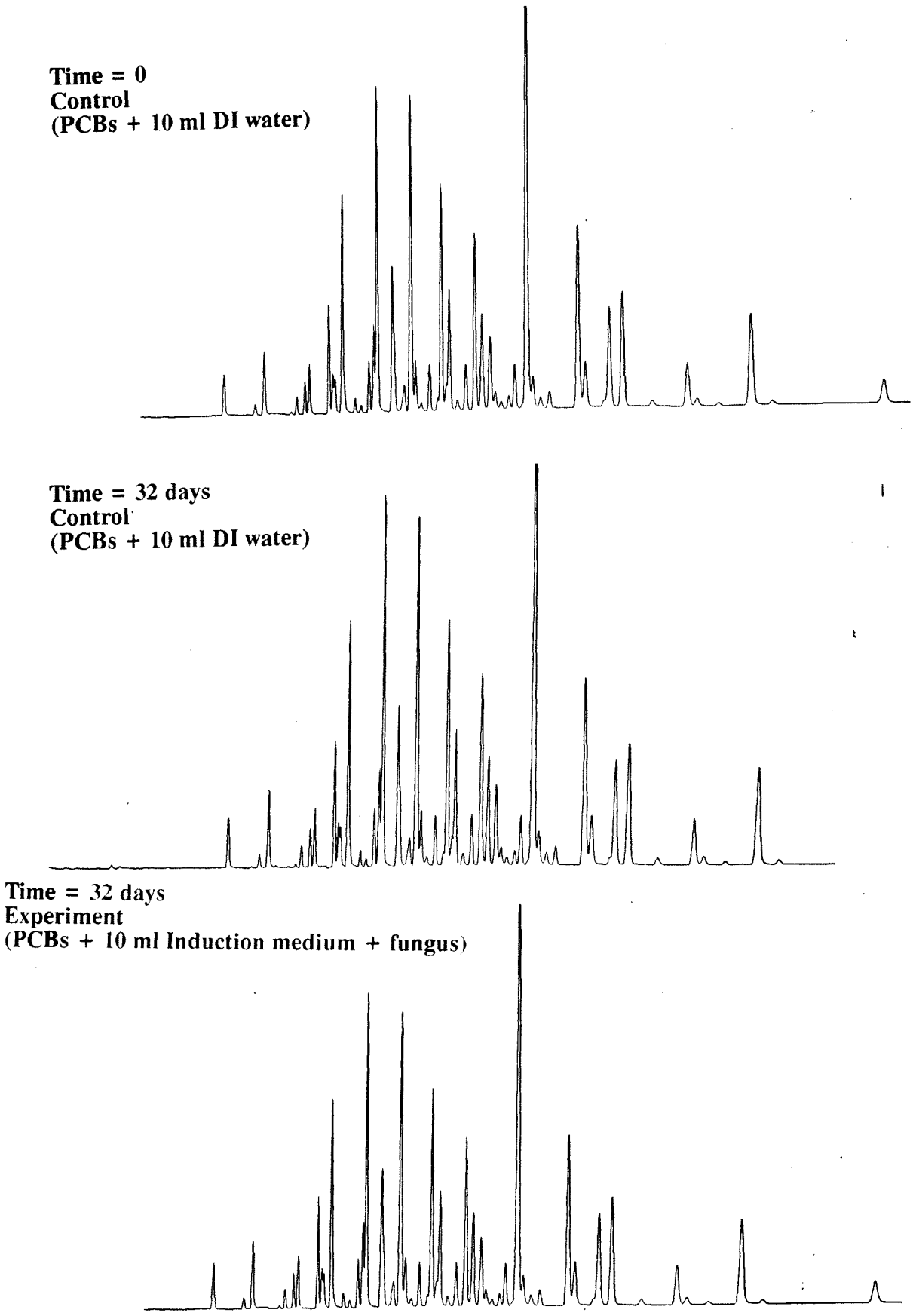


Figure 4.11 Chromatograms of Aroclor 1260 in Experimental Set# 1

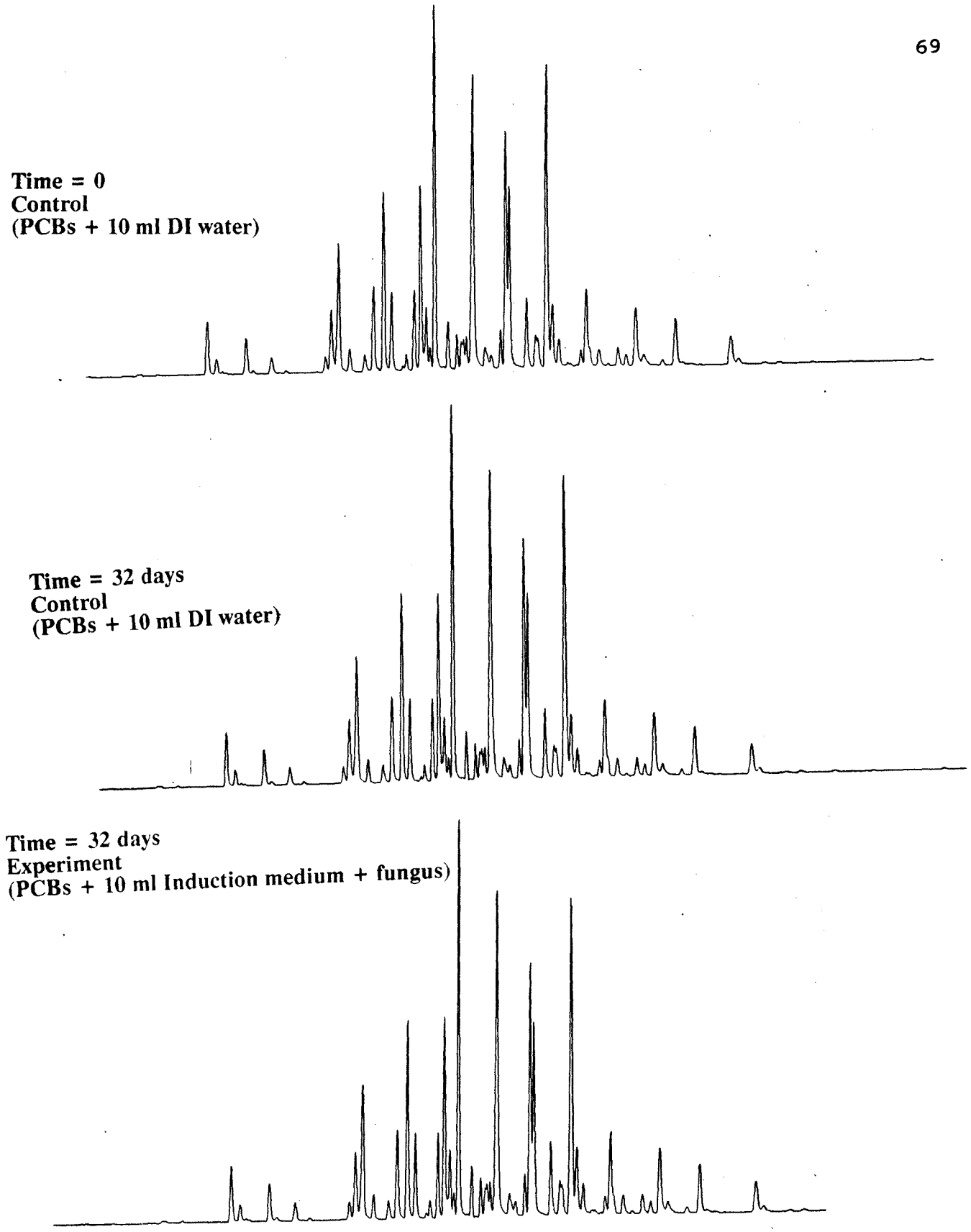


Figure 4.12 Chromatograms of Aroclor 1254 in Experimental Set# 1

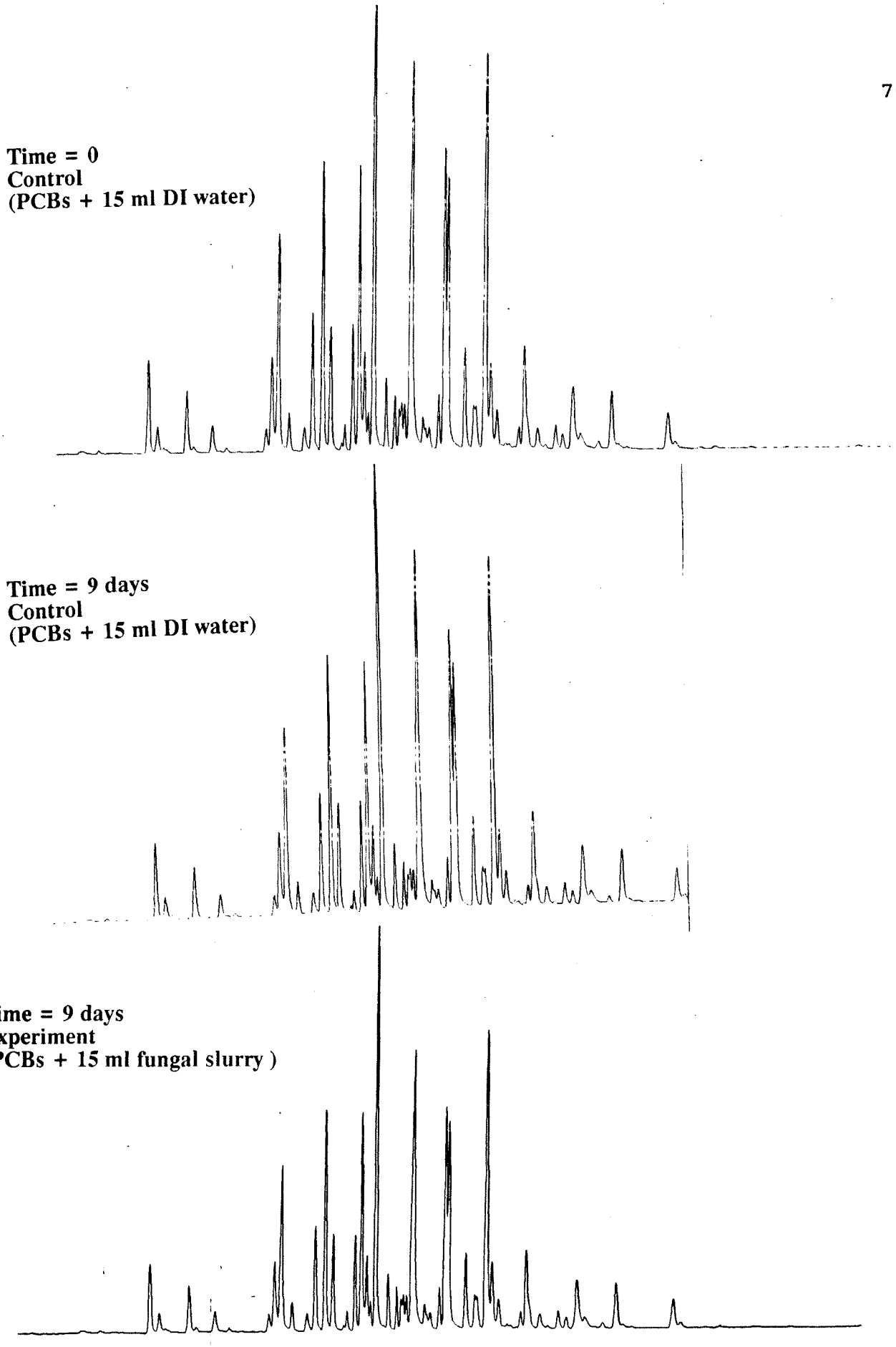


Figure 4.13 Chromatograms of Aroclor 1254 in Experimental Set# 2

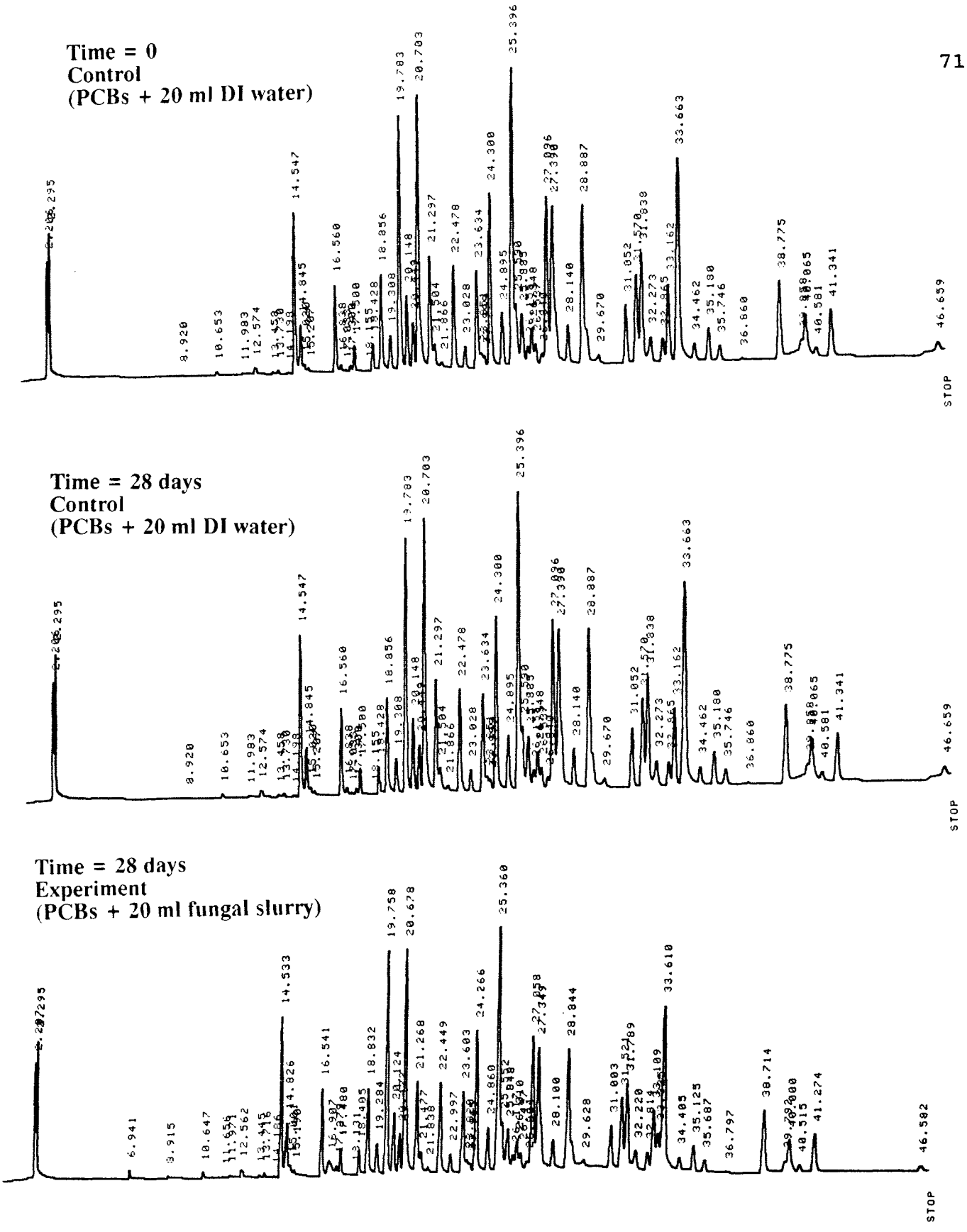


Figure 4.14 Chromatograms of Aroclor 1254 in Experimental Set# 3

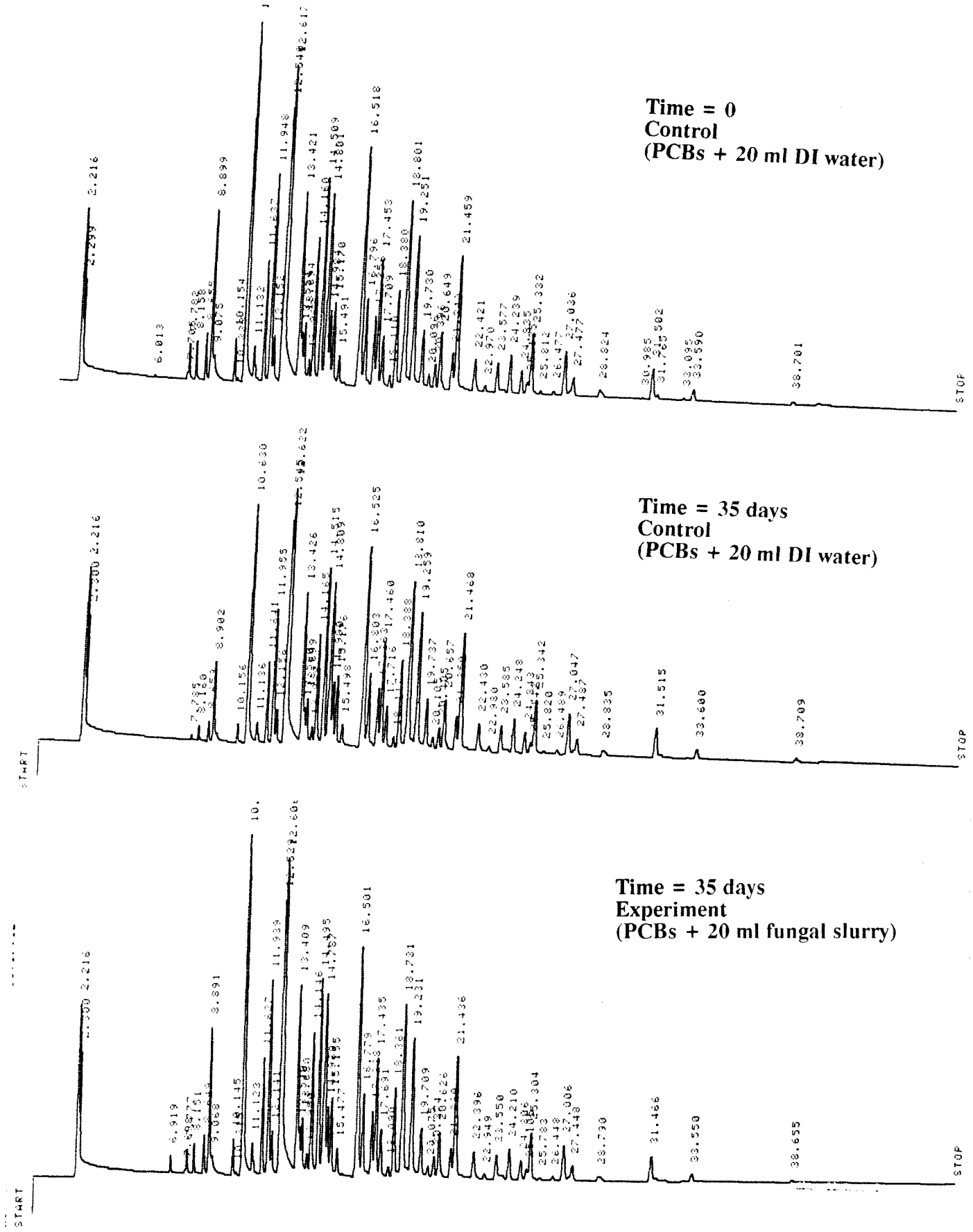


Figure 4.15 Chromatograms of Aroclor 1242 in Experimental Set# 4

CHAPTER 5
CONCLUSIONS

This research was conducted to determine the activity of the white rot fungus *P. chrysosporium* against PCBs in sand. From the results obtained, it can be conclusively said that the fungus is unable to degrade the PCBs in sand, at a concentration of 100 ppm (w/w PCB to sand), under a number of different experimental conditions.

There were some problems encountered during the experimental work, the main being the inability to fully recover the residual amounts of PCBs from the sand accurately. This was a consequence of the incomplete extraction of the PCBs from the sand/biomass matrix. However, despite the difficulty encountered during extraction, a comparison of the peaks in the GC chromatograms, provided a strong evidence of the inability of the fungus to biodegrade PCBs in sand. Even after changing some key parameters like nitrogen and glucose concentrations, amount of biomass, aeration, etc., the same negative results were obtained.

In order to further confirm the preliminary results obtained initially, a number of additional experiments were conducted. Firstly, the extraction efficiency of PCB from sand was studied independently (using sand contaminated with known amounts of PCB). These experiments showed that the extraction was reproducible but did not result in the full recovery of all the initial PCB.

Secondly, parallel experiments were conducted in which the fungus was exposed to PCB (in one set of bottles) or TCP (in separate set of bottles). Special attention was paid to ensure that the experimental conditions for the two compounds (PCB or TCP) were identical. To minimize any bias, the fungal inoculum for all the experiments came from the same batch. These experiments clearly showed that the fungus was able to attack TCP but showed no activity against PCBs. The degradation of TCP was determined on the basis of decrease in residual TCP concentrations as a function of exposure time to the fungus. In addition, the chloride ion was recovered in significant amounts, indicating that TCP degradation was indeed occurring. Control experiments were also run to positively confirm that the TCP disappearance was a result of fungal activity. Conversely, the results obtained from the parallel experiments in which the fungus was exposed to PCB, indicated that no PCB was degraded. These results were further confirmed by examining the chromatograms for the PCB before and after the treatment, which appeared to be nearly identical in all cases.

The results obtained in this work contradict some previous reports found in the literature (Bumpus et al., 1985; Eaton et al., 1985), which claim that the fungus is capable of attacking PCBs. However, a close examination of these reports showed that the evidence provided by these authors is questionable, since the alleged degradation is minimal, and can be explained by simply invoking experimental error rather than biodegradation.

Therefore, on the basis of all the evidence collected in this work, it can be concluded that *P. chrysosporium* is unable to attack PCB, at least in the significant concentrations tested in this work and under the experimental conditions under which these experiments were conducted.

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