BIOREMEDIATION OF PENTACHLOROPHENOL AND BLEACH PLANT EFFLUENT BY *TRAMETES VERSICOLOR* AND ITS EXTRACELLULAR FLUID, FOCUSED ON INTERMEDIATES AND PRODUCTS FORMED AND THE ROLE OF PROTEIN BINDING OF CHLORINATED COMPOUNDS IN A TWO-STAGE REACTOR SYSTEM

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DISSERTATION ABSTRACT

BIOREMEDIATION OF PENTACHLOROPHENOL AND BLEACH PLANT EFFLUENT BY *TRAMETES VERSICOLOR* AND ITS EXTRACELLULAR FLUID, FOCUSED ON INTERMEDIATES AND PRODUCTS FORMED AND THE ROLE OF PROTEIN BINDING OF CHLORINATED COMPOUNDS IN A TWO-STAGE REACTOR SYSTEM

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Bioremediation of pentachlorophenol (PCP) from aqueous solutions can provide valuable methods to remove other toxins from aqueous solutions, those that are more or less persistent or toxic than PCP. Understanding the mechanisms of remediation of PCP from aqueous solutions can provide information on the reactions involved in removing PCP from solution and the products resulting from the removal reactions. It is important to recognize if the products formed are less toxic or less bioavailable than the original compound being removed if the remediation technique is to be feasible and practical to use. There were several objectives of this study. One objective was to develop and optimize a novel two-stage bioreactor system that utilizes the white-rot fungus *Trametes*

versicolor and its extracellular fluids to effectively remove PCP from aqueous solutions. Use of this bioreactor system to treat an industrial significant effluent, bleach plant effluent, was another goal of this investigation. Throughout the study, an objective was to identify the reactions that were involved in remediation of PCP, other than direct action of the fungal material. Realizing the types of products formed by removing PCP from solutions was an important objective to determine if the products formed were either more or less harmful to the environment.

The extracellular fluid of two-stage bioreactor system developed was determined to remove over 96.6% of PCP from a feed containing of 2.56mg/L at 8 hours retention time. Long-term effectiveness and robustness to environmental fluctuations were found to be useful characteristics of the two-stage bioreactor system. Tetrachlorohydroquinone (TCHQ) was identified as an oxidative product of PCP, and was present in untreated BPE. When the bioreactor system was used to treat BPE about 90% of the PCP and TCHQ present was removed. Non-enzymatic reactions were found to proceed in reactor resulting in the formation of addition products, probably 2,3,4,5,6-pentachloro-4-(pentahchlorophenoxy)-2,5-cyclohexadien-1-one and 2,3,5,6-tetrachloro-4-(pentachlorophenoxy)-phenol. Protein produced by the immobilized *Trametes versicolor* was determined to be important in removing PCP and TCHQ from solution. PCP and TCHQ were removed from aqueous solution by reactor protein as a result of binding with the sulfhydryl groups in the protein.

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CHAPTER I

EXTRACELLULAR FLUID REACTOR FOR REMEDIATION OF PENTACHLOROPHENOL

ABSTRACT

A novel continuous extracellular fluid reactor (CEFR) was developed for remediation of pentachlorophenol (PCP). Used in series with a fluidized bed bioreactor (FR) system, which utilizes immobilized *Trametes versicolor*, the CEFR makes use of the extracellular fluid produced by *T. versicolor* in the FR to remediate PCP. The CEFR was fed the extracellular fluid produced in the FR and a fresh stream of PCP solution. The extracellular fluid was able to degrade over 96.6% of the initial concentration of 2.56mg/L in the combined feed streams at 8 hours retention time. The optimum pH for PCP degradation in the CEFR was between 3.5 and 4.0. Long term reactor studies showed that the CEFR was able to operate at these conditions for at least 50 days with over 89% removal of PCP. The CEFR was also robust to changes in the flow rate of the fresh stream of PCP solution while holding the flow rate of the extracellular fluid constant. Removal of PCP was shown to follow first-order kinetics. At 23^{o}C the maximum removal rate was 6.6 mg PCP/ g protein-hr and at 33^{o}C the maximum removal rate was 3.195 mg PCP / g protein-hr. Degradation of PCP in the CEFR was show to

proceed in a non-enzymatic fashion, producing addition products, verified by FTIR analysis of CEFR solution. The fungus used for the immobilized fungal reactor grown on plates preformed best when grown at 85^{o}F. The solution in the fungal reactor contained fungal cells. It was determined that most of the fungal material was removed in the settling basin and cells that may have flowed into the CEFR were not at a level that would be viable at the reactor conditions or contribute to PCP degradation.

INTRODUCTION

Pentachlorophenol (PCP) is a persistent toxic compound that has been listed a priority pollutant by the EPA since 1984. Recognizing the importance and difficulty in removing this compound from the environment, several researchers have investigated avenues by which to remove this pollutant. Fungi have been shown to have great potential in degrading several pollutants. Bioremediation of PCP by the basidiomycete *Trametes versicolor* has been shown to be highly effective. The mechanism of PCP remediation by fungi involves several enzymes. *T. versicolor* produces the inducible enzymes lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. Lignin peroxidase catalyzes the oxidation of lignin during lignin degradation (McAllister et al., 1996). It has also been found to lead to the oxidation of veratryl alcohol, phenanthrene, polycyclic aromatic hydrocarbons, phenolic pollutants and benzo[a]pyrene (Hammel et al., 1992; Bogan & Lamar, 1995; Aitken et al., 1989; Sanglard et al., 1986). Manganese peroxidase is capable of oxidizing Mn(II) to Mn(III) which then is able to oxidize phenolic substrates to phenoxy radicals (McAllister et al., 1996; Archibald et al., 1997). Manganese

peroxidase has been found to be involved in pulp bleaching, delignification and demethylation (Addlemann & Archibald, 1993). Laccase of *T. versicolor* is excreted in large amounts, along with small amounts of manganese peroxidase, under the proper inducing conditions (Archibald et al., 1990, Roy-Arcand & Archibald, 1991, Pallerla & Chambers, 1998). Laccase oxidizes substrate in one electron steps, resulting in polymerization and the formation of reactive quinones (Majcherczyk et al., 1998). Laccase has been shown to be involved in the degradation of phenolic compounds, chlorinated phenols, lignin, and polycyclic aromatic hydrocarbons (Jönsson et al., 1998, Dec & Bollag, 1990, Jönsson et al., 1998, Johannes et al., 1996, Majcherczyk et al., 1998).

Realizing the potential of the extracellular enzymes of *T. versicolor*, the primary objective of this portion of the study was to develop a novel continuous reactor system that makes use of extracellular fluid produced by *T. versicolor* to remediate PCP from aqueous streams. Pallerla and Chambers (1998) developed a continuous fungal bioreactor which utilizes polyurethane immobilized *T. versicolor*, which removed 99% of PCP feed to the reactor. Immobilization of the fungus allows for the separating of the fungal material and the treated stream. The treated stream was found to contain laccase, referred to as extracellular fluid. This laccase rich extracellular fluid was studied for its ability to degrade PCP in batch studies, compared to purified laccase, as well as for the development of the novel continuous reactor to treat PCP contaminated aqueous streams. The novel continuous reactor was studied for robustness to changes in reactor environment. Investigation was made into the types of products formed by PCP

treatment by the extracellular fluid enzymes. Studies were also conducted to determine if immobilized fungal cells are released from the polyurethane cubes in amounts that would produce a viable culture and contribute to the degradation of PCP. These studies will provide an additional reactor scheme to remediate pollutants from aqueous streams. Understanding some of the mechanisms of PCP removal insures that the products resulting from this reaction scheme are not more harmful to the environment than PCP.

LITERATURE REVIEW

Lignin Peroxidase and Manganese Peroxidase

Peroxidase enzymes catalyze the reduction of peroxides and the oxidation of substrate (Ikehata et al., 2004). Lignin peroxidase, also known as liginase, was first identified in 1983, as an extracellular oxygenase produced by *Phanerochaete chrysosporium*, and then recognized as a peroxidase (Linko, 1988). Lignin peroxidase is a heme-containing exoenzyme (Jönsson et al., 1989) initially found to catalyze the oxidation of lignin during depolymerization. Other studies have shown lignin peroxidase to be involved in the degradation of several organic pollutants such as phenanthrene, polycyclic aromatic hydrocarbons, phenolic pollutants and benzo[a]pyrene (Hammel et al., 1992; Bogan & Lamar, 1995;Aitken et al., 1989; Sanglard et al., 1986).

Manganese peroxidase (MnP) is a manganese dependent heme-containing peroxidase which oxidizes Mn(II) to Mn(III) when suitable Mn(III)-complexing compounds are present (Roy et al., 1994). The Mn(III) complexes formed are capable of catalyzing

delignification (Johannson & Nyman, 1987). MnP participates in bleaching of kraft pulp by attacking and partially degrading residual lignin (Paice et al., 1993). Wariishi et al. (1991) found MnP catalyzes the depolymerization of dehydrogenation polymerisate oligomers, lignin model compounds.

Johansson & Nyman (1987) provided evidence that *Trametes versicolor* excreted an inducible manganese(II) dependent peroxidase. Collins & Dobson (1996) reported that MnP in the culture medium of *Trametes versicolor* was capable of catalyzing the oxidization of phenanthrene and flourene, PAH's. This suggested that MnP may be capable of degrading a broader range of PAH's than LiP because phenanthrene and flourene have ionization energies above 8eV and LiP has catalyzed the ionization of PAH's with ionization energies up to 7.55eV (Hammel et al., 1986). These researchers did assay for LiP activity, however, they did not assay for laccase activity. The assay used to measure MnP activity used Mn(II) as a substrate. Höfer & Schlosser (1999) reported the ability of laccase to oxidize Mn(II) to Mn(III) , therefore it could be possible that laccase is responsible for some of the degradation of PAH's reported by Collins & Dodson (1996). However, in 1998, Majcherczyk et al., studied the ability of laccase from *T. versicolor* to degrade several PAH's and found laccase did not have the ability to degrade phenanthrene or flourene.

Lignin peroxidase was evaluated by Mileski et al. (1988) for its ability to degrade pentachlorophenol. Lignin peroxidase was purified from the extracellular fluid of *Phanerochaete chrysosporium* and mixed with hydrogen peroxide, dimethyl formamide

and PCP. Results showed that with the disappearance of 9.3μM PCP, 7.3μM of 2,3,5,6-tetrachloro-2,5-cyclohexadiene-1,4-dione appeared.

Veratryl alcohol (VA) is a common substrate of extracellular enzymes by white-rot fungi, used in assays of lignin peroxidase and to initiate expression of enzymes (Kirk et al., 1986; De Jong et al., 1994; Lundquist & Kirk, 1978; Dodson et al., 1987; Mester et al., 1995). Researchers have found VA to be an important co-factor for peroxidases to oxidize substrates. Chung & Aust (1995) found that VA increased conversion of PCP to its degradation product, tetrachlorobenzoquinone (TCBQ). In a reaction mixture containing VA, 45 mol% of PCP was converted to TCBQ and without VA only 8 mol% of PCP was converted to TCBQ. These researchers also determined that VA limits LiP inactivation. They suggest that VA is oxidized to vertryl aldehyde by LiP, forming the veratryl alcohol cation radical (VA*+) intermediate compound, which can react with the inactivated form of LiP, reverting it to its ferric, active state. These results are supported by findings of Tonon and Odier (1988), that hydrogen peroxide at physiological levels deactivates LiP and VA protects against such deactivation. Oxidation of substrates by white-rot fungi relies on the peroxidase enzymes, lignin peroxidase and manganese peroxidase (MnP), as well as secondary metabolites (Kirk, 1987). Veratryl alcohol is one such important secondary metabolite that is produced de novo by several white-rot fungi that excrete LiP (De Jong et al., 1994) including *Coriolus versicolor* (Dodson et al., 1987). Mester et al. (1995) realized the role of manganese in the oxidative system of white-rot fungi. These researchers studied white rot fungi *Bjerkandera* sp. Strain BOS55 and *Phanerochaete chrysosporium* BKM-F-1767 and the effects of manganese on MnP

expression, LiP expression and VA production de novo. The addition of Mn(II) greatly enhanced MnP production produced a response in *mnp* mRNA, suggesting that in white-rot fungus Mn(II) has a regulatory role at the molecular level. Addition of Mn(II) decreased the production of LiP overall, however it did not lower the response of *lip* mRNA. These researchers concluded that Mn(II) did not inhibit production of LiP, however when converted to Mn(III) it quickly deactivates LiP. Mn(II) was determined by this group to play a role in the biosynthesis of VA, which is greatly increased in the absence of Mn(II). This finding gives an explanation for the decrease in LiP activity in the presence of Mn(II). Since at higher levels of VA, LiP activity is protected, then when Mn(II) decreases production of VA de novo, LiP is deactivated at a greater rate.

Several studies have been performed on lignin peroxidase from *Phanerochaete chrysosporium*, verifying its ability to degrade lignin as well as the mechanism of catalytic action (Tein, 1987). Dodson et al. (1987) were the first researchers to demonstrate the production of lignin peroxidase by *Coriolus versicolor* (which is also referred to as *Trametes versicolor*). In 1987, Jönsson et al. isolated and purified lignin peroxidase isozymes from *Trametes versicolor*. Jönsson et al. (1989) used three lignin peroxidase isozymes and investigated the substrate specificity using synthetic dimeric models for lignin. These researches determined that the isozymes attacked both β-O-4 and β-1 lignin model compounds, verifying the ability of lignin peroxidase from *Trametes versicolor* to degrade lignin. They also determined one of the isozymes isolated from *Trametes versicolor* to be very similar to an isozyme isolated from *Phanerochaete chrysosporium.*

Ferulic acid, a humic acid precursor, and PCP were studied for co-polymerization by enzymes produced by *Phanerochaete chrysosporium* by Rüttimann-Johnson and Lamar (1996). In the presence of fungal extracellular enzymes, hydrogen peroxide and a detergent, they discovered that PCP was incorporated in a higher molecular weight material with ferulic acid as a co-substrate. Without ferulic acid as a co-substrate, PCP was cross-linked with itself, forming a higher molecular weight compound. The presence of ferulic acid, however, did enhance polymerization and the disappearance of PCP. The extracellular enzymes produced by *Phanerochaete chrysosporium* were determined to be LiP, MnP and laccase. All of these enzymes, in purified state, were found to be effective in catalyzing polymerization reactions. Experiments excluding hydrogen peroxide did not result in polymerization; neither did experiments excluding magnesium sulfate. These results indicate that at the conditions the experiments were performed, LiP and MnP played an integral role in catalyzing polymerization reactions and laccase did not catalyze polymerization.

Wang et al. (1999) explored the polymerization of PCP in water-acetonitrile mixtures, using LiP to catalyze the reaction. The activity of native LiP was increased by 60% with the addition of 1(v/v)% acetonitrile. This increase in activity was found to be due to enhanced solvent-substrate interactions and not an actual increase in enzyme activity. Higher concentrations of solvent resulted in the loss of enzyme activity, as expected due to the inhibitory nature of solvents. These researchers modified LiP with poly(ethylene glycol) to increase activity, stability and tolerance against organic solvent. Activity of modified LiP was the same as that of native LiP and remained soluble in aqueous

solution. At concentrations of acetonitrile as high as 10(v/v)%, the modified LiP retained over 90% activity, while native LiP only retained 50% activity.

Johansson & Nyman (1993a, b) isolated five forms of manganese peroxidase and sixteen forms of lignin peroxidase from Trametes versicolor. These researchers characterized a majority of these isozymes with respect to isoelectric point, molecular mass, and specific enzyme activity.

Reddy et al. (1998) investigated the degradation of chlorinated compounds by the extracellular enzymes of *Phanerochaete chrysosporium*, LiP and MnP, in order to understand the pathway of degradation. Purified LiP and MnP both catalyzed the oxidation of 2,4,6-trichlorophenol to yield 2,6-dichloro-1,4-benzoquinone. These enzymes also catalyzed the oxidation of 2,6-dichloro-1,4-dihydroxybenzene to yield 2,6-dichloro-1,4-benzoquinone. This suggests that 2,6-dichloro-1,4-dihydroxybenzene, a hydroquinone, is an intermediate compound between 2,4,6-trichlorophenol and 2,6-dichloro-1,4-benzoquinone. In the culture medium, with or without fungal cells, when 2,6-dichloro-1,4-dihydroxybenzene was added, metabolites 2-chloro-1,4-dihydroxybenzene, 2,6-dichloro-4-methoxyphenol, 2,6-dichloro-1,4-methoxybenzene, 3,5-dichloro-1,2,4-trihydroxybenzene, 5-chloro-1,2,4-trihydroxybenzene, 1,2,4-trihydroxybenzene, and 1,4-hydroquinone were detected. Reddy et al. (1998) proposed that intracellular reduction was responsible for the formation of 2,6-dichlorohydroquinone, which is subsequently reduced to 2-chlorohydroquinone from which trihydroxybenzene is formed, either directed or by the intermediate

chlorotrihydroxybenzene. 1,2,4-Trihydroxybenzene 1,2-dioxygenase was identified as the enzyme responsible for ring cleavage of trihydroxybenzene.

Laccase

Laccase is a copper containing oxidase, also referred to as a polyphenol oxidase, widely found in plants and microorganisms (Boyr,1975). Unlike peroxidases, laccase does not require the presence of hydrogen peroxide. When lacquer trees are damaged, laccase is recognized to catalyze the oxidation of phenols to free radical products, in the presence of air, which then polymerize to form a protective plastic (Boyr,1975). Laccase in fungi plays a role in the degradation of lignin (Boyr, 1975). This enzyme is able to depolymerize substrate when it is of high molecular weight, and catalyzes polymerization when substrate is of low molecular weight (Leonowicz et al., 1985). Laccase catalyzes the oxidation of substrate by the direct transfer of electrons from the reducing substrate to dioxygen (Boyr, 1975), simultaneously reducing dioxygen to water (Gardiol et al., 1996 & Garzillo et al., 2001) in one electron steps. Guillen et al. (2000) recently exhibited that laccase, in the presence of Fe^{3+} and a chelating agent, was involved in the formation of superoxide anion radical and hydroxyl radical through oxidation of hydroquinones derived from lignin. Miskowski et al. (1975) suggested that charge transfer in laccase involves sulfur and blue oxidases are known to have one cysteine. Proteins related to oxidases have exhibited the involvement of a sulfhydryl group in copper coordination (Boyr, 1975). Coll et al. (1993) determined that laccase contains four regions, which correspond to highly conserved copper-binding motifs and contain ten histidinyl residues

and one cysteinly residue. Briving et al. (1980) determined that laccase from *Polyporus versicolor* contains two internal disulfide bridges and one sulfhydryl group.

Laccase is a multi-copper containing enzyme with three different types of copper: Type I, Type II and Type III (Boyr, 1975). Fungal laccase contain one Type I Cu^{2+}, which is responsible for the blue color of the laccase enzyme. Type I Cu^{2+} is a unique chromophore present in the laccase enzyme which is blue when the enzyme is fully oxidized and losses color when reduced. Gardiol et al. (1996) developed a biosensor that was calibrated to give the response time as a function of specific changes in atmospheric oxygen concentration, as a change in color intensity using laccase. These researches were able to regenerate the laccase biosensor with four electron equivalents of ascorbate, the reducing substrate. During laccase catalysis, oxidation takes place at the Type I Cu^{2+}. One Type II Cu^{2+} is present in fungal laccase. This copper has high catalytic activity. It is the binding site for many anionic inhibitors, such as metal poisons and halides (Boyer, 1975). There are two Type III coppers (Type III Cu) present in fungal laccase, which exist as a tightly paired couple. The Type II Cu^{2+} is positioned near the Type III coppers, forming a tri-nuclear center (Tollin et al., 1993). The reduction of dioxygen to water takes place at the tri-nuclear center. Messerschmidt et al. (1993) proposed the molecular mechanism in laccase to be a sequential reduction of the coppers present. First the reaction is initiated with the substrate interacting with Type I Cu^{2+}, followed by the uncoupling of the Type III coppers with loss of the bridging hydroxyl ligand through the release of water; finally oxygen is bound to one of the Type III coppers, reducing the oxygen. Tollin et al. (1993) disagree with this mechanism, proposing that dioxygen

displaces the bridging hydroxyl before the tri-nuclear center is completely reduced. The presence of four copper atoms in laccase from basidiomycete fungi has been verified by Koroljova et al. (1999) and Gazillo et al. (2001).

Bollag & Leonowicz (1984) purified laccase from a number of basidiomycetes, ascomycetes, and deuteromycetes. *Trametes versicolor* was one of the basidiomycetes studied and was found to produce the highest levels of extracellular laccase, with induction by 2,6-xylidine. The activity of this laccase was found to be greatest at pH 5.2. Ong et al. (1997) isolated two laccase genes from *Trametes versicolor*, LaccI and LaccIV. LaccI is expressed during fungal growth and LaccIV is expressed after the introduction of an inducer. These researchers found the laccase produced by these genes contained highly conserved histidinyl and cysteinyl residues, similar to laccase isolated from other fungi.

Laccase from fungi have been the focus of many studies for their ability to catalyze oxidation, dechlorination, and polymerization reactions. Bollag & Liu (1985) investigated the copolymerization of halogenated phenols with a naturally occurring phenol, syringic acid, catalyzed by an extracellular laccase from the fungus *Rhizoctonia praticola.* Two types of reactions were found to occur with the phenols and syringic acid. One reaction lead to the formation of phenols covalently bound to an orthoquinone product of syringic acid which lead to the formation of quinonoid oligomers, which did not precipitate. The second type of reaction lead to phenols covalently bound to decarboxylated products of syringic acid which lead to the formation of phenolic

oligomers, which did precipitate. Reactions with PCP resulted in no detection of the formation of the quinonoid oligomers, but produced dimers, trimers, tetramers and pentamer of the phenolic oligomers. The molecular weights of the polymers obtained indicated that the binding of PCP to syringic acid products had to be by C-O linkages exclusively at the 1-O-4' position and that the phenolic oligomers produced consisted of one PCP molecule bound to several molecules of the syringic acid products.

Other studies have been conducted, using laccase from *R. praticola* to further the ability of this enzyme to catalyze reactions. Shuttleworth & Bollag (1986) used free and immobilized laccase and found it was capable of transforming 100% of the methoxyphenols and less than 10% of the chlorophenols studied. Syringic acid addition enhanced removal of the phenols investigated over three fold and removal was dependent on syringic acid concentration. Bollag et al. (1988) explored the detoxification of phenolic compounds by laccase of *R. praticola*. These researchers found laccase was able to detoxify 2,6-xylenol, 4-chloro-2-methylphenol and *p*-cresol. In the presence of syringic acid, o-cresol, and 2,4-dichlorophenol were detoxified. Laccase, with or without the addition of syringic acid, did not reduce the toxicity of p-chlorophenol or 2,4,5-trichlorophenol.

Dec & Bollag (1990) used laccase from *R. praticola* and *T. versicolor* to study the detoxification of substituted phenols by polymerization catalyzed by the enzyme. The laccase produced by *T. versicolor* was ten times more efficient than that produced by *R. praticola*. They found laccase catalyzed oxidative coupling of the substrate, during

which the substrate is dehalogenated forming free radicals, such as reactive quinones, which couple forming polymer. Dechlorination of 2,4-dichlorophenol was evident by the release of chloride atoms, up to 20% of the chlorine associated with 2,4-dichlorophenol. Polymerization was evident by precipitate forming during the reaction which, when subjected to gel permeation chromatography had an average molecular weight of 800. The precipitate is thought to be stable; however, since dehalogenation is associated with detoxification of phenols then if the polymer degrades it will release compounds less toxic than the parent compound. Dehalogenation and polymerization decreased with increasing number of halogens and increasing molecular weight.

Roy-Arcand and Archibald (1991) studied the oxidation, polymerization, and cleavage of phenolic compounds by laccase enzymes from *T. versicolor*. They isolated three laccases from the extracellular fluid of *T. versicolor*: laccase I, laccase II and laccase III. The extracellular fluid mixture was referred as crude laccase. Selected phenols present in E1-stage bleach plant effluent were studied for the effect that laccase has on the disappearance of each. Among the phenols studied 2,4,6-trichlorophenol, pentachlorophenol, 4,5-dichloroguaiacol, 4,5,6-trichloroguaiacol and tetrachloroguaiacol were decreased by 72%, 34%, 100%, 100% and 77% respectively when the effluent was treated with crude laccase for 30min. When effluent was treated with laccase I, laccase II or laccase III individually, slightly better results were obtained, with laccase III causing a more rapid reaction. The above mentioned chemicals were reacted with crude laccase, as a mixture and individually. The mixture of 800μM of chlorinated phenols treated with crude laccase released 540μM of Cl^- and consumed 100μM of O_2 in the first 30 min. of

reaction. During that time, 44% of PCP was consumed, more than twice the amount consumed when PCP was treated individually. When PCP was treated individually they found that during the initial stages (first 30 min.) of the reaction that no Cl^- was released while 17.5% of the PCP was consumed. However, after 17 hours, 6.6% of the total organochlorine was released and 24% of the PCP was consumed. Comparatively, 4,5-dichloroguaiacol released 74% of the total organochlorine and 88.1% of the substrate was consumed. This suggested to them that a phenoxy free radical was produced, which could lead to polymerization as opposed to mineralization. They suggested limiting the polymerization by laccase by increasing dechlorination using reductive means. They conducted an experiment using 2,2'azino-bis-ethylbenzothiazoline-6-sulfonate (ABTS) to increase PCP removal using laccase from *T. versicolor* and found that in the presence of ABTS 69% of PCP disappeared opposed to 0%-17% PCP disappearance without ABTS, but gave no indication of the products formed during these reactions

A laccase from *T. versicolor* was used to study the copolymerization of chlorinated phenols with co-substrates, guaiacol, caffeic acid, 2,6-dimethoxyphenol, syringic acid and ferulic acid by Roper et al. (1995). They found the most effective co-substrate for copolymerization with 2,4,5-trichlorophenol was 2,6-dimethoxyphenol. Without co-substrate in the presence of laccase, 8% removal of 2,4,5-trichlorophenol was obtained. When 2,6-dimethoxyphenol was added removal increased to 85%, an increase over 10 fold. They concluded that the extent of removal is dependent upon the type of co-substrate and the ratio of co-substrate to substrate. Co-precipitates containing

2,4,5-trichlorophenol and 2,6-dimethoxyphenol were formed during reactions with laccase. These researchers determined that polymerization reactions do not have to occur with enzyme present. They reacted 2,6-dimethoxyphenol with horseradish peroxidase and filtered the resulting mixture to exclude the enzyme. This filtrate was reacted with 2,4,5-trichlorophenol and co-precipitates formed, although at a much lower rate than with enzyme in the reaction. This suggests that during treatment with enzyme, 2,6-dimethoxyphenol was transformed to a reactive compound or free radical that was able to react with 2,4,6-trichlorophenol, possibly forming reactive oligomers by transferring the radical to the oligomer, continuing the polymerization reaction.

Laccase is believed to catalyze the reactions leading to polymerization. Ricotta and Bollag (1996) investigated the ability of laccase from *T. versicolor* to mineralize PCP, the other type of reaction that occurs during remediation of PCP. Growth media with an actively growing culture of *T. versicolor*, with and without the addition of laccase or denatured laccase were used to study the transformation of PCP during the growth of *T. versicolor*. In cultures with no added laccase significant mineralization of PCP occurred after 80% of the PCP had transformed. After 17 days about 21% of the PCP had been mineralized, indicated by the amount of $^{14}CO_2$ that had been released. Immediately after the addition of laccase significant mineralization was detected, however, at day 17 only 15.8% of PCP was mineralized. Reactions of PCP with laccase alone resulted in the formation of p-chloranil and o-chloranil but did release CO_2 at a rate that would be associated with mineralization. These researchers concluded that laccase does not play a

role in mineralization, but most probably plays a role in catalyzing the polymerization reaction.

Ullah et al. (2000a) evaluated the reaction of PCP with laccase from *Coriolus (Trametes) versicolor*. At a low concentration of PCP (25 μg/ml), all of the PCP was removed after 48 hours. At the higher concentration of 50μg/ml PCP, 65% of PCP was removed, and 45% of 200μg/ml PCP was removed. The products were found to be higher molecular weight polymers with tetrachlorobenzoquinones in trace amounts. In this study, no free chloride was detected, indicating mineralization had not occurred to any significant level. This led the researcher to evaluate the stability of the polymer formed and found it to be stable under aqueous conditions in alkali and in acid. In a simultaneous study, Ullah et al. (2000b) examined several solid growth substrates for *C. versicolor*. The greatest amount of laccase production by *C. versicolor* occurred when grown with wheat husk and wheat bran, Schlosser et al. (1997) reported similar results. The extracellular fluid produced under these conditions was able to reduce PCP in aqueous solution by 75% to 80%.

Bioreactor production of enzymes used in a subsequent continuous enzyme bioreactor

Crude enzymes have been used in batch reactors, semi-batch reactors and hollow-fiber reactors to produce useful products such as fructose and hexanal (Widjaja et al., 1999ab, Cass et al., 2000, Schade et al., 2003). However, these crude enzymes were harvested prior to reaction with substrate, not continuously produced. Gabalman et al. (1998)

developed a continuous reactor system to convert alcohols to aldehydes. The continuous reactor system consists of two reactors in series. The first reactor contains a continuous culture of yeast and methanol as the carbon source. The culture of yeast produces methylotrophic yeast cells, which contain methanol oxidase and catalase. These cells are present in the effluent and this effluent is fed to the second reactor. The second reactor is fed fresh feed of alcohol and oxygen, along with the first reactor effluent. Alcohols that could be present in the alcohol feed are ethanol, n-propanol, n-butanol, 2-chloroethanol, 2-bromoethanol, allyl alcohol, 2-buten-1-ol and benzyl alcohol. The alcohols are oxidized by methanol oxidase to their prospective aldehydes, which are removed from the effluent of the second reactor. The oxidation reaction yields hydrogen peroxide which is converted to water and oxygen by catalase. This is beneficial because it reduces any adverse side reactions caused by hydrogen peroxide and provides additional oxygen to be used in the oxidation reaction. In this case, the enzymes implemented to carry out the reaction were intracellular and yeast cells were present in the second reactor.

MATERIALS AND METHODS

Fungal growth

Trametes versicolor (ATCC 20869), the white-rot fungus used in this investigation, was obtained from the National Forest Products Laboratory in Wisconsin. The fungus was grown and maintained on malt agar (Difco) in a Petri dish.

Laccase Preparation

Crude laccase was obtained by inoculating 500ml flasks, containing 150ml growth medium, with four mycelia discs (6mm x 6mm) from actively growing fungal dishes. The growth medium was prepared by dissolving dextrose (40 g/L) and Difco Bacto-soytone agar (10 g/L) in distilled water and pH of the growth medium was adjusted to 4.8. The mycelia discs were crushed into fragmentation using a spatula. The flasks, stoppers, spatula used for transfer, and growth medium were all autoclaved at 121^{o}C for 20min. prior to use. The inoculated flasks were oxygenated for 1 min. with pure oxygen every twenty-four hours and continuously agitated in a rotary shaking incubator at 180-200 rpm. Extracellular laccase present in the growth solution, harvested on day 6 was defined as crude laccase. Purification of laccase was carried out by concentrating the extracellular fluid 10-fold by ultrafiltration and dialyzed against 30 mM sodium-acetate buffer at pH 5.0. The concentration of laccase in the resulting solution was approximately 4,000 units/ml using syringaldazine as substrate. This purified laccase was stored at 0^{o}C until use.

Measurement of Laccase Activity

Laccase activity was measured in 3-ml cuvettes containing 2.2 ml of 10 mM potassium phosphate buffer (pH 6.5), 0.3 ml of 0.216 mM syringaldazine solution, and 0.5 ml sample at 30^{o}C. Activity of laccase was measured as the ΔOD_{530nm}, with one unit of laccase able to produce a ΔOD_{530nm} of 0.001 per minute at pH 6.5 and 30^{o}C.

Measurement of Enzymatic Protein

The concentration of enzymatic protein in continuous extracellular fluid bioreactor was determined using the method by Dulley & Grieve (1975), and Lowry et al. (1951), using bovine serum albumin as the protein standard.

Gas Chromatography

The concentration of PCP in samples was determined by gas chromatography. Quantification and identification was performed after derivatization (acetylation). Sample (1 ml) was added to 9 ml distilled water in a 30ml beaker, with 25µl of 0.5mg/ml 2,6-dibromophenol solution added as an internal standard. The solution was adjusted to pH 7.0 with NaOH, followed by addition of 0.26ml of 0.6g/ml potassium carbonate. The pH was then adjusted to 11.6. The solution was transferred to a 30 ml conical flask and 0.5 ml acetic anhydride was added. The flask was shaken until no gas came out when venting. The acetylated compounds were extracted with 2ml hexane. The hexane layer was separated and stored in a clean, dry sample vial. A DB-5 fused silica capillary column (length, 15 m; ID,0.25 mm; film thickness, 0.25 µm, J&W) was installed on a Varian Model 3400 gas chromatograph equipped with a electron capture detector. Split injection (1:100) was used and the carrier gas was nitrogen. For analysis of PCP the injector temperature was set at 260^{o}C, the detector temperature was set at 325^{o}C. The column was held at 45^{o}C for 1 min, then ramped to 100^{o}C at 15^{o}C/min, increased to 165^{o}C at 2^{o}C/min, increased to 230^{o}C at 20^{o}C/min and held at 230^{o}C for 5 min. to remove any late eluted compounds. The volume of the sample hexane layer injected was 0.5 µl. The concentration of PCP in the biomass was determined by suspending the

mycelium in 0.25 N NaOH and fragmenting in a laboratory blender. The fragmented biomass was centrifuged and the supernatant was subjected to the treatment described above.

Extraction of Addition/Combination Products

Product stream from the enzyme reactor was collected and stored at 4^{o}C until processed. The stored solution was brought to room temperature and 250 ml of solution and 250 ml benzene were placed in a 1000 ml conical flask. The flask was shaken vigorously, with occasional venting, for 10 min and the two layers were allowed to separate. The benzene layer was placed in a 500 ml round bottom flask and evaporated to dryness in a Rotavap. This procedure was repeated, using the same round bottom flask, until 1000 ml of enzyme reactor solution was extracted. The dried residue was dissolved in benzene and methanol and analyzed by GC-MS and FTIR.

Continuous Extracellular Fluid Bioreactor Studies

The bioreactor system used to study PCP degradation is a two-stage system consisting of two multiphase bioreactors. The first stage reactor, developed by Pallerla & Chambers (1996), was a three phase fungal reactor with a working volume of 600 ml which utilizes polyurethane immobilized *T. versicolor*. The fungal reactor was aerated and fluidized by flowing compressed breathing air (BOC Gases) through an in-line air sterilizing membrane filter (pore size 0.2μm, Millex-FG$_{50}$, Millipore) and a glass dispersion tube (Ace Glass). A constant flow of feed solution was fed through an in-line micro-filter (pore size 0.22μm, Dominick) to ensure the feed solution was free of contaminants. The

reactor solution was constantly withdrawn and passed through a settling basin prior to being fed to the second stage reactor. The second stage reactor was a continuous extracellular fluid reactor (CEFR) with a working volume of 900ml. The CEFR was constantly stirred and aerated by flowing compressed breathing air (BOC Gases) through an in-line air sterilizing membrane filter (pore size 0.2μm, Millex-FG_{50}, Millipore) and a glass dispersion tube (Kimble) to provide oxygen for the enzymatic reactions. Fresh make-up feed to the CEFR was fed through an in-line micro-filter (pore size 0.22μm, Dominick) to ensure the feed solution was free of contaminants. All materials used in the reactor systems were autoclaved at 121^{o}C for 20 min prior to use.

Viable Fungal Material Studies

Malt agar plates were prepared using autoclaved 50g/L malt agar (Difco) at pH 5.0. Samples were taken from the FR, the settling basin and the CEFR and three drops of each solution was placed on a malt agar plate. A plug from a maintained agar plate of *Trametes versicolor* (ATCC 20869) was also plated on a fresh malt agar plate at the same time. The inoculated plates were allowed to grow for 4days at 85^{o}F.

Experimental Approach

The extracellular fluid, containing PCP degrading enzymes, was produced in the continuous fungal reactor, utilizing polyurethane immobilized *T. versicolor* as described by Pallerla & Chambers (1998), and continuously fed to the continuous extracellular fluid reactor as the source of enzyme. Fresh PCP solution was also continuously fed to the CEFR through a separate feed line (Figure 1). Reactions were carried out at different

temperatures, reactor pH's and residence times. At each condition studied, PCP concentration in the effluent and reactor pH was measured at least three times until the reaction reached steady state. The total extracellular protein was also measured. Samples were taken from the FR, the CEFR and settling basin and plated on malt agar plates.

RESULTS AND DISCUSSION

PCP Degradation in Continuous Extracellular Fluid Reactor

The results of continuous extracellular fluid reactions carried out at 23^{o}C are shown in Table 1. At the highest residence time of 8 hours, 96.6% of the initial concentration of 2.56mg/L of PCP was removed. Removal rate of PCP in the CEFR was much higher than earlier batch reactor results (Zhao, 2000). At a residence time of 2 hours in the continuous reactor the highest removal rate of 0.75mg/l-hr was achieved, compared to the highest removal rate in the batch reactor of 0.096 mg/l-hr. The increase in the removal rate in the CEFR could be due to several characteristics of the reactor. The enzyme is replenished continuously; therefore the enzyme present in the reactor has more activity. Batch studies showed that enzyme lost activity over time, resulting in lower removal of PCP. Another reason could be the formation of a reactive intermediate that may react with PCP, enhancing removal. Roper et al. (1995) showed that when chlorophenolic compounds are reacted with enzyme, a free radical is formed that is capable of polymerizing with chlorophenolics, even without enzyme present. There could be levels

of free radical compounds produced in the fungal reaction by action on PCP, which are present in the extracellular fluid fed to the CEFR.

CEFR Optimum pH

Reactor pH was studied to determine the effects of this important reactor variable on PCP degradation. Effects of pH in the extracellular fluid reactor are shown in Table 2 and Figure 2. The optimum pH for the CEFR was determined to be 3.5, compared to 4.0 in the batch reactor. The acidic shift in pH is most likely due to the fungal reactor extracellular fluid characteristics. In the batch reactor, PCP removal by the extracellular fluid was shown to be effective a pH as high as 10, compared to the CEFR, where no PCP removal was achieved above pH 6.3.

Effects of Incubation Temperature

Studies were carried out to determine what effect the incubation temperature of the *T. versicolor* malt agar plates has on effective incubation time, reactor pH and PCP removal. The ATCC and the USDA Forest Service suggest that malt agar plates to be grown at 75^{o}F. Previous incubation of plates occurred on laboratory bench tops at atmospheric temperatures. The southern region of the Unites States experiences very warm temperatures during summer months and mild temperatures during winter months. The reactors seemed to perform better during summer months than during winter months. A temperature controlled incubation chamber was utilized to control incubation temperature to verify the effect that incubation temperature has on fungal growth and reactor performance.

Table 3 shows that incubation at 75°F requires a 4 day longer growth period than incubation at 85°F. The CEFR has been shown to perform optimally at pH 3 to pH 4 shown in Figure 2, and the FR has been shown to operate best in the same pH range. The effect of incubation temperature of fungal plates on reactor pH in the FR and CEFR is shown in Table 4. When the fungal plates were incubated at 75°F, the resulting reactor pH's were outside of the optimum pH range for the FR and the CEFR. Incubation at 85°F resulted in reactor pH well within the useful pH range. Incubation temperature of 85°F improved the reduction of the concentration of PCP in the FR and CEFR by about 10%, shown in Table 5, while increasing percentage removal of PCP slightly. The higher incubation temperature of 85°F shortens incubation time by approximately half, results in a more effective reactor pH in both the FR and CEFR and improved removal of PCP, therefore this temperature was used for incubation in further experiments.

Long Term Reactor Characterization

Long term experiments were carried out to determine the effects of long term continuous operation on reactor conditions and PCP removal. The first samples were taken on the fifth day of operation and the reactor ran for 50 days, on which day the last sample was taken. Reactor pH, reactor protein concentration and reactor PCP concentrations were measured for each sample.

Reactor pH in both the continuous fungal and extracellular fluid reactors took two weeks to stabilize. The FR pH was approximately 3.9 during the reactor operation, shown in Figure 4A and Table 6. The CEFR pH was approximately 3.7 during the reactor

operation, shown in Figure 4B and Table 6. The pH in each reactor remained within the pH range determined to be optimum in previous studies.

In general, extracellular protein concentration in the FR increased steadily over time, shown in Table 6 and Figure 5A. On the fifth day of bioreactor operation, the protein concentration in the FR was 33μg/ml. Protein concentration increased at a rate of about 2.6μg/ml/d up to day 28 on which the protein concentration was 94μg/ml. Between day 36 and day 37, the fresh feed to the FR was stopped for several hours. On the 37th day, the protein concentration was measured as 357 μg/ml before fresh feed was resumed, a 280% increase from the concentration on day 28. Protein built up was due to active fungal protein production, without removal in the form of exit flow, since the flow rate of fresh feed is equal to the exit flow rate. On day 38, after flow of fresh feed the fungal reactor resumed, the protein concentration was measured as 125μg/ml. This concentration is in relation to the trend of protein concentration increase over time, excluding the sharp increase in protein concentration on day 37, of 3.6μg/ml/d. When flow was restored to the reactor, the excess protein was able to wash out of the reactor, returning the protein concentration to a quasi-steady state concentration. The overall increase in protein in the FR could be due to accumulation of protein. The fungus produced more extracellular protein than was removed from the reactor. During the time the fresh feed to the FR was halted for several hours, it is possible that some of the fungal cells died during this period and/or were released into the extracellular fluid, adding to the amount of measured protein.

Protein concentration in the CEFR, shown in Table 6 and Figure 5B, increased steadily over time at an overall rate of 0.6μg/ml/d. On day 5 of reactor operation, the protein concentration was measured as 24μg/ml. A sharp increase in protein concentration was measured on day 38 of 58μg/ml, up from 43μg/ml on day 37. On day 37 the protein concentration in the FR increased significantly and the sharp rise in protein in the CEFR followed the significant increase in protein concentration in the FR. By day 42, the protein concentration in the CEFR resumed to a quasi-steady state concentration of 41μg/ml. If the concentration of protein in the extracellular fluid fed to the CEFR was the same as that in the FR then, based on the flow rate ratio of extracellular fluid to fresh PCP solution fed to the CEFR of 2 to 1 the concentration of protein in the CEFR should be 66% of that in the FR. During the first two weeks of reactor operation, the concentration of protein in the CEFR was approximately 66% of that in the fungal reactor. However, from the third week on the concentration of protein in the CEFR was only about one-quarter of that in the FR. This suggests that a large fraction of the FR protein measured in the FR precipitated out in the settling basin.

Long Term PCP Removal in the FR and CEFR

PCP was used to induce enzyme production in the fungal reactor. The immobilized fungus degraded PCP by action of intracellular enzymes in the polymer encapsulated fungal cubes as well as extracellular fluid. The concentration of PCP in the FR rapidly decreased from the concentration in the feed of 2.5ppm down to 0.013ppm on the fifth day of reactor operation, shown in Table 7. After the pH stabilized the concentration of PCP during reactor operation, shown in Figure 6A, ranged between 0.041ppm and

0.30ppm, with the average concentration of PCP being 0.18ppm. During bioreactor operation, the PCP removal in the fungal reactor was 88% to 99.5% as shown in Figure 6B and Table 6. Pallerla and Chambers (1998) also achieved high removal rates over 99% in a fungal reactor utilizing polyurethane immobilized *T. versicolor* with initial PCP concentrations as high as 25ppm.

Fresh PCP solution and extracellular fluid produced in the FR was fed to the CEFR. On the fifth day of reactor operation, the concentration of PCP was 0.009ppm in the CEFR. The concentration of PCP in the CEFR increased at a steady rate of approximately 0.0054ppm/day to the highest concentration measured on day 49 of reactor operation of 0.28ppm, as shown in Figure 7A and Table 7. During CEFR operation the average PCP concentration was 0.173ppm. Reduction of PCP in the total feed remained between 89% and 99.6% as shown in Figure 7B and Table 7.

Robustness of Reactor to Changes in Reactor Characteristics

Experiments were carried out to determine the robustness of the CEFR to variations in reactor pH and the ratio of the flow rate of extracellular fluid fed to the CEFR to the flow rate of the fresh PCP solution to the CEFR. The flow rate of extracellular fluid to the CEFR was held constant, while the flow rate of fresh PCP solution was changed. This change directly impacts the retention time in the CEFR. At each residence time studied, the pH was changed to determine if the optimum pH for the reaction changes with the ratio of the flow rate of extracellular fluid fed to the CEFR to the flow rate of the fresh PCP solution to the CEFR.

During initial reactor acclimation and stabilization, the residence time in both the fungal reactor and the CEFR was set at 24 hrs. After reactor stability was achieved, the residence time in both reactors was dropped to 12 hr and held constant in the fungal reactor throughout reactor operation. The residence time in the CEFR was varied in the following order: 12 hr, 10 hr, 8 hr, and 6 hr. This cycle was repeated two more times, with three total cycles, for a total of 6 weeks of reactor operation. The reactor was held at each residence time for six residence times. During cycle 1; the pH was set at different pH values than at cycle 2 and 3, which were set at the same pH.

Figure 8 exhibits how well the reactor returned to similar conditions at the same residence times during cycles 2 and 3. The reactor pH was held constant at 3.5 during cycles 2 and 3. At the residence time of 6 hr, the rate of PCP removal and the steady state PCP concentration returned to the same levels during cycle 2 as in cycle 3. Rate of PCP removal, at residence times 8 hr and 12 hr was slightly lower during cycle 3 than cycle 2. At residence time of 10 hr the removal rate was slightly higher in cycle 2 while the steady state concentration of PCP returned to same as that in cycle 3. This indicates that the extracellular fluid enzyme remained active throughout the duration of reactor operation and the reactor was robust to environmental changes.

The optimum pH for reaction in the continuous extracellular fluid was determined to be 3.5 in earlier experiments. In earlier studies, the flow rate ratio of the extracellular fluid fed to the CEFR to that of the fresh PCP solution fed to the reactor was approximately 2 to 1, with the residence times in both the fungal reactor and the CEFR being the same. In this set of experiments, the residence times in the CEFR were changed while keeping the

residence time in the fungal reactor constant. Experiments were carried out to determine if changing the flow rate ratio, thusly changing the ratio of enzyme to PCP, has an effect on optimum pH of the reaction. Table 8 shows that the optimum reaction pH was higher than 3.5, ranging from 3.8 to 4.0, at flow rate ratios less than 2 to 1.

Kinetic Model Development

Continuous extracellular fluid reactor experiments were conducted at 33^{o}C to develop a kinetic model for PCP removal. Residence time was used as an evaluating parameter. Table 7 shows the results for the reactions at 23^{o}C and 33^{o}C that were used to determine parameters of the kinetic model.

The CEFR was well mixed and modeled as a CSTR. The following equation is the dynamic mass balance of PCP for the reactor:

$$V\frac{dS}{dt} = F(S_o - S) - r_s V \qquad (1)$$

where:

$\frac{dS}{dt}$ = PCP accumulation in the reactor, mg/L-hr

V = reactor void volume, L

S_o = reactor feed PCP concentration, mg/L

S = reactor outlet PCP concentration, mg/L

r_s = rate of PCP removal, mg/L-hr

F = volumetric feed rate, L/hr

At steady state, the accumulation term, $\frac{dS}{dt} = 0$. The rate of PCP removal equation becomes:

$$0 = F(S_o - S) - r_s V \qquad (2)$$

Or

$$r_s = \frac{F(S_o - S)}{V} \qquad (3)$$

PCP removal rate was calculated using equation 3.

After steady state conditions had been reached, PCP removal was described by the Michaelis- Menten equation:

$$r_p = \frac{V_m S}{K_m + S} \qquad (4)$$

where:

r_p = rate of PCP removal, mg PCP/g protein-hr

V_m = maximum PCP removal rate, mg PCP/g protein-hr

K_m = Michaelis-Menten constant, mg/L

Rearrangement of the Michaelis-Menten equation into the slope-intercept form gives:

$$\frac{1}{r_p} = \frac{K_m}{V_m}\frac{1}{S} + \frac{1}{V_m} \qquad (5)$$

Equation 5 using data presented in Table 6 is plotted as the Lineweaver-Burk plot, shown in Figure 9, to determine V_m and K_m. At 23^{o}C, the maximum removal rate, V_m, was determined to be 6.588 mg PCP / g protein-hr, the Michaelis-Menten constant, K_m, was determined to be 0.179 mg/L with the first order rate constant determined as 36.8 g

protein^{-1} hr^{-1}. At 33^{o}C, V_m was determined to be 3.195 mg PCP / g protein-hr, K_m was determined to be 0.127 mg/L with the first order rate constant determined as 25.2 g protein^{-1} hr^{-1}.

The experimental rates were compared with the rates calculated from the by the Michealis-Menten constants in equation (4). Figures 9 and 10 show the predicted values are in good agreement with the experimental data in the range of conditions studied. Data was also collected at 27^{o}C to verify the results fall within the range expected by the model equation, also shown in Figure 10. Based on these findings, the Michealis-Menten equation sufficiently describes PCP degradation by the extracellular enzymes at low PCP reaction concentrations.

Formation of Products due to Extracellular Fluid Reactions

Several researchers have shown that several polychlorinated phenols, when reacted with enzyme, form reactive compounds that are capable of forming oligomers with other polychlorinated phenols (Leonowicz et al., 1985, Bollag & Liu, 1985, Dec & Bollag, 1990, Roy-Arcand & Archibald, 1991, Roper et al., 1995). Experiments were carried out to determine if possible polymer products were formed from enzyme reactions with PCP. Reactor solution was collected during operation in 1 gal containers and stored at 4^{o}C until analyzed. Organic products were extracted from stored reactor solution with hexane and concentrated. The concentrated hexane extracted compounds were analyzed by FTIR. Figure 11 shows the FTIR obtained for authentic PCP, TCHQ, TCBQ and the bioreactor sample. From the figure, it is obvious that the product formed in the reactor is not PCP,

TCHQ or TCBQ. While PCP, TCHQ and TCBQ are most likely present in the sample, the concentration of the compounds FTIR identified sample is far greater. Gattrell & MacDougall (1999) published FTIR data for PCP, p-chloranil, o-chloranil and a product formed from PCP one-electron oxidation. Several peaks obtained for both the bioreactor sample and the PCP one-electron oxidation samples were in common. These peaks are centered around 800-830 cm^{-1}, 1100 cm^{-1} and 1110 cm^{-1}. The wide peak centered around 800 cm^{-1} is significant because it represents a symmetrical C-O-C stretch (Dollish et al., 1974 & Colthup et al., 1990). This is significant because Gattrell & MacDougall proposed the product formed from PCP one-electron oxidation to be 2,3,4,5,6-pentachloro-4-pentachlorophenoxy-2,5-cyclohexadienone or 2,3,4,5,6-pentachloro-2-pentachlorophenoxy-3,5-cylcohexadienone. Shown in Figure 12, these compounds are dimers with C-O-C linkages. The reaction product or products identified by FTIR contain a C-O-C linkage, likely formed from combination of PCP with a free radical formed by the enzyme reaction.

The concentration of PCP was measured in samples that were collected from the extracellular fluid reactor and stored at 4^{o}C for approximately 30 days. These concentrations were compared to the concentration of PCP fresh from the reactor. Table 9 shows the concentration of PCP collected from the reactor on a specific date compared to the stored effluent collected during the same time period. The concentration of PCP decreased about 80% during storage. This indicates that PCP under went further transformation, even though no longer under enzymatic treatment. Enzymes present in the stored solution are assumed to be inactive because the solution was stored at 4^{o}C, and

previous studies on the extracellular fluid enzymes show the enzymes lose activity after 240hr, these samples were stored for approximately 720 hr. The most likely scenario for PCP transformation is that reactive compounds were formed in the reactor and PCP continued to react with these compounds over time. The FTIR data obtained by extraction of stored samples supports this conclusion.

Viable Fungal Material Study

Fungal cells are expected to be released from the polyurethane cubes periodically due to friction of mixing, release and transport of viable cells into the reactor media from the polymer matrix or sloughing of dead cells. In order to determine the viability of fungal cells in the reactor system and whether cells are present in all stages of the reactor system a viable fungal material study was conducted by inoculating solutions from the FR, the CEFR and the settling basin. These inoculations were compared to a plate of *T. versicolor* obtained from a maintained plate. All of the plates were inoculated and grown under the same conditions. After 4 days of growth the plates were photographed and are shown in Figure 13. The cultures that formed from the samples taken from the FR, settling basin and CEFR were white in color, just as the plate of *T. versicolor*. The growth that resulted from the FR solution had a well defined center of growth, however, the reactor samples did not produce the thick mat of hyphae of the *T. versicolor* plate. The growth patterns of the reactor samples were most likely due to the spread of the liquid sample as opposed to the growth of the fungus, which is symmetrical on the *T. versicolor* plate, except in the FR sample that had a well defined center of growth. This study also was used to determine if contaminants were present in the reactor system.

Based on the color and morphology of the cultures grown from the reactor samples and the absence of any other types of organisms present, the cells are most likely those of *T. versicolor*. However, since the, growth is not as robust as that of the *T. versicolor* culture plate, it can be concluded that free *T. versicolor* cells are present in the reactor system, but are not highly viable at the reactor conditions (low sugar content and presence of inhibitor compounds). In the CEFR these free fungal cells contribute little, if any, to the degradation or removal of PCP from the reactor solution.

CONCLUSIONS

The proposed novel continuous extracellular fluid reactor (CEFR) makes use of extracellular fluid produced by *Trametes versicolor* exposed to PCP, in a continuous fungal reactor. Advantages of using this extracellular fluid as an enzyme source include directly using the extracellular fluid without further purification, continuous renewal of enzyme source, and the presence of reactive compounds, produced in the fungal reactor from PCP, capable of reacting with PCP in the CEFR. These advantages enhance the PCP removal rate while minimized loss of enzyme activity between production in the fungal reactor and use in the CEFR. Enzymatic and non-enzymatic removal of PCP from this system was verified.

Reactor pH was found to have an important effect on removal efficiency with the useful pH range being between pH 3 and pH 4. Incubation temperature of the malt agar plates was determined to have a significant effect on the length of incubation time and reactor

pH. The Michealis-Menten kinetics model was shown to adequately describe enzymatic degradation of PCP at the conditions employed in the CEFR.

Biological material was determined to be free in solution in the FR by the viable fungal material study. This material was determined to be *T. versicolor* and not any other contaminating materials. Based on the morphology of the samples taken from the bioreactor system, compared to a robust culture of *T. versicolor*, it can be concluded that the fungal cells are not highly viable at the reactor conditions. In the CEFR the lack of a well defined center of growth indicates that any free cells do not play a large role in PCP degradation. PCP degradation can be largely contributed to the action of the immobilized *T. versicolor* and the reactions of the extracellular fluid.

Table 1: PCP degradation performance in the CEFR
Temperature: 23^{o}C

RT (hr)	Flow rate (ml/hr)	Outlet pH	Inlet PCP (mg/L)	Outlet PCP (mg/L)	Removal Rate (mg PCP/L.hr)	PCP% Removal
8	112.5	3.727	2.556	0.0865	0.3079	96.6
6	150	3.723	2.573	0.1694	0.4141	93.4
4	225	3.755	2.600	0.3511	0.5673	86.5
3	300	3.765	2.693	0.5863	0.7167	78.2
2	450	3.783	2.920	1.4291	0.7456	51.1

Note: 1. Data shown is an average of three measurements
2. Removal rate = (Inlet PCP – Outlet PCP)/ RT

Table 2: The effects of pH in the CEFR

Outlet pH	Inlet PCP (mg/L)	Outlet PCP (mg/L)	Removal Rate (mg PCP / L.hr)	% PCP Removal
6.4	2.7171	2.9750	0	0
4.9	2.7556	1.0283	0.3838	62.7
3.9	2.7236	0.8194	0.4231	69.9
3.3	2.6913	0.6221	0.4598	76.9
2.4	2.6853	0.7734	0.4249	71.2
2.4	2.7196	1.0866	0.3629	60.0
1.9	2.7304	1.1793	0.3447	56.8

Note: 1. Residence time: 4.5 hr

2. Temperature: 23^{o}C

3. Data presented is an average of three measurements.

4. Removal Rate = (Inlet PCP – Outlet PCP)/RT

Table 3: Effect of incubation temperature on length of time of incubation to sufficient growth of *T. versicolor* on malt agar plates

Incubation Temperature	Incubation Time to Sufficient Growth
75°F	9 days
85°F	5 days

Table 4: Effect of incubation temperature on reactor pH

Incubation Temperature	Day of Reactor Operation	FR pH	CEFR pH
75°F	18	4.911	4.092
	21	4.908	4.167
	22	4.728	4.045
	29	4.838	4.217
	32	4.792	4.315
	Average	4.835	4.167
85°F	16	3.908	3.685
	21	3.947	3.743
	27	3.886	3.717
	28	3.930	3.732
	37	3.857	3.736
	Average	3.906	3.723

Table 5: Effect of incubation temperature on concentration of PCP in reactors

Incubation Temperature	Day of Reactor Operation	[PCP] mg/L FR	CEFR
75°F	18	0.091	0.115
	22	0.138	0.168
	32	0.232	0.190
	Average	0.154	0.157
	% Removal	93.4%	93.9%
85°F	15	0.156	0.095
	21	0.041	0.107
	32[1]	0.179	0.187
	Average	0.139	0.144
	% Removal	94.4%	94.4%

Note: 1. Data points are an average of data collected on day 27 and 37.

Table 6: Long term reactor characteristics in the FR and the CEFR

	Day of Reactor operation	Reactor pH	Reactor Protein (μg/ml)
FR			
	5	4.62	33.3
	7	4.77	45.8
	15	3.91	52.8
	21	3.95	69.9
	27	3.89	97.1
	28	3.93	94.1
	37	3.86	357
	38	4.08	125
	42	3.92	180
	43	3.94	199
	49	3.90	173
	50	3.92	172
CEFR			
	5	3.90	23.7
	7	3.96	23.4
	15	3.70	31.1
	21	3.74	19.1
	27	3.72	30.0
	28	3.73	34.7
	37	3.74	43.0
	38	3.72	57.8
	42	3.72	40.9
	43	3.70	45.7
	49	3.70	44.0
	50	3.69	44.0

Table 7: Long term reactor PCP Removal in the FR and the CEFR

	Day of Reactor operation	Reactor pH	Reactor [Protein] (μg/ml)	[PCP] (mg/L)	% Reduction of PCP
FR					
	5	4.62	33.3	0.013	99.5
	15	3.91	52.8	0.156	93.8
	21	3.95	69.9	0.041	98.4
	27	3.89	97.1	0.141	94.3
	37	3.86	357	0.217	91.3
	42	3.92	180	0.219	91.2
	49	3.90	173	0.296	88.2
CEFR					
	5	3.90	23.7	0.009	99.6
	15	3.70	31.1	0.095	96.4
	21	3.74	19.1	0.107	95.8
	27	3.72	30.0	0.161	93.8
	37	3.74	43.0	0.215	91.8
	42	3.72	40.9	0.177	93.3
	49	3.70	44.0	0.283	89.4

Note: Data points presented are an average of three sample measurements

Table 8: Summary of PCP removal performance in the CEFR

Reaction Temperature (^{o}C)	RT (hr)	Inlet PCP (mg/L)	Outlet PCP (mg/L)	Inlet Protein (g protein/L)	Reactor Protein (g protein/L)	Removal Rate (mg/ g. protein hr)	%PCP Removal
23	8	2.5559	0.0866	0.2083	0.1431	2.1531	96.6
	6	2.5731	0.1694	0.1966	0.1318	3.1371	93.4
	4	2.6001	0.3511	0.1860	0.1220	4.6500	86.5
	3	2.6926	0.5863	0.2116	0.1425	5.0119	78.2
	2	2.9204	1.4294	0.2003	0.1318	5.6485	51.1
33	12	2.5371	0.0813	0.2408	0.1639	1.2483	96.8
	8	2.5542	0.1517	0.2586	0.1785	1.6823	94.1
	6	2.5730	0.2415	0.2441	0.1722	2.2567	90.6
	5	2.6306	0.5171	0.2243	0.1595	2.6502	80.3
	4	2.7288	1.0253	0.2348	0.1607	2.6503	62.4

Note: 1. Data reported is an average of three measurements; 2. Removal Rate= (Inlet PCP-Outlet PCP)/Reactor Protein/RT

Table 9: Effects of changes in ratio of extracellular fluid to fresh PCP on optimum reaction pH

Flow Rate Ratio	Fresh Feed pH	Reactor pH	Outlet PCP (mg/L)	PCP Removal Rate
2	4	4.107	0.904	0.145
	3.8	3.678	0.764	0.167
	3.5	3.578	0.735	0.171
1.25	4	3.860	0.629	0.200
	3.5	3.556	0.987	0.186
0.8	3.8	3.768	0.919	0.216
	3.5	3.515	1.261	0.193
0.5	3.8	3.768	1.074	0.260
	3.5	3.541	1.486	0.222

Note: 1. Flow rate ratio = flow rate of extracellular fluid to CEFR / flow rate of fresh PCP solution to CEFR

2. Flow rate of extracellular fluid was held constant at 50ml/hr
3. Flow rates of fresh PCP solution to CEFR at flow rate ratios: 2, 1.25, 0.8 and 0.5, were: 25 ml/hr, 40 ml/hr, 62.5 ml/hr, and 100 ml/hr respectively.
4. Data presented is an average of three measurements
5. Removal rate = (Inlet PCP-Outlet PCP)/RT

Table 10: Effect of storing reactor effluent on PCP concentration

	Collection Date(s)	PCP Conc. (mg/L)
Samples collected directly from reactor		
	6/17	0.194
	6/20	0.375
	7/5	0.867
Samples stored at 4^{o}C for ~ 30 days		
	6/13-6/18	0.0406
	6/18-6/20	0.0806
	7/2-7/5	0.192

Notes: 1. Data presented is an average of three measurements

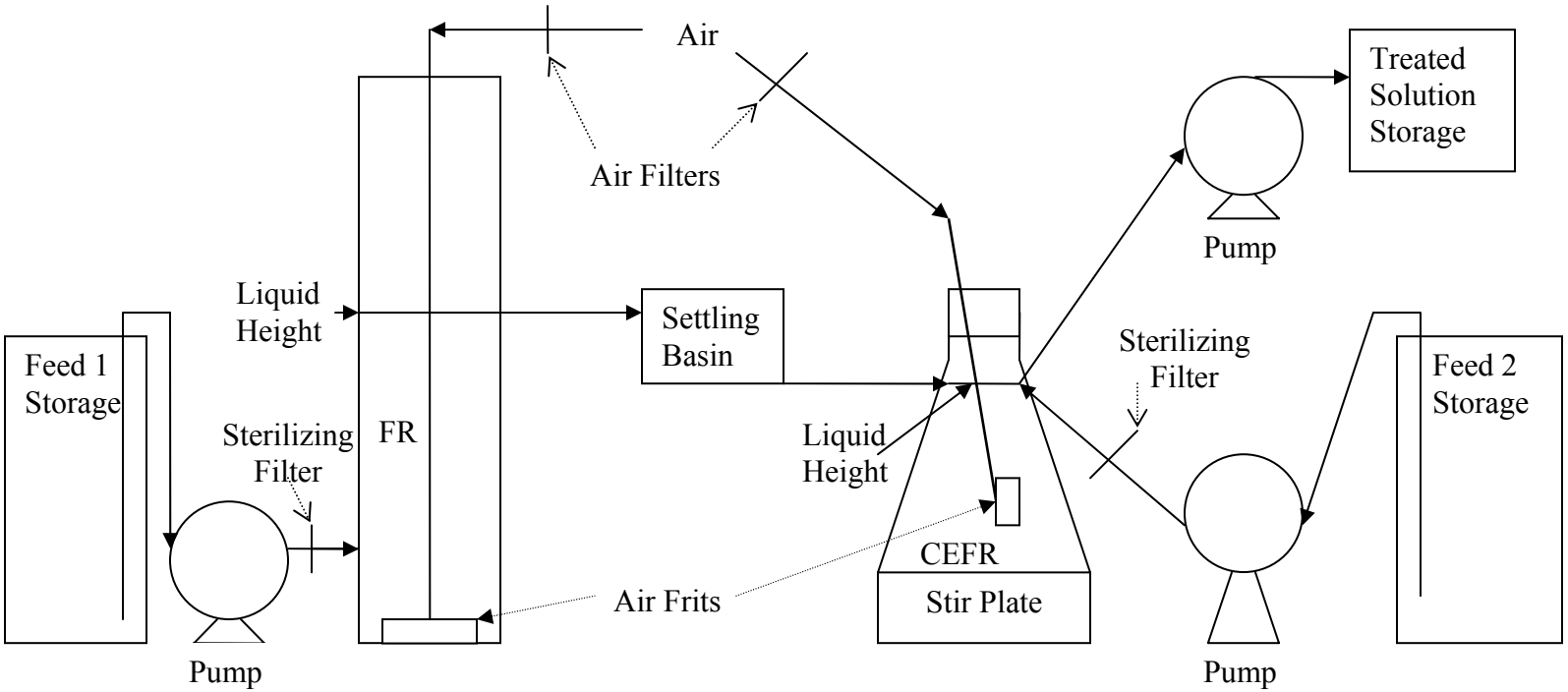


Figure 1: Schematic diagram of continuous fungal reactor in series with continuous extracellular fluid reactor

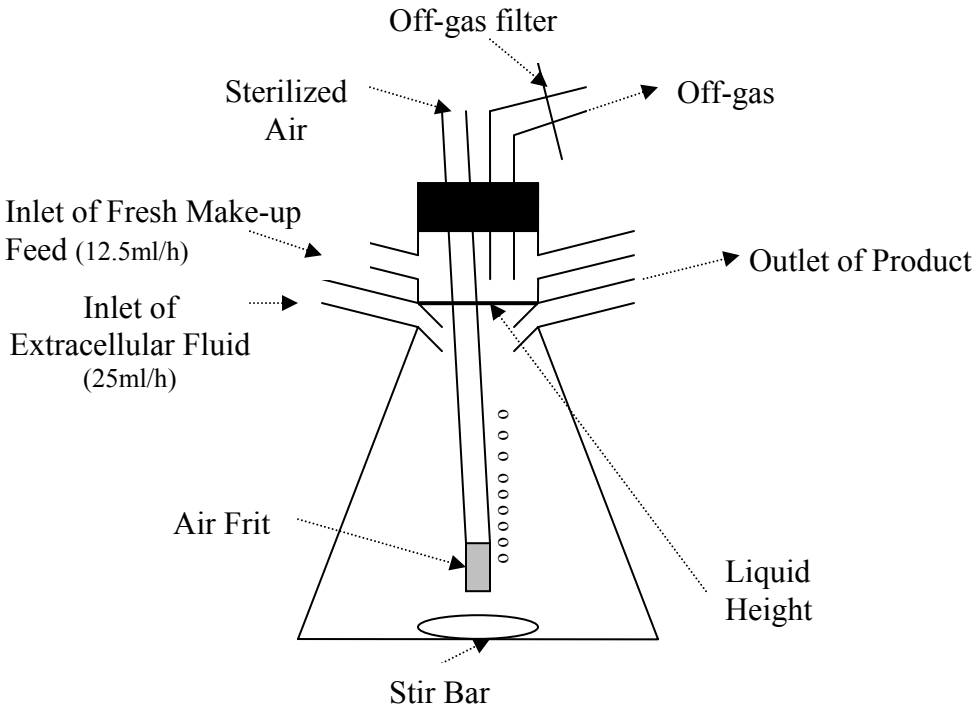


Figure 2: Detailed schematic of continuous extracellular fluid reactor

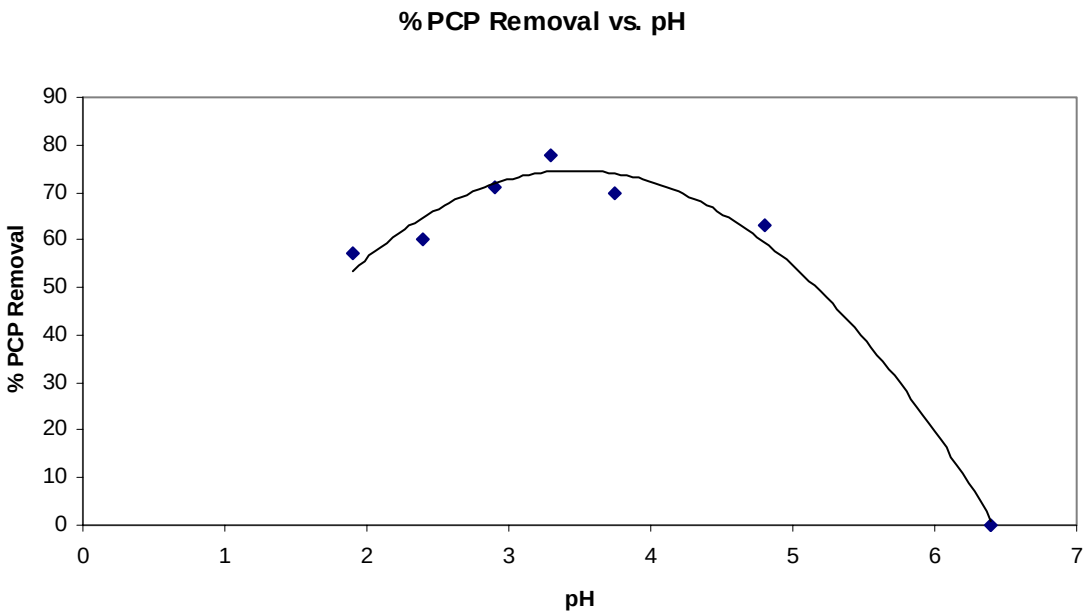


Figure 3: The effect of pH on PCP degradation in a CEFR

Note: 1. Residence time: 4.5 hr

2. Temperature: 27^{o}C

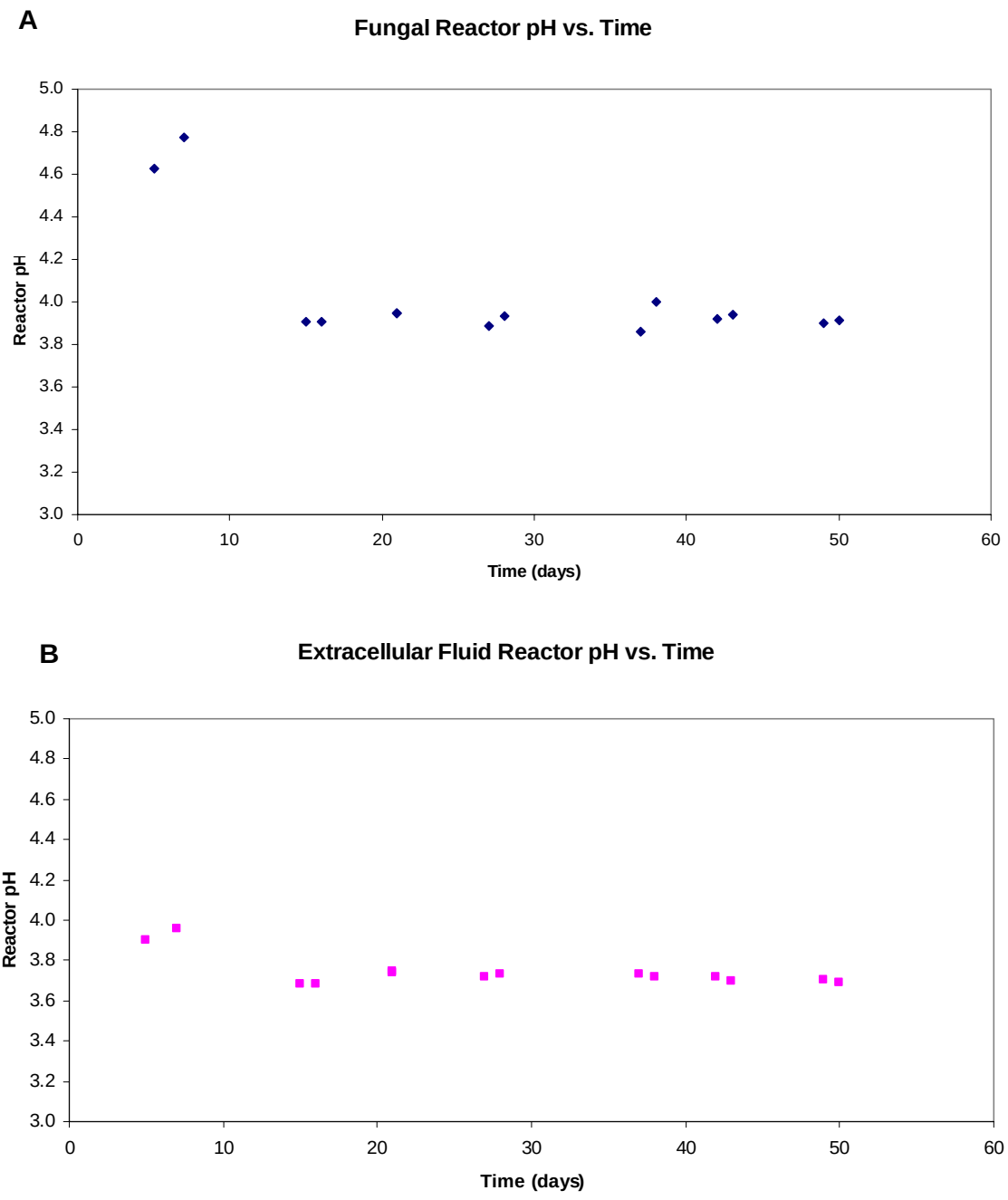


Figure 4. Reactor pH in fungal reactor (A) and reactor pH in extracellular fluid reactor (B) during reactor operation ◆-Reactor pH in fungal reactor; ■-Reactor pH in extracellular fluid reactor

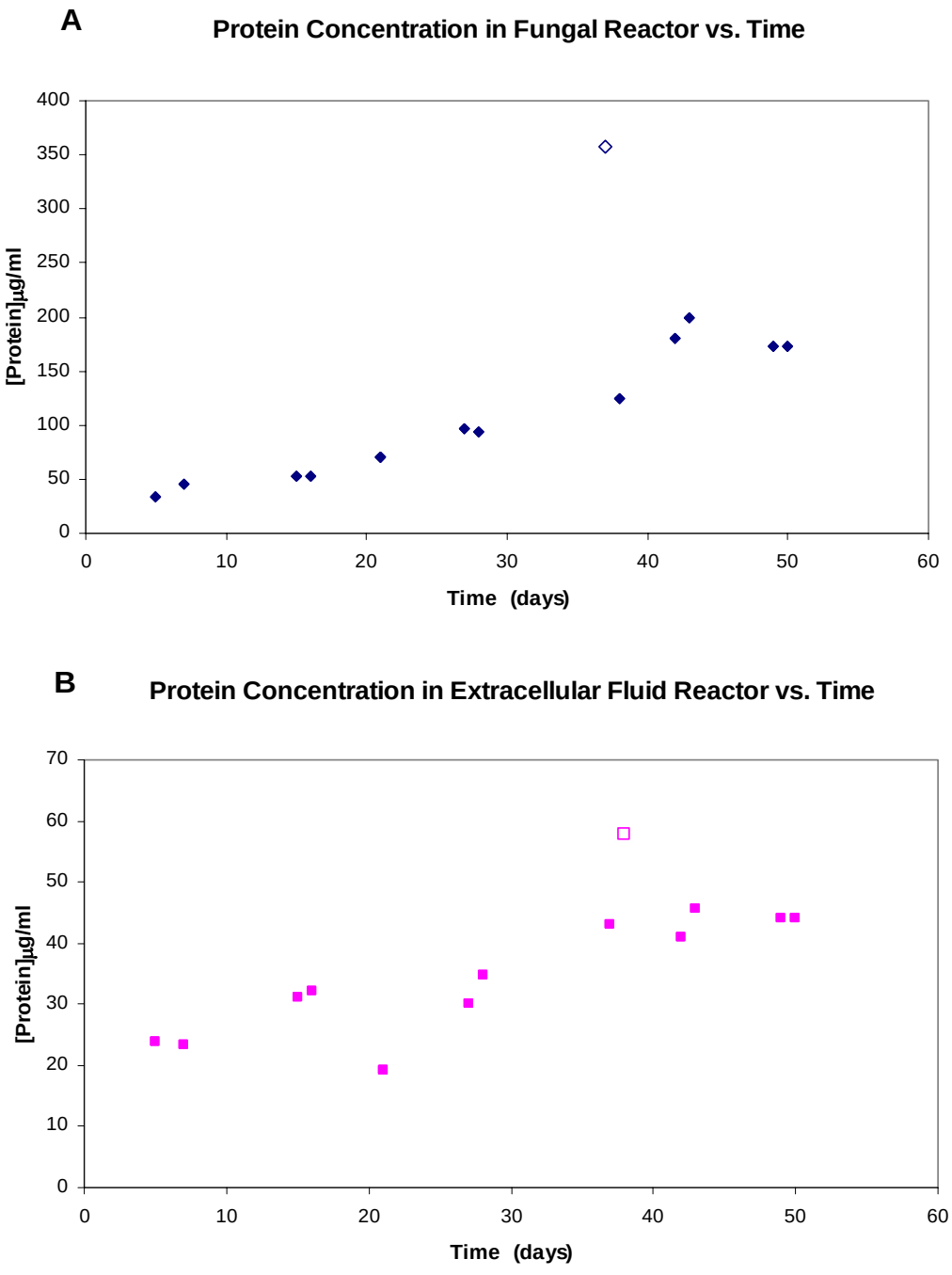


Figure 5. Protein concentration in fungal reactor (A) and protein concentration in extracellular fluid reactor (B) during reactor operation ◆-Protein concentration in fungal reactor; ■-Protein concentration in extracellular fluid reactor

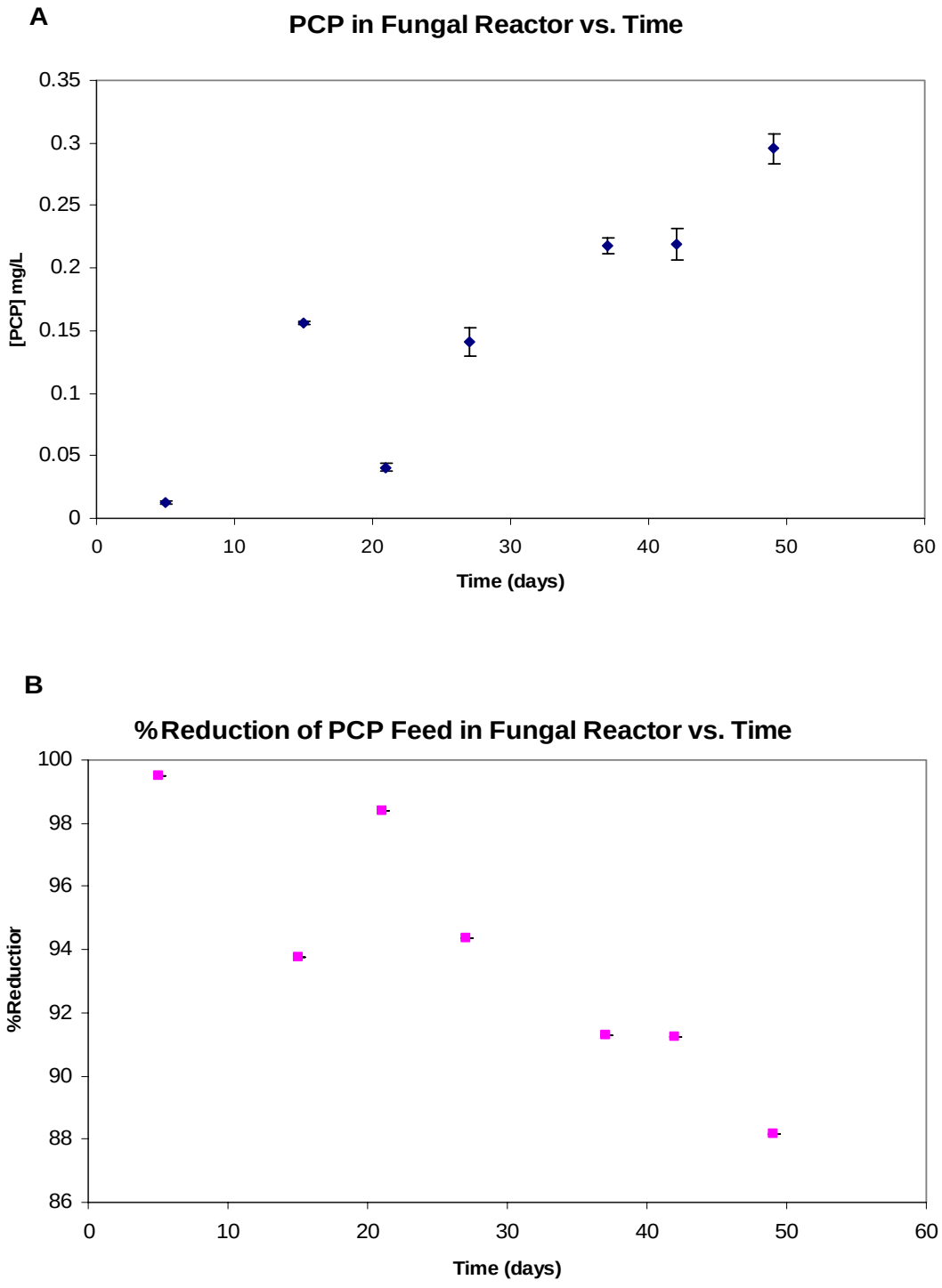


Figure 6. Concentration of PCP in fungal reactor (A) and %Reduction of PCP in fungal reactor (B) during reactor operation ◆-Concentration of PCP in fungal reactor; ■-%Reduction of PCP in fungal reactor

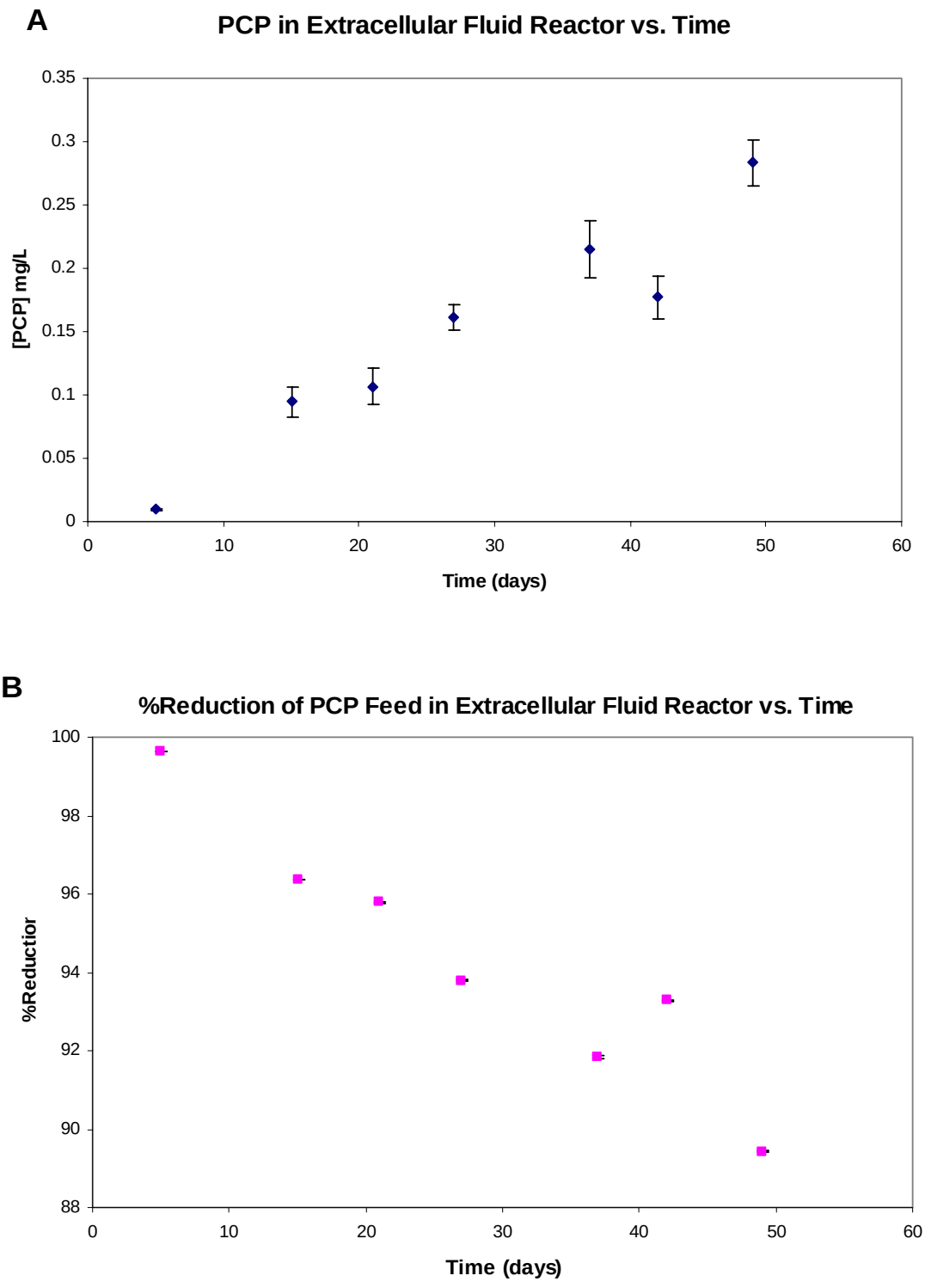


Figure 7. Concentration of PCP in extracellular fluid reactor (A) and %Reduction of PCP in extracellular fluid reactor (B) during reactor operation ◆-Concentration of PCP in extracellular fluid reactor; ■-%Reduction of PCP in extracellular fluid reactor

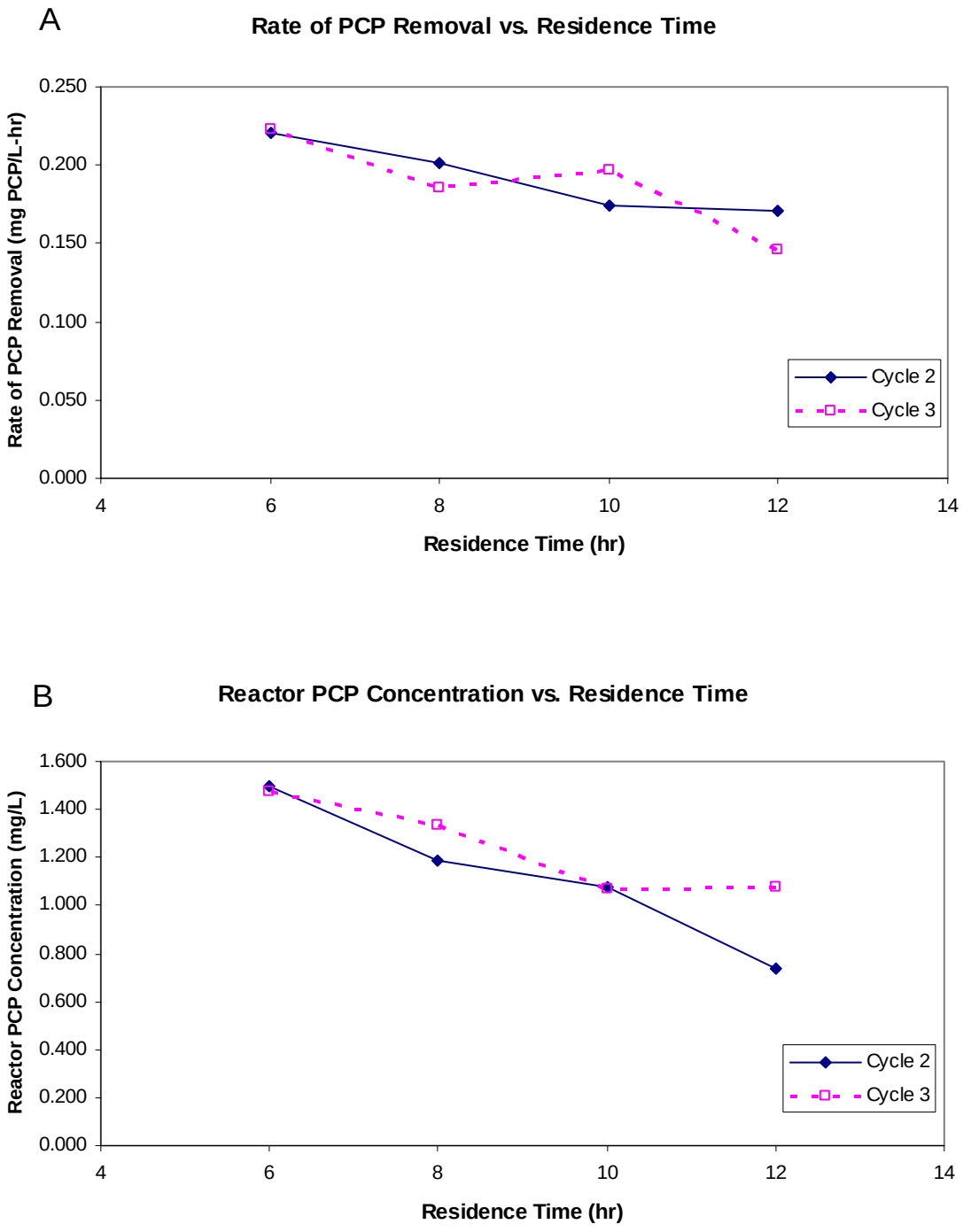


Figure 8: Effects of variations in the continuous extracellular fluid reactor on rate of PCP removal and PCP concentration. (A) Rate of PCP removal during CEFR operation and (B) concentration of PCP in CEFR during operation ◆-Cycle 2 ; □-Cycle 3.

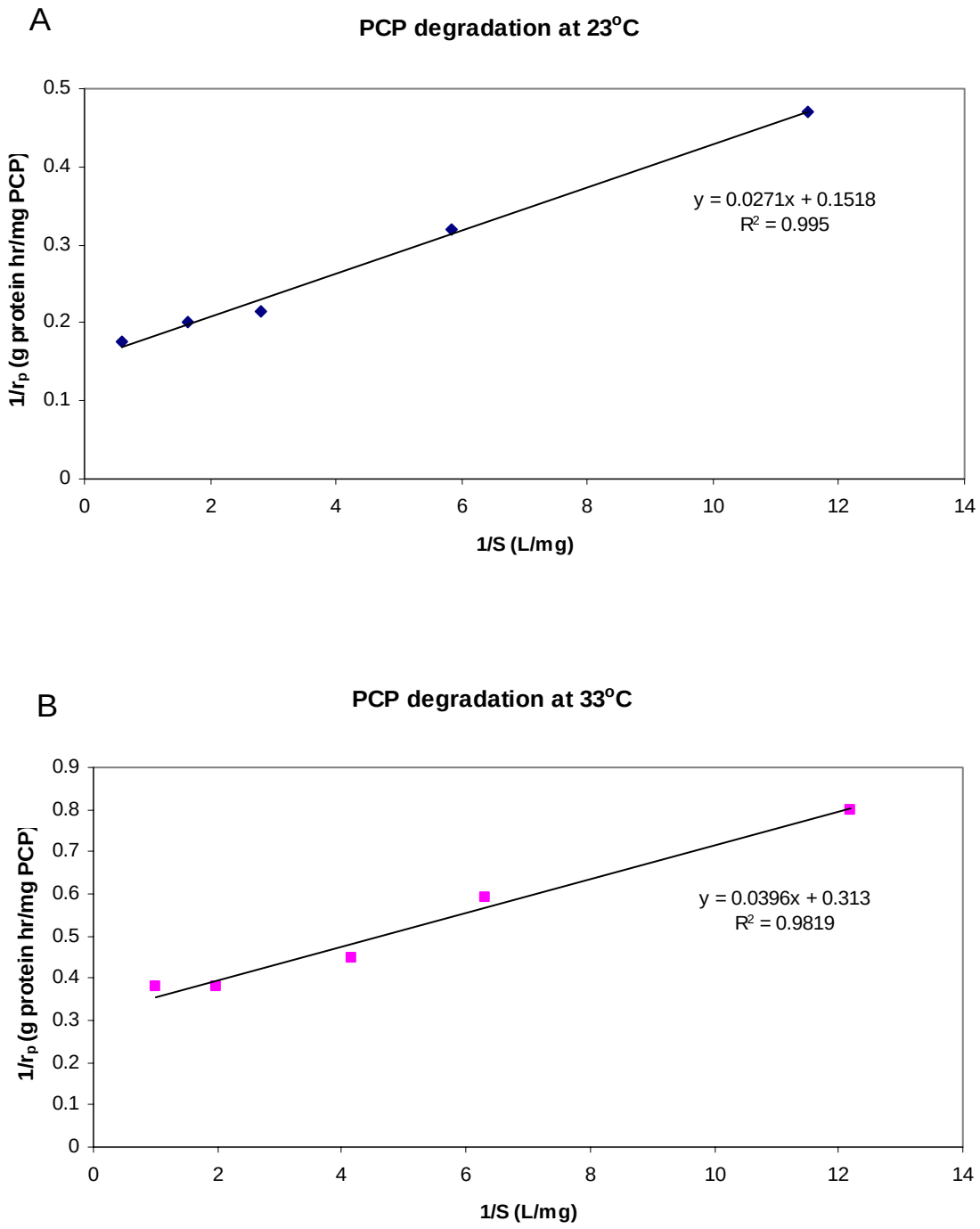


Figure 9. Lineweaver-Burk plot for enzyme kinetics of the CEFR (A) at 23°C (B) and at 33°C ◆-Inverse of the rate of removal at 23°C; ■-Inverse of the rate of removal at 33°C reactor. Note: Data collected by R. Zhao

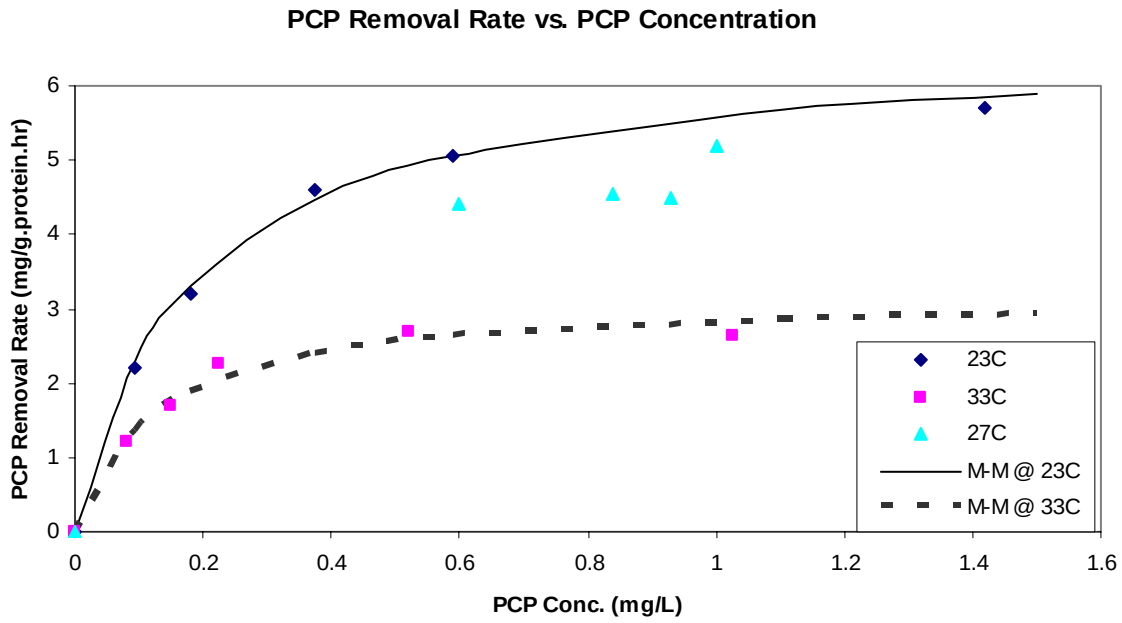


Figure 10: Applicability of Michealis-Menten model for PCP biodegradation in continuous extracellular fluid reactors ◆-CEFR PCP concentration at 23^{o}C; ■-CEFR PCP concentration at 33^{o}C; ▲-CEFR PCP concentration at 27^{o}C. Note: Data collected by R. Zhao

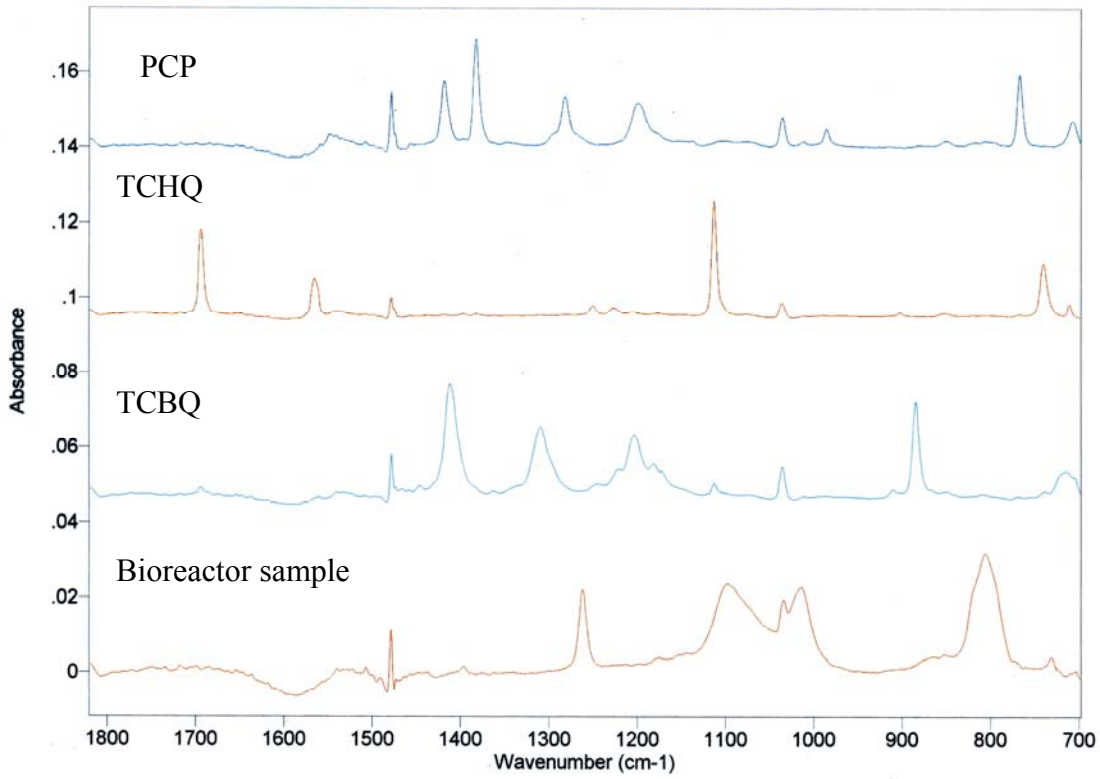
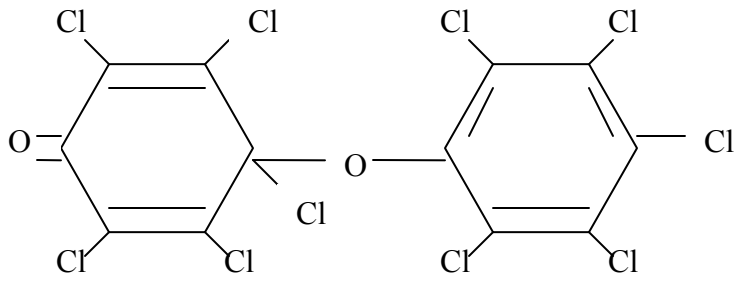
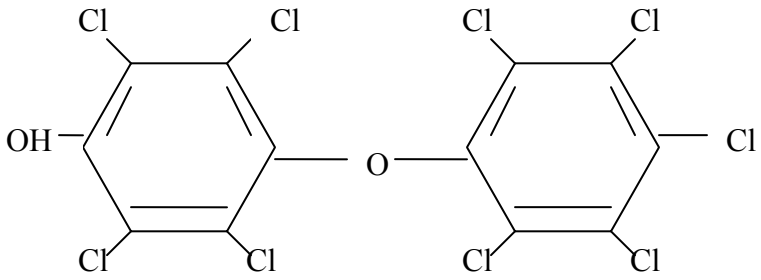


Figure 11: FTIR identification of bioreactor products compared to pure compounds.



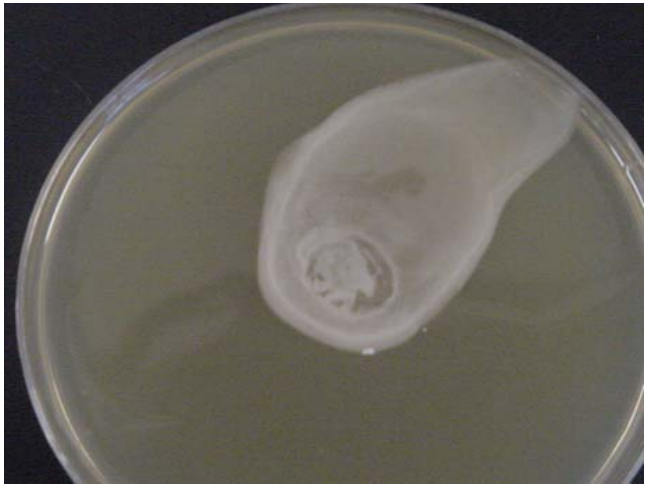
2,3,4,5,6-pentachloro-4-(pentachlorophenoxy)-2,5-cyclohexadien-1-one



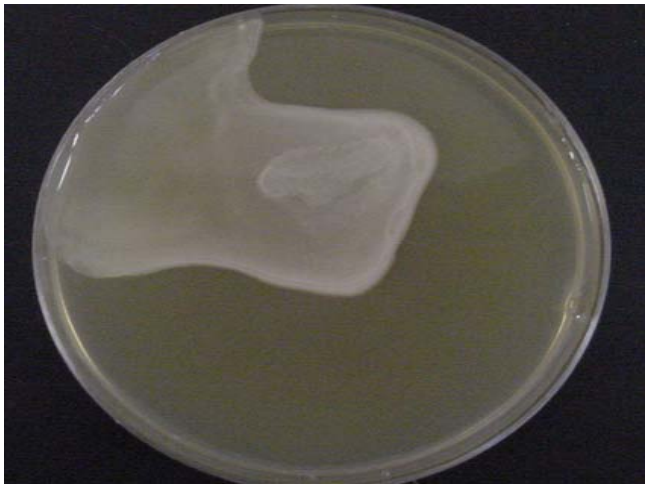
2,3,5,6-tetrachloro-4-(pentachlorophenoxy)-phenol

Figure 12: Structures of possible reaction products in the effluent of the continuous extracellular fluid reactor.

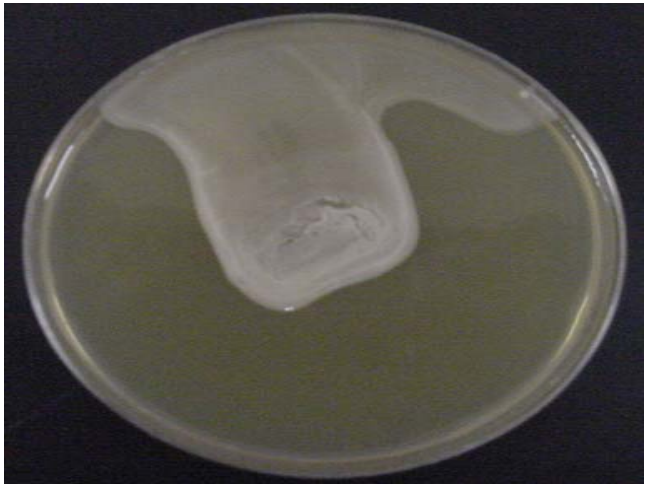
Malt agar plate of *T.versicolor*



Fungal Reactor



Continuous Extracellular Fluid Reactor



Settling Chamber

Figure 13: Samples taken from bioreactor system and a malt agar plate of *T.versicolor* plated on malt agar

CHAPTER II

PCP REACTIVE INTERMEDIATE IDENTIFICATION AND PROTEIN BINDING

ABSTRACT

A two-stage bioreactor was studied to identify the mechanisms of pentachlorophenol (PCP) degradation in aqueous streams. A reactive intermediate produced by remediation of PCP, tetrachlorohydroquinone (TCHQ) was identified in reactor solutions at concentrations of about 10% of the amount of PCP present in the reactor solutions. Reactor protein was determined to play a role in removal of PCP from solution. PCP and TCHQ were found to bind to protein, with PCP binding in greater amounts. The concentration of apparent protein bound PCP was much less than the concentration of PCP free in solution and was strongly dependent on the concentration of protein present in the reactor. Incubation temperature of the fungal plates used for fungal immobilization in the FR had a significant effect on apparent protein binding of PCP and TCHQ, with more occurring when the fungal plates were incubated at 85^{o}F. More protein binding occurred in the FR compared to the CEFR, most likely because the protein in the system is produced in the FR and is more abundant than the protein in the CEFR.

INTRODUCTION

Pentachlorophenol has been used as a biocide, herbicide, pesticide and wood preservative (Banerji et al., 1993). The EPA has listed PCP a priority pollutant and has banned its use in consumer products since 1984 (Jackson & Bisson, 1990). Contamination with PCP is present in soil and water throughout the world, mainly due to wood treatment and pesticide use, entering either by direct spraying or leaching out of treated wood products. Abandoned wood treatment plants in northern California (Daniels, 1994), Montana (Jackson & Bisson, 1990), and Alberta, Canada (Pollard et al., 1993) have been studied for PCP contamination. Agricultural sites documented to be contaminated with PCP include a potato field and grassland area in Taipei City, Taiwan (You & Liu, 1996) and rice fields in Japan (Donaldson & Miller, 1997). Soil surrounding in use utility poles have also been found to be contaminated with PCP (Murarka et al., 1995, EPRI, 1995). Ground water can be contaminated by PCP leaching out of soil. Humans have been exposed to PCP in several ways, by occupational exposure at chemical plants or wood treatment sites by inhalation of vapors or contaminated dust, through the food chain by ingesting contaminated animals or food grown in contaminated soil as well as contaminated drinking water, by contact with contaminated soil or water, and through deodorants and repellants (Pollard et al., 1994, Veningerová et al., 1994, Wang & Lin, 1995). PCP has been detected in the urine of occupational exposed workers as well as the general population; with the occupational exposed workers have about three times the amount as the general population (Veningerová et al., 1994). Tam et al. (1999) detected PCP in the urine of 85% of U.S. volunteers and in the serum of Dutch volunteers.

PCP is toxic to microbial life in soils and in aquatic systems as well as mammals. Salminan and Haimi (1999) found that soils contaminated with PCP have lower amounts of ATP, less CO_2 evolution (by biological activity) and increased amount of NH_4^+-nitrogen (suggesting less decomposition). The flora of the soil is also changed due to the disappearance of microorganisms affected by the toxicity of PCP and the appearance of microorganisms not affected by PCP toxicity. In aquatic systems, PCP contamination results in the rapid decline of daphnia, autotrophic phytoplankton and dissolved oxygen (Schauerte et al., 1982). Toxicity of PCP to mammals is due to its ability to lead to the uncoupling of mitochondrial oxidative phosphorylation (Weinbach, 1954), liver disease (Rizzardini & Smith, 1982), and DNA single strand breakage (Witte et al., 1985, & Wang & Lin, 1995). The LD_{50} (lethal dose, 50% kill) of PCP given orally to rats is 27 mg/kg. However the oxidative products tetrachlorobenzoquinone (TCBQ) and tetrachlorohydroquinone (TCHQ) are much less toxic when taken orally. The LD_{50} of TCHQ is 3319 mg/kg, and the LD_{50} of TCBQ is 4000 mg/kg. Mammalian metabolism of PCP results in the formation of the reactive compounds TCHQ and TCBQ which have been shown to directly cause DNA breakage (Witte et al., 1985) and bind to liver (Lin et al., 1993) and blood proteins (Waidyanatha, 1994). However, when taken orally, TCHQ and TCBQ are metabolized in such a way not to cause as extreme toxic effects as oral administered PCP.

Bioremediation of PCP from the environment is very important due to the widespread contamination and toxic effects of this chemical. Fungal bioremediation has shown great promise in removal of PCP from aqueous solution (Pallerla & Chambers, 1998). The

extracellular enzymes produced by white-rot fungus have also been effective in the removal of PCP (Jönsson et al., 1989, Dec & Bollag, 1990). A bioremediation system, consisting of two bioreactors in series, was studied to determine the reaction intermediates formed from the biodegradation of PCP by the fungus *Trametes versicolor* and its extracellular enzymes. The first reactor in the bioreactor system, termed the fungal reactor (FR), utilizes polyurethane immobilized *T. versicolor* (Pallerla & Chambers, 1998) to produce extracellular fluid containing degradative enzymes when exposed to PCP. The extracellular fluid from the FR is fed to the second reactor, termed the continuous extracellular fluid reactor (CEFR), to act on PCP freshly fed to the CEFR. This bioreactor system has been shown to remediate over 99% of PCP. The objectives of this portion of the study were to determine the reaction intermediates formed from PCP, and to determine if protein binding of the PCP and the reaction intermediates in the two stage bioreactor system occurs and to what extent protein binding plays a role in removing PCP from aqueous solutions. Further understanding of the mechanisms by which PCP is removed can show that when PCP is removed from solution it is either in a less toxic form, such as addition reactions, or is entrapped, such as by protein binding.

LITERATURE REVIEW

Reaction Intermediates

Oxidative treatment of PCP has been shown to result in the formation of tetrachlorobenzoquinone (TCBQ), also known as chloranil, which can be readily reduced to tetrachlorohydroquinone (TCHQ). Fang et al. (1998) determined that oxidative treatment of PCP in aqueous solution by γ-radiolysis resulted in the initial products formed being 1,2-TCBQ and 1,4-TCBQ, which were able to undergo further hydrolysis. Ho and Bolton (1998) reported that the intermediates formed during photolysis of PCP are polychlorinated phenols and polychlorinated benzoquinones.

In studies carried out utilizing enzymes produced by *P. chrysosporium,* namely LiP and MnP, the quinone undergoes subsequent reductive dechlorination (Reddy & Gold, 2000). In the presence of hydrogen peroxide, LiP can oxidize PCP to TCBQ. However, probably due to the low level of laccase present in the extracellular enzyme system of *P. chrysosporium*, the polymerization reaction is not noticed to any significant degree. These enzymes produced by this fungus led to the dechlorination of all five chlorine atoms from PCP. Enzymatic conversion of PCP to TCHQ by LiP was also confirmed by Chung & Aust (1995).

Grey et al. (1998) used extracellular laccase produced by *Trametes versicolor* to study the enzymatic degradation of 2-chlorophenol. They determined that the primary degradation product formed was 2-chloro-1,4-benzoquinone. Formation of the quinone correlated

with extracellular laccase activity. Ricotta & Bollag (1996) used extracellular laccase produced by *Trametes versicolor* to study the enzymatic degradation of PCP. These researchers determined that the primary degradation products formed from PCP were 1,2-TCBQ and 1,4-TCBQ.

TCBQ has been the subject of studies carried out to develop a PCP or phenol assay, utilizing the reductive nature of TCBQ (Brown et al., 1994; Cybulski et al., 1999; Labra-Espina et al., 2000). Brown et al. (1994) used another copper-containing enzyme, tyrosinase, to study the recyclablity of TCBQ to TCHQ and back using NADH as a reducing agent. Tyrosinase is known as polyphenol oxidase and can catalyze polymerization reactions with phenols as substrates, similar to laccase. The use of excess NADH reduces quinones formed from phenols to their reduced state. Polymerization is reduced because the quinones are in their reduced form. The tyrosinase then oxidizes the reduced quinone back to the oxidized state and NADH again reduces the quinone. This cycle can continue until all NADH is used. Inactivation of tyrosinase is also reduced, which has been attributed to the reduction of quinones and reaction intermediates. They also determined that NADH does not react with polymer products, indicating that the quinone polymer cannot be recycled as the quinone monomer can.

Research sponsored by the Biotechnology Research Institute, National Research council in Canada, carried out by Cybulski et al. (1999) and Labra-Espina et al. (2000) used bilirubin oxidase to carry out the recycling of TCBQ. Bilirubin oxidase is a copper-containing enzyme, but does not lead to the polymerization of quinones as tyrosinase and

laccase do. When Cybulski et al., evaluated enzymes to use for substrate recycling, they determined that tyrosinase and laccase lead to an unsatisfactory detection level of the quinone, suggesting polymerization occurred.

Protein Binding

Laccase catalyzes the removal of chloride atoms from PCP, resulting in reactive quinone compounds. These compounds, TCHQ and TCBQ, are highly reactive. They are known to bind with protein's sulfhydryl groups (Renner & Mücke, 1986). In biological systems, PCP is metabolized into these reactive compounds, which bind with proteins such as hemoglobin, albumin, DNA and liver tissue. This binding is believed to play a role in the toxicity and carcinogenicity of PCP. Many chlorinated ring compounds, when introduced into biological systems, are metabolized into PCP and its secondary metabolism results in reactive quinones, which bind to protein. Several studies on the protein binding of PCP, precursor molecules, and metabolites support the above statements and are further discussed below.

PCP has been shown to form conjugates with sulfur containing compounds. Akitake & Kobayashi (1975) exposed goldfish to PCP and recovered approximately 54% of initial PCP as conjugated PCP. The conjugate was determined to be pentachlorophenylsulfate, sulfate bound PCP, biosynthesized by the goldfish. When the conjugate was hydrolyzed, by heating under acidic conditions, PCP was released. Lilienblum (1985) reported on the formation of pentachlorophenol glucuronide in rat and human liver microsomes. Pentachlorophenol glucuronide was determined to be stable at neutral conditions and

readily hydrolyzed at pH 4-6, releasing PCP. Reigner et al. (1991) found that administration of PCP to rats resulted in pentachlorophenol glucuronide and tetrachlorohydroquinone glucuronide excreted in the urine.

Shuttleworth & Bollag (1986) observed that the removal of several phenols was enhanced by the addition of protein to laccase catalyzed reactions. The addition of bovine serum albumin increased the removal of 2,6-dimethylphenol, 4-chloro-2-methylphenol and a-naphthol. Increased removal depended on the concentration of bovine serum albumin added, however no removal was observed with additional protein if no active laccase was present in the reaction mixture.

Rizzardini and Smith (1982) studied the metabolism of hexachlorobenzene (HCB) in rats. Historically, HCB has been used as a fungicide. In the mid 1950's, many people in southeastern Turkey ate HCB treated seed wheat and developed a chronic porphyria, with some people still showing signs twenty years later. Porphyria is caused by the inhibition of hepatic uroporphyrin decarboxylase and is characterized by the urinary excretion of uroporphyrin. In rats, given HCB, this enzymatic inhibition is linked to the metabolism of HCB. The metabolites found in urine and feces of HCB fed rats were found to be pentachlorophenol, tetrachlorohydroquinone, and pentachlorothiophenol. Pentachlorothiophenol is formed from pentachlorophenyl-N-acetylcysteine during hydrolysis. High levels of the development of porphyria were directly related to high levels of metabolites in urine and feces. Female rats excreted porphyrin at significantly higher levels then males and pre-pubertal rats excreted the highest levels of porphyrin.

These comparisons seem to be related to the rate of metabolism of the animal assuming females have a faster metabolism than males and pre-pubertal rats have a faster metabolism than adult rats. The presence of pentachlorothiophenol, formed from pentachlorophenyl-N-acetylcysteine, is likely derived from a dechlorinated HCB that bound with a sulfur containing amino acid.

Van Ommen et. al. (1986) studied the microsomal metabolism of HCB, to further understand the covalent binding to protein using rat microsomal protein. They found PCP to be the main metabolite of HCB with TCHQ detected in small amounts. Metabolites bound to microsomal protein represented approximately 10% of HCB metabolized. Assuming that covalent binding during HCB metabolism causes the inhibition of the liver enzyme, uroporphyrin decarboxylase, led these investigators to examine the nature of covalent binding during HCB metabolism. They determined covalent binding did not originate from an intermediate formed during conversion of HCB to PCP but rather it originates from the conversion of PCP to tetrachlorobenzoquinone. PCP was found to bind to protein; however, the majority of the metabolites bound to protein were tetrachlorobenzoquinone and tetrachlorosemibenzoquinone (TCSBQ), an intermediate formed during the conversion of PCP to TCBQ. Further studies by Van Ommen et al. (1988) found covalent binding of TCBQ to protein to be very rapid and non-enzymatic. Covalent binding was inhibited using ascorbic acid, glutathione and cysteine. Ascorbic acid was able to completely inhibit binding by reducing TCBQ and TCSBQ to TCHQ. Loss of binding due to the addition of ascorbic acid was accompanied by an increase of TCHQ. The addition of

glutathione and cysteine completely inhibited binding, but did not result in an increase of TCHQ. Glutathione and cysteine are able to inhibit binding by forming adducts with TCBQ. Van Ommen et. al. (1988) determined that glutathione reacting with TCBQ results in highly conjugated molecules, with at least three glutathione molecules bound to one molecule of TCBQ. This shows that one TCBQ is capable of binding to several protein molecules.

The metabolism of pentachlorobenzene (PeCB) by rat liver microsomes was investigated (den Besten et. al., 1989). These researchers determined metabolism was dependent on cytochrome- P450 activity. Major metabolites resulting from microsomal PeCB metabolism were PCP and 2,3,4,6-tetrachlorophenol. Minor metabolites were 2,3,4,5-tetrachlorophenol and 2,3,5,6-tetrachlorophenol, with 1,2-tetrachlorohydroquinone and 1,4-tetrachlorohydroquinone as secondary metabolites. A significant amount of metabolites, 5-15%, was covalently bound to protein. Protein binding was inhibited by the addition of ascorbic acid and glutathione. Ascorbic acid inhibited binding 60-70% and was accompanied by an increase in TCHQ, as seen by other researchers. Glutathione inhibited binding significantly by reacting with 36% of metabolites; however substantial covalent binding still occurred. Due to the fact that both ascorbic acid and glutathione inhibited binding to a large extent, but not completely, leads to the conclusions that quinones are responsible for most binding, however, other metabolites or intermediates are accountable for covalent binding. These compounds are most likely formed during conversion of PeCB to PCP.

Several researchers have investigated the metabolism of PCP in rats by studying urine of animals exposed to PCP (Renner & Hopfer, 1990; Reigner et al, 1990, 1991). Renner & Hopfer (1990) determined the metabolites of PCP present in urine of rats dosed with PCP to be 2,3,4,5-tetrachlorophenol, 2,3,4,6-tetrachlorophenol, 2,3,5,6-tetrachlorophenol, tetrachlorocatechol, tetrachlororesorcinol, tetrachlorohydroquinone, trichlorohydroquinone, tetrachlorobenzoquinone and trichloro-1-4-benzoquinone. The major metabolite was TCHQ, excreted as a conjugate. Tetrachlorobenzoquinone and trichlorohydroquinone were found in trace amounts. They determined from their results that the pathway of PCP biotransformation in warm-blooded animals to be conjugation, reductive dechlorination, hydrolytic dechlorination and oxidation. When samples were subjected to oxidation using $K_2Cr_2O_7$, the concentration of the quinone forms increased while the hydroquinone forms decreased. When samples were reduced using SO_2 the concentration of the quinone and hydroquinone decreased and water-soluble hydroquinone sulphonic acids appeared. Reigner et al. (1990 & 1991) determined that metabolism of PCP in rats leads to PCP conjugation with glucuronic acid, the oxidation of PCP resulting in TCHQ, and TCHQ forming conjugates with glucuronic acid and sulfate.

Several researchers have investigated the binding of PCP by way of its metabolite, TCHQ, to albumin and hemoglobin in rats and mice (Waidyanatha et al., 1994, 1996). Waidyanatha et al (1994) studied binding to albumin and hemoglobin by performing experiments *in vivo* and *in vitro*. Tetrachlorobenzoquinone was reacted with rat blood and it was verified that adducts were formed by TCBQ reacting with the sulfhydryl

groups of albumin and hemoglobin. Adduct formation with albumin was about three times that of adduct formation with hemoglobin *in vitro*. Adduct formation *in vivo* was studied by administering PCP at several concentration to rats. Adduct formation with albumin was about ten times that of adduct formation with hemoglobin *in vivo.* This led the researchers to the theory that following PCP metabolism, TCBQ reacts with albumin inside liver cells. This could be possibly be explained by diffusion limitations into red blood cells, limiting binding to hemoglobin, or diffusion limitation out of liver cells, increasing binding to albumin. Waidyanatha et al. (1996) also determined that after TCBQ binds to a protein molecule, it is possible for that mono-substituted TCBQ to continue to react with protein and/or non-protein thiols (such as glutathione) until all chlorine atoms have been substituted with protein. Mono-, di- and tri- substituted TCBQ adducts were identified with mono-substituted TCBQ present in amounts much higher than the di- or tri- substituted TCBQ. Waidyanatha et al. were able to verify that tetrachloro-1,2-benzosemiquinone and tetrachloro-1,4-benzosemiquinone also react with cysteinyl residues.

Lin et al. (1993, 1997, 1999) investigated the formation of adducts with liver protein due to PCP metabolism. The production of multi-S-substituted adducts of TCBQ, tetrachloro-1,2-benzosemiquinone and tetrachloro-1,4-benzosemiquinone were studied by analyzing adducts formed with liver cytosolic and nuclear proteins. Following PCP metabolism *in vivo* and *in vitro*, hepatic production of multi-S-substituted adducts of TCBQ, tetrachloro-1,2-benzosemiquinone and tetrachloro-1,4-benzosemiquinone were identified. Mono substituted TCBQ adducts accounted for a large fraction of adducts

present. Formation of multi-S-substituted adducts with protein as well as non-protein thiols was indicated by large quantities of di- and tri-substituted adducts after only 30 seconds with liver homogenate, which probably contained glutathione, where as di- and tri-substituted adducts were formed at a much slower rate with isolated protein containing no glutathione. This observation led the researchers to postulate that protein-protein cross-links could be formed *in vivo*. Tsai et al. (2001, 2002) found PCP metabolism in rat and mice livers resulted in the significantly (>98%) more covalent binding in liver nuclei protein than liver cytosol, hemoglobin and serum albumin. This suggests that PCP metabolism that leads to covalent binding to larger molecules preferential to smaller molecules.

Yo-Chan et al. (1997) administered PCP to rats to investigate the binding potential of PCP to cellular macromolecules and glutathione. Metabolites formed from PCP were able to bind to serum albumin and hepatic protein. Hepatic protein binding increased in relation to cytochrome P450 activity, while binding to serum albumin decreased. Binding to DNA and hemoglobin was not observed. TCBQ was determined to be one of the most reactive PCP metabolites, rapidly forming conjugates with glutathione. These researchers concluded that cytochrome P450 metabolized PCP into reactive metabolites that rapidly form conjugates with neighboring proteins as well as non-protein sulfhydryl. This binding seems to occur prior to binding with DNA or hemoglobin.

Plant material is also a site of binding for toxic chemicals such as PCP. An investigation to study metabolites of PCP and the covalent binding in cell suspension cultures of wheat

was undertaken by Schafer & Sandermann (1988). By studying the incorporation of metabolized PCP into the insoluble residue fraction, they determined that the lignin cell wall is the main site of covalent binding and the primary metabolite is tetrachlorocatechol, the main metabolite found to bind to protein. Whole plants reported to incorporate PCP or its metabolites are rice, corn, wheat, alfalfa and rye. In whole wheat and wheat cultures, binding occurs through tetrachlorocatechol, where in rice binding occurs through tetrachlorocatechol and tetrachlorobenzoquinone.

Webb et al (2001) isolated *Saccharomonospora viridis* strains from mushroom compost that exhibited the ability to degrade PCP. Using shake flask cultures, these researchers found that *S. viridis,* a thermotolerant/thermophilic actinomycete, degraded PCP to undetectable levels. PCP degradation by S. viridis was unique in that it produced no partially dechlorinated phenols and chloroanisoles that were extractable by non-polar solvents such as n-hexane. These researchers determined, by following radiolabelled PCP, that 10% of the original PCP remained in the biomass while 88% was found in the supernatant, of which, all remained in the aqueous phase after solvent extraction of the supernatant. By using alkaline hydrolysis to release PCP transformation products remaining the aqueous phase, Webb et al liberated TCHQ and found TCHQ formation coincided with the disappearance of PCP. The highest concentration of TCHQ found corresponded with undetectable concentrations of PCP. When these researchers added a protein synthesis inhibitor to the culture prior to PCP, no PCP was removed. When the protein synthesis inhibitor was added to a PCP-metabolizing culture, PCP removal

ceased. PCP removal was determined to be growth dependent and required continuous protein production.

MATERIALS AND METHODS

Bioreactor System

The bioreactor system was a two-stage system consisting of two multiphase bioreactors. The first stage reactor was a three phase fluidized bed fungal reactor with a working volume of 600 ml which utilizes polyurethane immobilized *T. versicolor*. The fungal reactor was aerated and fluidized by flowing compressed breathing air (BOC Gases) through an in-line air sterilizing membrane filter (pore size 0.2μm, Millex-FG_{50}, Millipore) and a glass dispersion tube (Ace Glass). A constant flow of feed solution was fed through an in-line micro-filter (pore size 0.2μm, Dominick) to ensure the feed solution was free of contaminants. The reactor solution was constantly withdrawn from the fungal reactor and passed through a hold-up basin, a 250 ml polyethylene sample container with 100 ml working volume, where biomass and insoluble high molecular weight compounds precipitate out of the extracellular fluid prior to being fed to the second stage reactor. The second stage reactor was an enzyme reactor with a working volume of 1000ml. The enzyme reactor was constantly stirred and aerated by flowing compressed breathing air (BOC Gases) through an in-line air sterilizing membrane filter (pore size 0.2μm, Millex-FG_{50}, Millipore) and a glass dispersion tube (Kimble) to provide the necessary oxygen for the enzymatic reactions. Fresh make-up feed to the enzyme reactor was fed through an in-line micro-filter (pore size 0.2μm, Dominick) to

ensure the feed solution was free of contaminants. Enzyme reactor product was continuously withdrawn and held in a 1gallon storage container until full, then stored at 4^{o}C until processed and analyzed. The design of each reactor was modified by adding an air venting system, consisting of a glass tube, silicon tubing and a sterile air venting filter (pore size 0.2μm, Millex-FG_{50}, Millipore), to prevent contamination of the reactors. All materials used in the reactor systems were autoclaved at 121^{o}C for 20 min prior to use. The first stage fluidized bed fungal reactor system has been successfully used in a number of studies dealing with bleach plant effluent detoxification and decolorization by Pallerla and Chambers (1996,1997ab, 1998) and Samojedny (2000).

Feed Composition

A 10 mg/ml stock solution of PCP was prepared by dissolving PCP (Sigma) in ethanol. The feed to the PCP degrading fungal reactor was a solution of distilled water with PCP added to a final concentration of 2.5 mg/L. The feed solutions to the fungal reactors consisted of 0.6% dextrose, 0.16% $(NH_4)_2SO_4$, 0.3% K_2HPO_4, and 0.2% KH_2PO_4 (w/v) to provide carbon, nitrogen and phosphorus (Bergbauer et. al., 1991). A vitamin supplement final concentration was 0.2 mg/L each of D-pantothetic acid, riboflavin, folic acid, pyridoxal, thiamine-HCl, nicotinic acid and biotin was added to fungal feed solutions. Similarly, a mineral salt supplement was added to the final concentrations of 0.1 μM $NiCl_2$, 5μM $CoCl_2$, 20 μM $MnCl_2$, 5μM $ZnCl_2$, 1 μM $CuSO_4$, 40 μM Na-citrate, 20 μM $FeSO_4$, and 0.5 μM $(NH_4)_6Mo_7O_{24}$. The pH of the fungal feed reactor solution was adjusted to pH 5 with HCl.

The make-up feed to the PCP degrading enzyme reactor was a solution of distilled water with PCP added to a final reactor inlet concentration of 2.5 mg/L. The makeup feed solution to the enzyme reactors consisted of 0.16% $(NH_4)_2SO_4$, 0.3% K_2HPO_4, and 0.2% KH_2PO_4 (w/v). The pH of the makeup feed solution was adjusted to pH 3.5 with HCl.

Fungal growth

Trametes versicolor (ATCC 20869), the white-rot fungus used in this investigation, was obtained from the National Forest Products Laboratory in Wisconsin every six to twelve months. Cultures of *T. versicolor* were prepared by transferring the fungus to malt agar Petri dishes. The fungus was allowed to grow at 75^{o}F for 5-7 days. Mycelia pellets were obtained by inoculating six 250ml flasks, each containing 75ml growth medium, with 3-4 mycelia discs from actively growing fungal dishes. The growth medium was prepared by dissolving dextrose (40 g/L) and Difco Bacto-soytone agar (10 g/L) in distilled water. The pH of the growth medium was adjusted to 5.0 with 1M HCl. The flasks, stoppers, spatula used for transfer, and growth medium were all autoclaved at 121^{o}C for 20min. prior to use. The inoculated flasks were oxygenated for 1 min. with pure oxygen every twenty-four hours and continuously agitated in a rotary shaking incubator at 180-200 rpm. After 5 days, the mycelia pellets were 3-4 mm diameter and were filtered from the growth media by vacuum filtration and washed with distilled water.

Immobilization of mycelia pellets

Filtered and washed mycelia pellets were placed in a Waring blender along with distilled water and fragmented by pulsing the blender 5 times for 5 seconds each time, resulting in

a fungal slurry. The fungus was immobilized by vigorously mixing the fungal slurry (approx. 100 ml) with the polyurethane prepolymer (HYPOL 2000, Hampshire Chemical Corporation) at a 1:1 ratio and 2% (v/v) surfactant (DABCO DC193, Air Products & Chemical, Inc.) in a polypropylene beaker for 1 min. The foam was spread onto Parafilm in a thin layer and allowed to dry for 30 min. at room temperature. The foam was cut into cubes (4x4x4mm) and placed in 1000 ml volumetric cylinder to the volume mark of 200 ml and 500 ml of growth medium was added. The growth medium was prepared by dissolving dextrose (4 g/L) and Difco Bacto-soytone agar (1 g/L) in distilled water. The pH of the growth medium was adjusted to 4.8 with 1M HCl. The growth medium and all other materials used were all autoclaved at 121^{o}C for 20min. prior to use. The cylinder, containing the fungal cubes and growth media, was aerated to facilitate growth and mixing. The cubes were allowed to grow for 48 hours to insure a viable immobilized culture prior to exposure to the feed solution.

Extraction of Unbound PCP and Metabolites

Samples (5ml) were removed from the fungal reactors using a syringe and from the enzyme reactors using a glass pipet and placed in 25 ml volumetric flasks. To samples of either fungal reactor fluid or enzyme reactor fluid, 1 ml of 1M ascorbic acid and 25 μl of 10 mg/ml tribromophenol was added as an internal standard for gas chromatography. The sample pH was adjusted to 2.5 with 2.5 M HCl and extracted twice with 5 ml diethyl ether to remove nonconvalently bound molecules. The ether layers were combined and subjected to the derivatization procedure described below.

Cleavage of Protein Bound Metabolites

The method of Raney ® nickel cleavage of protein adducts was adapted from methods of McDonald et. al. (1993) and Waidyanatha et. al. (1994) for the measurement of hemoglobin and albumin adducts of benzoquinone and tetrachlorobenzoquinone. This method involves protein digestion, followed by solvent extraction to remove compounds that are not sulfur bound to protein amino acids. Raney ® nickel, a nickel-aluminum reducing catalyst, is then used to catalyze cleavage of carbon-sulfur bonds. This releases sulfur bound PCP, TCHQ and TCBQ. Ascorbic acid is used at several points in the procedure to maintain an acidic reaction environment and to act as a reducing agent to keep cleavage products in reduced form. Samples (5ml) were removed from the fungal reactors using a syringe and from the enzyme reactors using a glass pipet and placed in 25 ml volumetric flasks. To samples of either fungal reactor fluid or enzyme reactor fluid, 1.5 ml of 1M ascorbic acid and 1.5 ml of 1M 1, 3-bis[*tris*(hydroxymethyl)-methylamino]propane buffer (pH 7.0) were added and the pH was adjusted to 7 with 2.5 M HCl. The protein was digested by adding 10µl Pronase XIV (Pronase E, 5mg/ml) and heated at 37^{o}C for 4 hours with constant stirring. After protein digestion, 1.5 ml of 1M ascorbic acid in 1mM diethylenetriaminepentaacetic acid was added and the pH was adjusted to 1-2 with HCl. The sample was extracted twice with 10 ml diethyl ether to remove lipophilic and nonconvalently bound molecules. To the protein aqueous layer, 25 ml of 10 mg/ml tribromophenol was added as an internal standard. The sample was reacted with Raney® nickel for 10 min at 23^{o}C in a rotary shaker then centrifuged to stop the reaction and remove the Raney® nickel. The supernatant was transferred to a clean vial; the pH was adjusted to 2.5 and was extracted twice with 10 ml diethyl ether. The

ether layers were combined and subjected to the derivatization procedure described below.

Derivatization of PCP and Metabolites

The method of heptafluorobutyrylimidazole (HFBI) was adapted from the methods of McDonald et al. (1993) and Waidyanatha et al. (1994) for the measurement of hemoglobin and albumin adducts of benzoquinone and tetrachlorobenzoquinone. Reaction of quinones with HFBI adds a heptafluoroacyl group to the quinone, increasing the volatility and sensitivity for detection by gas chromotagrahy. Due to the sensitivity of the HFBI derivation technique, all glassware used for derivatization was washed by ultrasonication for 30min and rinsed with distilled water followed by HPLC grade acetone and dried at 105^{o}C for at least one hour (Niss & Poulson, 1988). Ether layers were evaporated under nitrogen to approximately 2 ml and treated with anhydrous Na_2SO_4 to remove any residual moisture; the dried ether was transferred to a clean 3 ml reaction vial and evaporated to complete dryness under nitrogen. Adding 100µl HFBI to the sample, capping vial tightly and heating at 85^{o}C for 1 hour completed the derivatization. Derivatized samples were cooled to room temperature and 1 ml of hexane was added. Derivatized samples were stored at minus 4^{o}C until ready for analysis.

Gas Chromatography Analysis

The concentration of PCP and TCHQ in samples was measured by gas chromatography. A DB-5 fused silica capillary column (length, 15 m; ID,0.25 mm; film thickness, 0.25 µm, J&W) was installed on a Varian Model 3400 gas chromatograph equipped with a

electron capture detector. Split injection (1:100) was used and the carrier gas was nitrogen. For analysis of derivatized PCP and TCHQ the injector temperature was set at 270^{o}C, the detector temperature was set at 320^{o}C. The column temperature programs for analysis of PCP and TCHQ were identical. The column was held at 45^{o}C for 1 min, then ramped to 150^{o}C at 10^{o}C/min, held at 150^{o}C for 4 min, ramped to 230^{o}C at 20^{o}C/min and held at 230^{o}C for 5 min. to remove any late eluted compounds. To maintain GC/ECD performance, solvent was injected between each sample run. During the solvent run the injector was set at 270^{o}C, the detector temperature was set at 330^{o}C, and the column was held at 45^{o}C for 1 min and ramped to 250^{o}C at 20^{o}C/min and held at 250^{o}C for 5 min. Prior to analysis by GC/ECD samples were brought to room temperature and washed three times with 0.5 ml distilled water to remove any unreacted HFBI. The hexane layer was removed and placed in a clean, dry sample vial. The volume of the sample hexane layer injected was 0.5 µl.

Protein Measurement

Protein concentration present in the reactors was determined using the Modified Lowry's Protein Measurement Kit (Sigma). The Lowry Reagent solution was prepared by adding 40 ml distilled water to a bottle of Lowry Reagent and inverted to mix. The Lowry Reagent solution was stored at room temperature. The Folin and Ciocalten's Phenol Reagent Working solution was prepared by emptying a bottle of Folin and Ciocalten's Phenol Reagent into the supplied amber glass bottle. The empty bottle of Folin and Ciocalten's Phenol Reagent was rinsed with 10 ml distilled water and the rinse added to the amber glass bottle. An additional 80 ml distilled water was added to the amber bottle

and the solution was mixed well. The Folin and Ciocalten's Phenol Reagent Working solution was stored at room temperature. A 400μg/ml protein standard solution was prepared by adding 5 ml distilled water to the supplied protein vial and the vial was gently swirled to dissolve the protein. The protein standard solution was stored at 4^{o}C up to 3 months.

The Micro Lowry, Peterson's Modification procedure was followed to determine protein concentration. Protein standard solutions were prepared of the following concentrations; 0, 50, 100, 200, 300 and 400 μg/ml. To an appropriately labeled test tube, 1 ml of sample or protein standard solution and 1 ml of Lowry's Reagent solution were added and the test tubes were vortexed and allowed to stand at room temperature for 20 min. Folin and Ciocalten's Phenol Reagent Working solution (0.5ml) was added to each test tube and the test tubes were immediately vortexed after addition and allowed to stand at room temperature for 30 min. The solutions were transferred to clean disposable cuvettes and the absorbance was measured at 750nm. A standard curve was obtained by plotting absorbance (nm) versus standard protein concentration (μg/ml). Protein concentration was determined from the standard curve.

Experimental Approach

An in-depth study was conducted to determine the reactive intermediate formed from PCP during fungal and enzymatic treatment using *T. versicolor*, as well as the role of protein binding in PCP bioremediation from aqueous solution. Products formed by *T. versicolor* fungal and enzymatic treatment of PCP contaminated aqueous streams were

studied by running the fungal and enzyme reactor at 24 hr residence times continuously for 7 weeks. The concentrations of PCP and TCHQ in the fungal reactor (FR) and the continuous extracellular fluid reactor (CEFR) were determined by GC/ECD. Protein bound PCP and TCHQ in the FR and CEFR were also determined, following the Raney Nickel procedure and measured by GC/ECD. Reactor pH and protein concentration were measured for each sample taken.

RESULTS AND DISCUSSION

Reactor Environmental Characteristics

The first samples were taken on the fifth day of operation and the reactor ran for 50 days, on which day the last sample was taken. Reactor pH in both the continuous fungal and extracellular fluid reactors took two weeks to stabilize. The FR pH was approximately 3.9 during the reactor operation, shown in Figure 14A and Table 11. The CEFR pH was approximately 3.7 during the reactor operation, shown in Figure 14B and Table 11. The pH in each reactor remained within the pH range determined to be optimum in previous studies.

In general, protein concentration in the FR increased steadily over time. On the fifth day of bioreactor operation, the protein concentration in the FR was 33.2μg/ml. Protein concentration increased at a rate of 2.6μg/ml/d up to day 28 on which the protein concentration was 94.1μg/ml. Between day 36 and day 37, the fresh feed to the FR was stopped for several hours. On the 37th day, the protein concentration was measured as

357 μg/ml before fresh feed was resumed, a 280% increase from the concentration on day 28. Protein built up was due to active fungal protein production, without removal in form of exit flow, since the flow rate of fresh feed is equal to the exit flow rate. On day 38, after flow of fresh feed the fungal reactor resumed, the protein concentration was measured as 125μg/ml. This concentration is in relation to the trend of protein concentration increase over time, excluding the sharp increase in protein concentration on day 37, of 3.6μg/ml/d. When flow was restored to the reactor, the excess protein was able to wash out of the reactor, returning the protein concentration to a quasi-steady state concentration. Protein concentration in the FR during reactor operation is shown in Figure 15A. The overall increase in protein in the FR could be due to accumulation of protein. The fungus produced more extracellular protein than was removed from the reactor. During the time the fresh feed to the FR was halted for several hours, it is possible that some of the fungal biomass died during this period and was released into the extracellular fluid, adding to the amount of measured protein.

Protein concentration in the CEFR increased steadily over time at an overall rate of 0.6μg/ml/d. On day 5 of reactor operation, the protein concentration was measured as 23.7μg/ml. A sharp increase in protein concentration was measured on day 38 of 57.8μg/ml, up from 43μg/ml on day 37. On day 37 the protein concentration in the FR increased significantly and the sharp rise in protein in the CEFR followed the significant increase in protein concentration in the FR. By day 42, the protein concentration in the CEFR resumed to a quasi-steady state concentration of 40.9μg/ml. Protein concentration in the FR during reactor operation is shown in Figure 15B. If the concentration of protein

in the extracellular fluid fed to the CEFR was the same as that in the FR then, based on the flow rate ratio of extracellular fluid to fresh PCP solution fed to the CEFR of 2 to 1 the concentration of protein in the CEFR should be 66% of that in the FR. During the first two weeks of reactor operation, the concentration of protein in the CEFR was approximately 66% of that in the fungal reactor. However, from the third week on the concentration of protein in the CEFR was only about one-quarter of that in the FR. This suggests that a large portion of the protein measured in the FR precipitated out in the hold-up basin. This portion could be biomass, supporting the theory that the extracellular fluid fed to the CEFR contains enzyme, not residual biomass material.

PCP Removal in the FR and CEFR

PCP was used to induce extracellular enzyme production in the fungal reactor. The immobilized fungus degraded PCP by action of intracellular enzymes as well as extracellular enzymes. The concentration of PCP in the FR rapidly decreased from the concentration in the feed of 2.5ppm down to 0.013ppm on the fifth day of reactor operation, shown in Table 11. After the pH stabilized the concentration of PCP during reactor operation, shown in Figure 16A, ranged between 0.041ppm and 0.296, with the average concentration of PCP being 0.178ppm. During bioreactor operation, the PCP removal in the fungal reactor was 88% to 99.5% as shown in Figure 16B and Table 12. Pallerla and Chambers (1998) also achieved high removal rates over 99% in a fungal reactor utilizing polyurethane immobilized *T. versicolor* with initial PCP concentrations as high as 25ppm.

Fresh PCP solution and extracellular fluid produced in the FR was fed to the CEFR. On the fifth day of reactor operation, the concentration of PCP was 0.0095ppm in the CEFR. The concentration of PCP in the CEFR increased at a steady rate of approximately 0.0054ppm/day to the highest concentration measured on day 49 of reactor operation of 0.28ppm, as shown in Figure PCP 17A and Table 11. During CEFR operation the average PCP concentration was 0.173ppm. Reduction of PCP in the total feed remained between 89% and 99.6% as shown in Figure 17B and Table 12.

Reactive Intermediate Identification

Tetrachlorobenzoquinone (TCBQ) has been shown to be a metabolite of PCP in biological systems (Renner & Hopfer, 1990; Reigner et al, 1990, 1991), a degradation product of PCP by enzymatic systems (Chung & Aust, 1995, Reddy & Gold, 2000) and an oxidative product in other oxidation processes (Fang et al., 1998, Ho & Bolton, 1998). TCBQ was suspected to be the reactive intermediate formed by treatment of PCP with extracellular fluid produced by *T. versicolor*. Reduction of TCBQ yields TCHQ, which could occur due to the acidic reactor environment or by the addition of a reducing agent. In these studies, the antioxidant ascorbic acid was added to reactor samples to reduce TCBQ to TCHQ and to maintain the compound in reduced form. Therefore, all values given for TCHQ express the total concentration of TCBQ and TCHQ present in the samples.

Concentrations of TCHQ in the FR, shown in Figure 18A and Table 11, remained between 0.009ppm and 0.032ppm, with the average concentration being 0.02ppm. The

concentration of TCHQ in the FR did not appear to be immediately affected by the dramatic increase in protein concentration on the thirty-seventh day of operation when the concentration was 0.02ppm, however on the forty-second day the concentration of TCHQ reached its highest level of 0.032ppm. By the last day of FR operation the TCHQ concentration returned close to the average value. The low concentration of TCHQ in the FR is expected due to the highly reactive nature of this compound.

In the CEFR, the concentration of TCHQ, shown in Figure 18B and Table 11, remained between 0.012ppm and 0.018ppm throughout reactor life, with the average concentration being 0.015ppm. Interestingly, plotting the percent change in TCHQ from the amount in the FR to the concentration in the CEFR against time, shown in Figure 19, indicates that the concentration of unbound TCHQ in the CEFR was often less than that in the FR, on average 26% less. On the fifth day of reactor operation, the concentration of unbound TCHQ in the CEFR was 25.5% less than that in the FR. During weeks 2, 3 and 4 of reactor operation, the concentrations of unbound TCHQ were higher in the CEFR than in the FR, 10%, 37% and 1% respectively. In weeks 5, 6, and 7 of reactor operation, the concentrations were 7.9%, 51.3%, and 46.2% less than that in the FR respectively. This suggests that while the concentration of unbound TCHQ in the FR changed during reactor operation, the CEFR was able to maintain an essentially constant concentration of TCHQ regardless of inlet concentration of unbound TCHQ.

Effect of Incubation Temperature on TCHQ concentration

Shown in Table 13, the incubation temperature of the fungal agar plates had no effect on the concentration of TCHQ in the FR and the CEFR. The reactor concentration of TCHQ remained approximately the same in the FR and the CEFR and at the two different incubation temperatures, reiterating the highly reactive nature of this compound.

Dependence of Remediation on Reactor Protein Concentration

PCP in the FR exhibited a slight dependence on protein concentration, shown in Figure 20A. At protein concentrations less than 100μg/ml, the concentration of PCP in the FR remained between 0.012ppm and 0.16ppm, which coincides with 94%-99.5% reduction in PCP, exhibited in Figure 20B. At protein concentrations 170mg/ml and higher, the concentration of PCP remained between 0.22ppm and 0.3ppm, coinciding with reduction of PCP between 88.2% and 91.3%. The higher concentrations of protein occurred after the sharp increase in protein on day 37. Since the additional protein could be due to residual biomass in the extracellular fluid, then lower removal rates are likely due to lower levels of active biomass present in the reactor. In the CEFR, where protein concentration did not vary as greatly as in the fungal reactor, a stronger dependence of PCP on protein was exhibited, shown in Figure 21A. Overall, the relationship between PCP and protein concentration was 0.0075mg PCP/mg protein. At the lowest protein concentration, 19μg/ml, PCP in the fungal reactor was reduced by 95.8%, shown in Figure 21B. The greatest reduction of PCP in the CEFR of 99.6% occurred at a protein concentration of 24μg/ml. The lowest reduction of 89% occurred at the highest protein concentration of 44μg/ml. Stronger dependence of PCP removal on protein

concentration in the CEFR supports the concept that residual biomass is not present in the CEFR and the protein concentration in the CEFR represents active extracellular protein then in the FR. In future experimental work, it is planned to monitor the amount of extracellular enzymatic activity in the FR, CEFR as well as the settling basin during reactor operation

The concentration of TCHQ with respect to protein concentration in the FR is shown in Figure PCP 22A and the concentration of TCHQ with respect to protein concentration in the CEFR is shown in Figure PCP 22B. Unlike PCP the concentration of TCHQ in the bioreactors exhibited no dependence on protein concentration.

Based on the concentration of PCP in the feed to the FR of 2.5ppm, the measured concentration of PCP and protein concentration, removal of PCP in the FR were determined as mg PCP removed / mg protein and shown in Figure 23A and Table 12, with respect to time. At lower reactor protein concentration in the FR, PCP removal averaged 0.045mg PCP removed / mg protein. When the protein concentration rose significantly on day 37 PCP removal decreased sharply to 0.006 then remained at 0.013 mg PCP removed /mg protein throughout the duration of reactor operation. PCP removal in the CEFR, shown in Figure 23B with respect to time, were based on total flow rate of unbound PCP in the total feed to the CEFR, concentration of unbound PCP and protein concentration in the CEFR. The total flow rate of unbound PCP included the flow rate of PCP in the extracellular fluid and the flow rate of PCP in the fresh feed solution. The removal rate of PCP in the CEFR varied during operation, averaging 0.8mg PCP

removed/mg protein. The removal rate of PCP in the CEFR followed a inverse relationship with protein, following a similar trend as that in the FR, however the dependence was stronger in the CEFR.

Protein binding of PCP and TCHQ in the FR and CEFR

Protein binding of PCP and TCHQ has been well documented to occur in biological systems (Akitake & Kobayashi, 1975, Van Ommen et al., 1988, Waidyanatha et al., 1994, 1996, & Tsai et al., 2001, 2002) and in plant material (Schafer & Sandermann, 1988). In biological systems, protein binding of chlorinated compounds has adverse effects (Van Ommen et al., 1986). Utilizing protein binding of PCP and TCHQ in bioremediation could be advantageous in removing these compounds. To study what role protein binding has in this fungal bioremediation system the concentrations of protein bound PCP and TCHQ were determined using the Raney Nickel sulfur bound cleavage method. This method selectively cleaves sulfur bounds, releasing PCP and TCHQ.

The concentration of apparent protein bound PCP in the FR with respect to time is shown in Figure 24A and Table 14. During the first three weeks of bioreactor operation, the concentration of apparent protein bound PCP remained relatively constant, between 0.01ppm and 0.014ppm, averaging 0.012ppm. On day 28, the concentration of apparent protein bound PCP rose sharply to 0.083ppm, a 590% increase from the average concentration measured the first 3 weeks of operation. On day 38, one day after the protein concentration sharply increased in the fungal reactor, the concentration of apparent protein bound PCP was 0.028ppm, a 133% increase from the average

concentration measured the first 3 weeks of operation. For the duration of reactor operation the apparent protein bound PCP concentration remained between 0.023ppm and 0.029ppm. The concentration of apparent protein bound PCP in the CEFR with respect to time is shown in Figure PCP 24B and Table 14. During the first three weeks of bioreactor operation, the concentration of apparent protein bound PCP remained relatively constant, between 0.01ppm and 0.015ppm. On day 28, the concentration of apparent protein bound PCP rose markedly to 0.055ppm, a 267% increase from the concentration measured on day 21. The concentration of apparent protein bound PCP decreased for the duration of operation. The concentrations of apparent protein bound PCP in the two reactors were comparable during reactor operation. For the first three weeks of reactor operation, the concentrations were about equal. In the fourth week, when a disturbance in flow occurred, the concentrations in both reactors increased significantly, then resumed to similar values by the middle of the fifth week. In the final weeks of operation, the concentration of bound PCP in the CEFR was 24%- 40% of that in the FR, and resumed to concentrations measured in the first three weeks. These findings are most likely due to protein build up in the FR, making more protein available for binding, whereas protein concentration in the CEFR slightly increased over time, keeping the level of protein available throughout reactor operation relatively constant.

Concentration of apparent protein bound TCHQ in the FR with respect to time is shown in Figure 25A and Table 14. The concentration of apparent protein bound TCHQ in the fungal reactor fluctuated remaining between 0.0008ppm and 0.0029ppm, averaging 0.0015ppm.

The concentration of apparent protein bound TCHQ in the CEFR with respect to time is shown in Figure PCP 25B and Table 14. In the CEFR, the concentration of apparent protein bound TCHQ fluctuated between the low concentration of 0.0006ppm and the high concentration of 0.0036ppm, averaging 0.0017ppm. The concentrations of apparent protein bound TCHQ in the FR and CEFR were about the same during the first three weeks of reactor operation. In the fourth week, when flow disturbance occurred, the concentration in the FR was twice that as in the CEFR. During the weeks of operation after the increase in protein the concentration of apparent protein bound TCHQ was higher in the CEFR than in the FR.

After the sharp increase in protein concentration occurred in the FR, the concentration of apparent protein bound TCHQ in the CEFR was higher than in the FR, opposite of apparent protein bound PCP in the reactors. Enzymatic activity may play a greater role in forming TCHQ protein conjugates than PCP protein conjugates. Apparent protein binding of PCP in both reactors over time was greater than that of TCHQ. In the FR, the concentration of bound TCHQ was 2%-20% of that of bound PCP. In the CEFR, the concentration of bound TCHQ was 2%-29% of that of bound PCP. This suggests that PCP binding may occur at a faster rate than the enzymatic formation of TCHQ or the apparent protein binding of TCHQ and that TCHQ may be reacting with other components in the reactors.

Studies carried out in most biological systems found that while PCP did form conjugates with sulfur the majority of sulfur binding in systems involving PCP, as the initial compound or as a metabolites, results from binding with TCHQ (Van Ommen et al., 1986; de Besten et al., 1989; Reigner et al., 1990, 1991; Waidyanatha et al., 1994, 1996; Lin et al., 1993, 1997, 1999). However one of the simpler biological systems studied, the goldfish, resulted in substantial levels of PCP conjugation with sulfur containing compounds (Akitake & Kobayashi, 1975). Fungal remediation in this reaction system seems to favor binding of PCP to sulfur containing compounds and other mechanisms of removal.

Dependence of Reactor Protein Concentration on PCP and TCHQ Protein Binding

Protein bound PCP in the FR with respect to protein concentration is shown in Figure PCP 26A. Protein bound PCP showed a positive relationship with protein concentration. At protein concentrations between 46μg/ml and 70μg/ml, the concentration of protein bound PCP remained between 0.01ppm and 0.014ppm. At higher protein concentrations between 125μg/ml and 199μg/ml the concentration of protein bound PCP remained between 0.023ppm and 0.029ppm. The sharp increase in protein bound PCP occurred at a protein concentration of 94μg/ml. Protein bound PCP in the CEFR with respect to protein concentration is shown in Figure PCP 26B. Protein bound PCP in the CEFR did not exhibit any relationship with protein concentration.

Protein bound TCHQ in the FR, at protein concentrations 45-94μg/ml, exhibited no correlation with protein concentration, as shown in Figure 27A. At protein

concentrations above 94μg/ml, the concentration of apparent protein bound TCHQ followed a downward trend with increasing protein concentration. Protein bound TCHQ in the CEFR generally increased with protein concentration, shown in Figure 27B. Protein binding of PCP seems to be much more dependent on protein concentration rather than enzymatic activity, whereas protein binding of TCHQ seems to be more dependent on enzymatic activity rather than protein concentration. This can be expected since TCHQ is formed from enzymatic action on PCP resulting in protein binding and protein binding of PCP depends more on reactor environment, such as pH and available protein. The different types and sizes of the protein present in the FR and CEFR probably play a large role in apparent binding. Tsai et al. (2001, 2002) conducted experiments, which suggest covalent binding due to PCP metabolism is preferential towards larger protein molecules. The FR definitely contains higher molecular weight material, including protein, than the CEFR, therefore the CEFR contains a type of protein more likely to be involved in covalent binding due to PCP metabolism.

Effect of incubation temperature on protein binding of PCP and TCHQ in the FR and CEFR

The concentration of protein bound PCP in the FR and CEFR were significantly higher, about three times, when the fungal plates were incubated at the higher incubation temperature of 85^{o}F, shown in Table 15. Protein binding of TCHQ in the FR was twice as high when the fungal plates were incubated at 85^{o}F, however incubation temperature did not have an effect on the concentration of apparent protein bound TCHQ in the CEFR, shown in Table 16. Since protein binding of TCHQ and PCP to protein seems to

be strongly dependent on the amount of reactor protein these results suggest that the higher incubation temperature results in a fungal culture that is more active and produces more protein. Increased protein results in more removal of PCP, which could explain why the reactor concentration of PCP was lower with fungus incubated at 85^{o}F.

Ratios of unbound compounds to protein bound compounds

Comparing the concentration of unbound compounds to protein bound compounds gives an indication of how much sulfur binding plays a role in removing PCP and TCHQ from solution. Figure 28A and Table 17 show the ratio of PCP to protein bound PCP in the FR with respect to time. The ratio of PCP to protein bound PCP in the FR fluctuated during operation, remaining between 1.1 and 15, averaging 6.9. Figure 28B and Table 17show the ratio of PCP to protein bound PCP in the CEFR with respect to time. In general, the ratio of PCP to protein bound PCP increased over time in the FR, averaging 12.3. This indicates that protein binding plays a significant role in PCP removal since over time PCP removal decreases. The ratio of PCP to protein bound PCP in the CEFR was approximately twice that as in the FR, supporting the hypothesis that protein present in the reactor may play a much greater role in protein binding of PCP than enzymatic activity.

Figure 29A and Table 17 show the ratio of TCHQ to protein bound TCHQ in the FR with respect to time. The ratio of TCHQ to protein bound TCHQ in the FR changed over time in an oscillatory fashion, remaining between 6.7 and 17.3, averaging 11.2. The ratio of TCHQ to protein bound TCHQ in the CEFR remained between 5.0 and 27.2, averaging

13, but varied significantly during reactor operation, shown in Figure 29B. Since the ratio of unbound TCHQ to protein bound TCHQ does not follow any significant trend overtime, protein binding TCHQ is likely dependent on reactive compound formation. Generally, the ratio of TCHQ to protein bound TCHQ was higher in the FR than in the CEFR, indicating that binding is more prevalent in the CEFR, supporting dependence on enzymatic activity for TCHQ protein binding.

Effect of Incubation Temperature on Ratio of Unbound Compounds to Protein Bound Compounds in the FR and CEFR

When the fungal plates were incubated at the lower temperature the average ratios of PCP to protein bound PCP in the FR and CEFR was markedly higher then when incubated at the higher temperature, shown in Table 18. These findings are a result of the higher concentrations of reactor PCP and the lower concentrations of protein bound PCP when the fungal plates were incubated at 75^{o}F. The ratios of TCHQ to protein bound TCHQ varied dramatically in the FR and CEFR when the fungal plates were incubated at the lower temperature. On average the ratios of TCHQ to protein bound TCHQ were lower when the fungal plates were incubated at the higher temperature. In the FR these results are dependent on the higher concentrations of protein bound TCHQ when using fungal plates incubated at 85^{o}F. In the CEFR, the average ratio excluding the highest point of 317 results in an average ratio that is slightly lower when fungal plates were incubated at 75^{o}F. This finding is a result from the fact that the average concentration of reactor TCHQ and the average concentration of protein bound TCHQ was not affected by incubation temperature. Protein binding of TCHQ in the CEFR did not exhibit a

dependence on reactor protein or fungal incubation temperature, suggesting the reactive TCHQ follows a different reaction pathway, such as addition product formation or polymerization.

Fraction of Actively Bound Protein

Binding of PCP and TCHQ to the protein was investigated using the principles of the binding of drugs to protein (Scatchard, 1949; Klotz, 1971). Using the principles of Scatchard (1949) and Klotz (1971) depicting protein binding of drugs, it was concluded that the binding of PCP and TCHQ is sufficiently strong and that the amount of available active protein in equilibrium with the unbound and protein bound PCP and TCHQ is quite small. Therefore, PCP and TCHQ are strongly bound to the protein and binds to almost all of the available active protein binding sites. Based on these findings, the fractions of reactor protein involved in binding of PCP and TCHQ were determined using the following relationships:

$$\phi_{PCP} = PCP_{bound} / \text{Reactor Protein} \quad (1)$$

$$\phi_{TCHQ} = TCHQ_{bound} / \text{Reactor Protein} \quad (2)$$

$$\phi_{Total} = (PCP_{bound} + TCHQ_{bound}) / \text{Reactor Protein} \quad (3)$$

where:

ϕ_{PCP} = fraction of reactor protein bound to PCP

ϕ_{TCHQ}=fraction of reactor protein bound to TCHQ

ϕ_{Total}= fraction of reactor protein bound to PCP and TCHQ

PCP_{bound} = molar concentration of bound PCP

$TCHQ_{bound}$ = molar concentration of bound TCHQ

Reactor Protein = molar concentration of reactor protein assuming molecular weight of 100,000.

Prior to the disturbance in flow, resulting in an increased fungal reactor protein concentration, the fraction of protein involved in binding PCP and TCHQ was much higher than after the flow was resumed, shown in Table 19. The fraction of protein involved in binding PCP in the CEFR was also much higher prior to the flow disturbance than after the flow was resumed. The fraction of protein involved in binding of TCHQ remained essentially constant, but since ϕ_{PCP} is the dominant term, then ϕ_{Total} was significantly higher prior to the flow disturbance than after flow was resumed. The increase in protein likely resulted from decreased viability of the fungal cells immobilized in the polyurethane cubes and these cells were probably sloughed off of the immobilized cubes which resulted in higher protein content in the extracellular fluid. However, the excess protein did not bind PCP and TCHQ as well as the protein prior to the flow disruption. In addition to a large portion of the excess reactor protein present in the reactor due to the flow disturbance, active protein may also have been lost due to decreased production of active protein and interference of active protein by coagulation with inactive protein.

Remediation Fate of Initial PCP

T. versicolor, enzymes, and proteins act upon PCP. In these experiments, the concentrations of reactor PCP, reactor TCHQ, protein bound PCP, and protein bound TCHQ were determined. These components represent part of the reactions occurring in

the bioreactors. The mole percent of the PCP in the feed solution that is accounted for as unbound PCP, unbound TCHQ, protein bound PCP and protein bound TCHQ were determined. Initially, only a small percentage of PCP in feed to the fungal reactor was accounted for as unbound PCP, unbound TCHQ, protein bound PCP and protein bound TCHQ, shown in Figures 30A and 30B and Table 20. Over time the total mole % accounted for increased from 1.8% up to 13.4%. However the largest fraction of the total accounted for could be attributed to reactor PCP, which increased during reactor operation. The mole % of initial PCP accounted for as PCP increased from 0.5% to 12% during reactor operation. Reactor TCHQ in the FR was, on average 0.8% of the mole % of initial PCP accounted for. The mole % of initial PCP accounted for as protein bound PCP averaged 1.1%, ranging between 0.4% and 3.3%. Protein bound TCHQ accounted for very small fraction of feed PCP, ranging from 0.034% to 0.12%, averaging 0.065%. During the operation of the FR, the fraction of initial PCP accounted for as unbound and protein bound PCP and TCHQ increased, with unbound PCP responsible for the largest fraction. Bound PCP and TCHQ, and unbound TCHQ accounted for less than 1.5% of initial PCP. Because of the presence of the fungal material in the FR, intracellular reaction of PCP to non-chlorinated or lesser chlorinated compounds is believed to be responsible for the largest fraction of PCP removal in the FR.

Initially only a small percentage of PCP in feed to the CEFR was accounted for as PCP, TCHQ, apparent protein bound PCP and apparent protein bound TCHQ, shown in Figure 31A, 31B and Table 21. The total mole % of initial PCP accounted for in the CEFR increased from 1.5% to 11.5% during reactor operation, averaging 7.1%. The mole % of

initial PCP accounted for as PCP in the CEFR steadily increased throughout the duration of reactor operation to 10.5%. Reactor PCP mole % is the largest fraction of the total mole % accounted for. The mole% of initial PCP accounted for as TCHQ remained relatively constant, ranging from 0.66% to 0.74%, averaging 0.70%. From week 1 to 3, unbound TCHQ was second largest fraction of total PCP feed. Protein bound PCP accounted for 0.8% of initial PCP on average, ranging from 0.4 to 2.14%. Protein bound TCHQ accounted for very small fraction of feed PCP, ranging from 0.027% to 0.15%, averaging 0.069%. In the CEFR, over time, as in the FR, a larger fraction of the initial PCP was accounted for as unbound and apparent protein bound PCP and TCHQ, mostly as unbound PCP. The protein bound components, PCP and TCHQ and the reactive component, unbound TCHQ accounted for about 1% of the initial PCP. These results indicate that which protein binding plays a role in PCP removal, a large fraction of PCP is removed from solution by some other mechanism. One such mechanism, discussed in the previous chapter, is polymerization. These results support the conclusions reached that the concentration of the compound identified by FTIR, most likely an addition, oligomer or polymer product, is much greater than PCP or TCHQ in the CEFR. These results also indicate that addition and polymerization reactions occur at a greater rate than apparent protein binding.

CONCLUSIONS

Bioremediation of PCP by *Trametes versicolor* and its extracellular fluid results in over 99% remediation of PCP with the formation of reactive compounds TCBQ and TCHQ and protein binding of PCP and TCHQ. The concentration of TCHQ in the CEFR remained very low and relatively constant during operation, independent of feed PCP concentration, feed TCHQ concentration, reactor protein or fungal plate incubation temperature. PCP binds to protein at a higher concentration than TCHQ and exhibits a strong dependence on reactor protein concentration. Incubation temperature of the fungal plates had a significant effect on protein binding of PCP in the FR and CEFR and a lesser effect on protein binding of TCHQ. Binding of PCP to protein was about three times higher in the FR and CEFR when fungal plates where incubated at 85^{o}F compared to 75^{o}F and binding of TCHQ in the FR was about twice as high, with no change in the CEFR. The binding parameters of PCP and TCHQ suggests that binding of TCHQ is stronger than binding of PCP, however a larger fraction of protein seems to be available for binding of PCP in the FR. The type and size differences in reactor protein in the FR and CEFR are likely responsible for the increased apparent binding in the FR. Protein binding represents a small percentage of the reactions leading to remediation of PCP from aqueous solutions.

Table 11: PCP and TCHQ in the FR and the CEFR

	Day of Reactor operation	Reactor pH	Reactor [Protein] (μg/ml)	[PCP] (mg/L)	[TCHQ] (mg/L)
FR					
	5	4.62	33.3	0.013	0.018
	15	3.91	52.8	0.156	0.014
	21	3.95	69.9	0.041	0.009
	27	3.89	97.1	0.141	0.016
	37	3.86	357	0.217	0.019
	42	3.92	180	0.219	0.032
	49	3.90	173	0.296	0.025
CEFR					
	5	3.90	23.7	0.009	0.014
	15	3.70	31.1	0.095	0.016
	21	3.74	19.1	0.107	0.012
	27	3.72	30.0	0.161	0.016
	37	3.74	43.0	0.215	0.018
	42	3.72	40.9	0.177	0.016
	49	3.70	44.0	0.283	0.013

Note: Data points presented are an average of three sample measurements

Table 12: Summary of PCP removal performance in the FR and the CEFR

Day of Reactor operation	Inlet [PCP] (mg/L)	Reactor [PCP] (mg/L)	Reactor [Protein] (μg/ml)	PCP Removal (mg PCP / mg Protein)	%Reduction of PCP
FR					
5	2.5	0.013	33.3	0.075	99.5
15	2.5	0.156	52.6	0.045	93.8
21	2.5	0.041	69.9	0.035	98.4
27	2.5	0.141	97.1	0.024	94.3
37	2.5	0.217	356	0.006	91.3
42	2.5	0.219	180	0.013	91.2
49	2.5	0.296	173	0.013	88.2
CEFR					
5	2.511	0.0095	23.7	0.105	99.6
15	2.596	0.095	31.1	0.081	96.4
21	2.527	0.011	19.1	0.127	95.8
27	2.588	0.161	30.0	0.081	93.8
37	2.633	0.214	43.0	0.056	91.8
42	2.634	0.177	40.9	0.060	93.3
49	2.680	0.283	44.0	0.055	89.4

Note: Data points presented are an average of three sample measurements

Table 13: Effect of Incubation Temperature on Concentration of TCHQ in FR and CEFR

Incubation Temperature	Day of Reactor Operation		[TCHQ] mg/L FR	CEFR
75°F	18		0.013	0.016
	22		0.023	0.021
	32		0.021	0.013
		Average	0.019	0.017
85°F	15		0.019	0.016
	21		0.010	0.017
	32[1]		0.018	0.017
		Average	0.016	0.017

Note: 1. Data points are an average of data collected on day 27 and 37.

Table 14: Protein bound PCP and TCHQ in the FR and the CEFR

	Day of Reactor operation	Reactor pH	Reactor [Protein] (μg/ml)	$[PCP]_{bound}$ (mg/L)	$[TCHQ]_{bound}$ (mg/L)
FR					
	7	4.77	45.8	0.011	0.0013
	16	3.91	52.8	0.010	0.0029
	21	3.95	69.9	0.014	0.0008
	28	3.93	94.1	0.083	0.0020
	38	4.00	125	0.028	0.0017
	43	3.94	199	0.029	0.0012
	50	3.92	173	0.023	0.0009
CEFR					
	7	3.96	23.4	0.010	0.0017
	16	3.70	32.2	NA	NA
	21	3.74	19.1	0.015	0.0006
	28	3.73	34.7	0.055	0.0009
	38	3.72	57.8	0.035	0.0036
	43	3.70	45.7	0.007	0.0021
	50	3.70	44.0	0.009	0.0010

Note: Data points presented are an average of three sample measurements

Table 15: Effect of Incubation Temperature on Concentration of Protein Bound PCP in FR and CEFR

Incubation Temperature	Day of Reactor Operation		$[PCP]_{bound,}$ (mg/L) FR	CEFR
75°F	16		0.0056	0.0053
	21		0.0108	0.0053
	29		0.0155	0.0182
		Average	0.0106	0.0096
85°F	16		0.0103	N/A
	21		0.0142	0.0151
	28		0.0832	0.0554
		Average	0.0359 (0.0174[1])	0.035

Note: 1. Average does not include highest value.

Table 16: Effect of Incubation Temperature on Concentration of Protein Bound TCHQ in FR and CEFR

Incubation Temperature	Day of Reactor Operation		[TCHQ] $_{bound}$ (mg/L) FR	CEFR
75°F	16		0.0004	0.0005
	21		0.00007	0.00007
	29		0.0014	0.0015
		Average	0.0006	0.0007
85°F	16		0.0029	N/A
	21		0.0008	0.0006
	28		0.0020	0.0009
		Average	0.002	0.0008

Table 17: Ratios of PCP to protein bound PCP and TCHQ to protein bound TCHQ in FBR and CEFR

Day of Reactor Operation	$[PCP]_{unbound}/[PCP]_{bound}$		$[TCHQ]_{unbound}/[TCHQ]_{bound}$	
	FBR	CEFR	FBR	CEFR
5-6	1.1	0.9	14	8
15-16	15	N/A	5	N/A
21	2.9	7	11	19
27-28	1.7	2.9	8	17
37-38	7.8	6.2	11.5	5
42-43	7.5	26	26.4	7.4
49-50	13	31	28.4	13.4
Average	6.9	12.3	14.9	11.6

Table 18: Effect of Incubation Temperature on Concentration of Protein Bound TCHQ in FBR and CEFR

Incubation Temperature	Day of Reactor Operation	$[PCP]_{unbound}/[PCP]_{bound}$ FBR	$[PCP]_{unbound}/[PCP]_{bound}$ CEFR	$[TCHQ]_{unbound}/[TCHQ]_{bound}$ FBR	$[TCHQ]_{unbound}/[TCHQ]_{bound}$ CEFR
75°F	16-18	16	22	32	34
	21-22	13	32	305	317
	29-32	15	11	15	8
	Average	15	21	117 (24)	120 (21)
85°F	15-16	15	N/A	7	N/A
	21	3	7	12	29
	27-28	2	3	8	18
	Average	7	5	9	24

Table 19: Fraction of apparent protein bound to PCP and TCHQ in the FR and the CEFR

	ϕ_{PCP}	ϕ_{TCHQ}	ϕ_{Total}
FR			
Before Flow Disturbance	0.075	0.013	0.088
After Flow Disturbance	0.054	0.003	0.057
CEFR			
Before Flow Disturbance	0.30	0.013	0.31
After Flow Disturbance	0.069	0.015	0.084

Table 20: Distribution of initial PCP in FR accounted for as PCP, protein bound PCP, TCHQ and protein bound TCHQ

Days of	Mole % of initial PCP accounted for				
Reactor Operation	PCP	PCP $_{bound}$	TCHQ	TCHQ $_{bound}$	Total
5-6	0.5	0.5	0.8	0.06	1.8
15-16	6.2	0.4	0.6	0.1	7.4
21	1.6	0.6	0.4	0.03	2.6
27-28	5.7	3.3	0.7	0.08	9.7
37-38	8.7	1.1	0.8	0.07	10.7
42-43	8.8	1.2	1.4	0.05	11.4
49-50	12	0.9	1.1	0.04	13.9

Table 21: Distribution of initial PCP in CEFR accounted for as PCP, protein bound PCP, TCHQ and protein bound TCHQ

Days of Reactor Operation	Mole % of initial PCP accounted for				
	PCP	PCP $_{bound,}$	TCHQ	TCHQ $_{bound,}$	Total
5-6	0.4	0.4	0.6	0.07	1.4
15-16	3.6	N/A	0.7	N/A	4.3
21	4.2	0.6	0.5	0.03	5.4
27-28	6.2	2.1	0.7	0.04	9.0
37-38	8.2	1.3	0.7	0.15	10.3
42-43	6.7	0.3	0.6	0.09	7.7
49-50	11	0.3	0.5	0.04	11.5

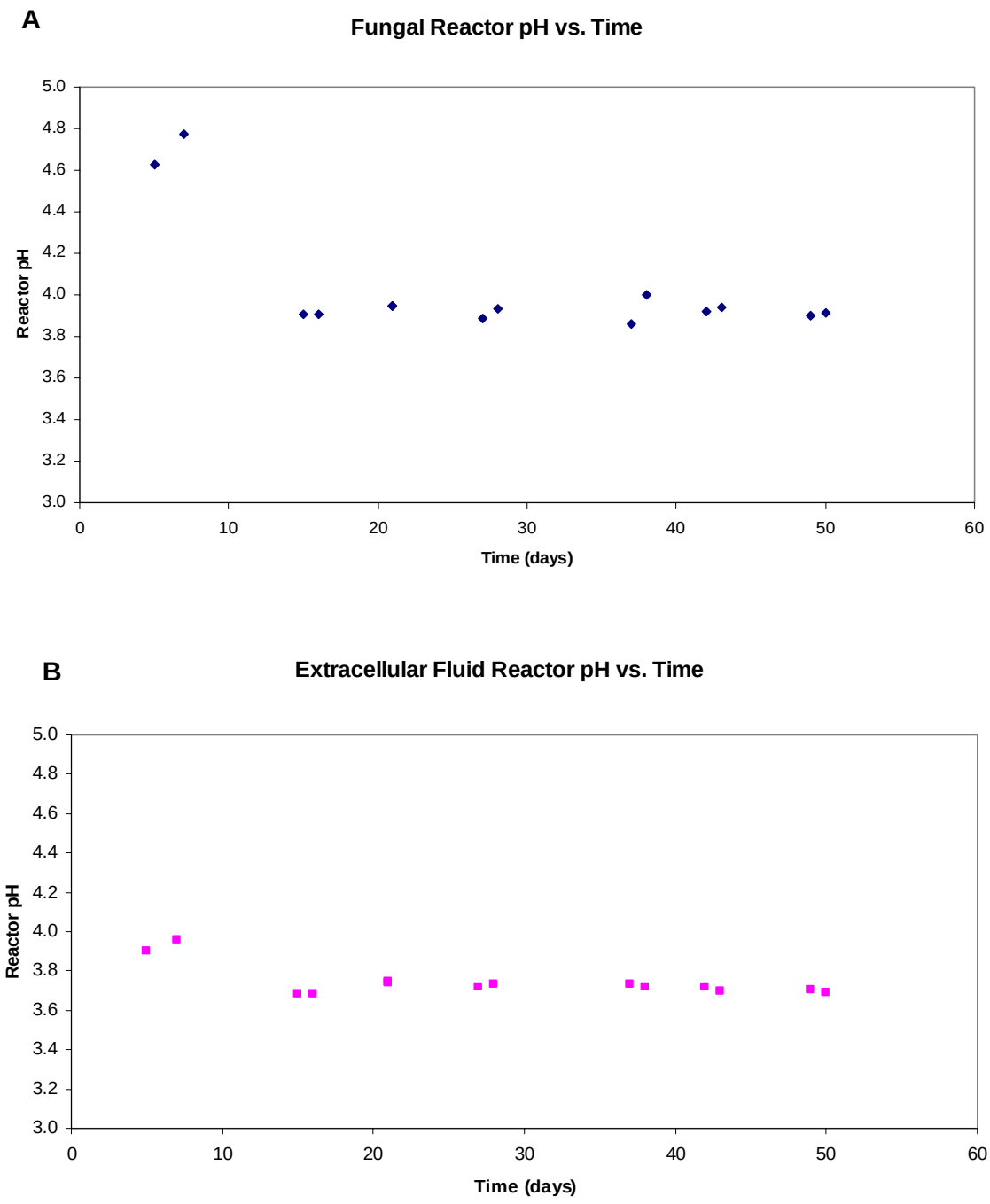


Figure 14. Reactor pH in fungal reactor (A) and reactor pH in extracellular fluid reactor (B) during reactor operation ◆-Reactor pH in fungal reactor; ■-Reactor pH in extracellular fluid reactor

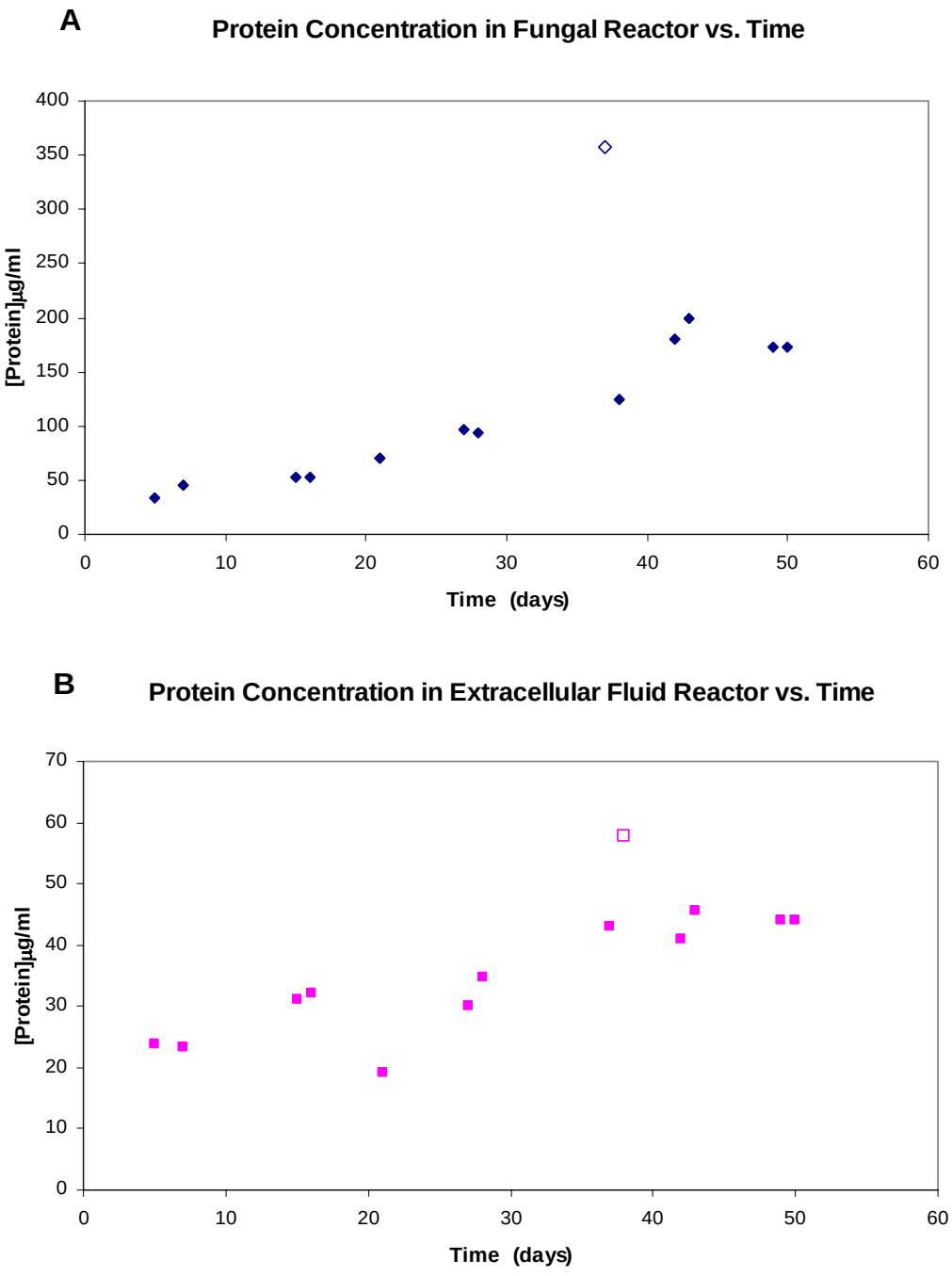


Figure 15. Protein concentration in fungal reactor (A) and protein concentration in extracellular fluid reactor (B) during reactor operation ◆-Protein concentration in fungal reactor; ■-Protein concentration in extracellular fluid reactor

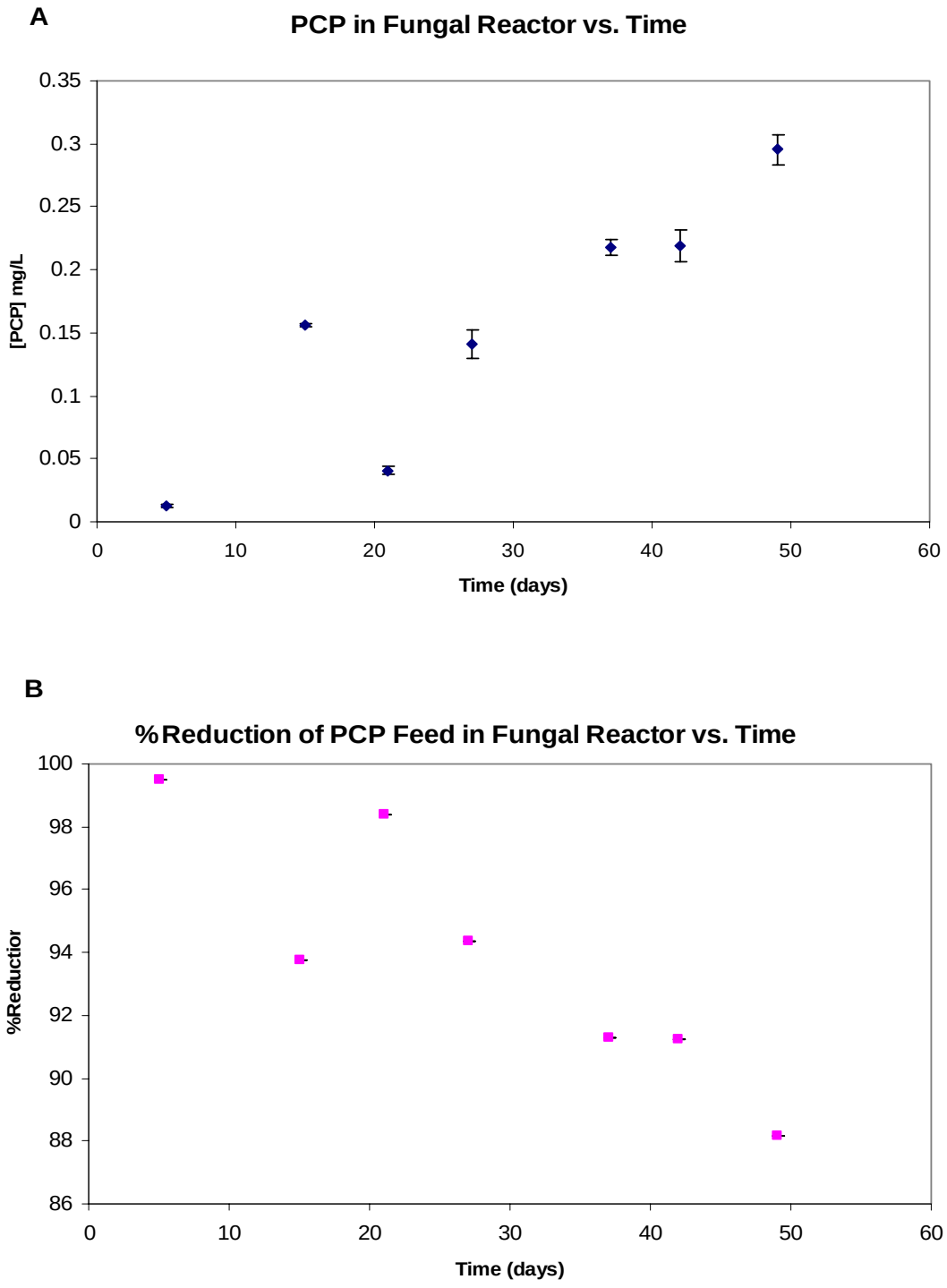


Figure 16. Concentration of PCP in fungal reactor (A) and %Reduction of PCP in fungal reactor (B) during reactor operation ◆-Concentration of PCP in fungal reactor; ■-%Reduction of PCP in fungal reactor

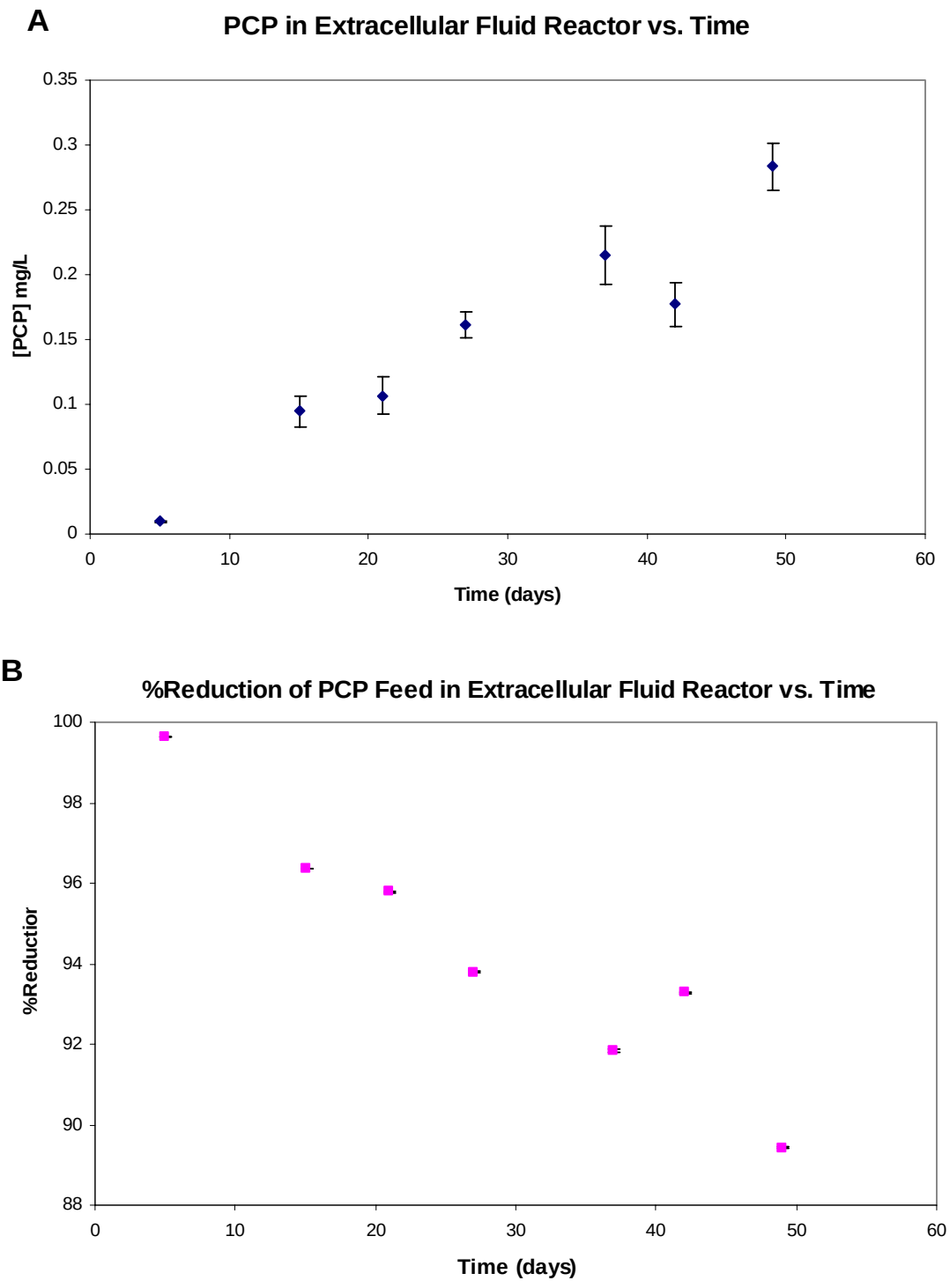


Figure 17. Concentration of PCP in extracellular fluid reactor (A) and %Reduction of PCP in extracellular fluid reactor (B) during reactor operation ◆-Concentration of PCP in extracellular fluid reactor; ■-%Reduction of PCP in extracellular fluid reactor

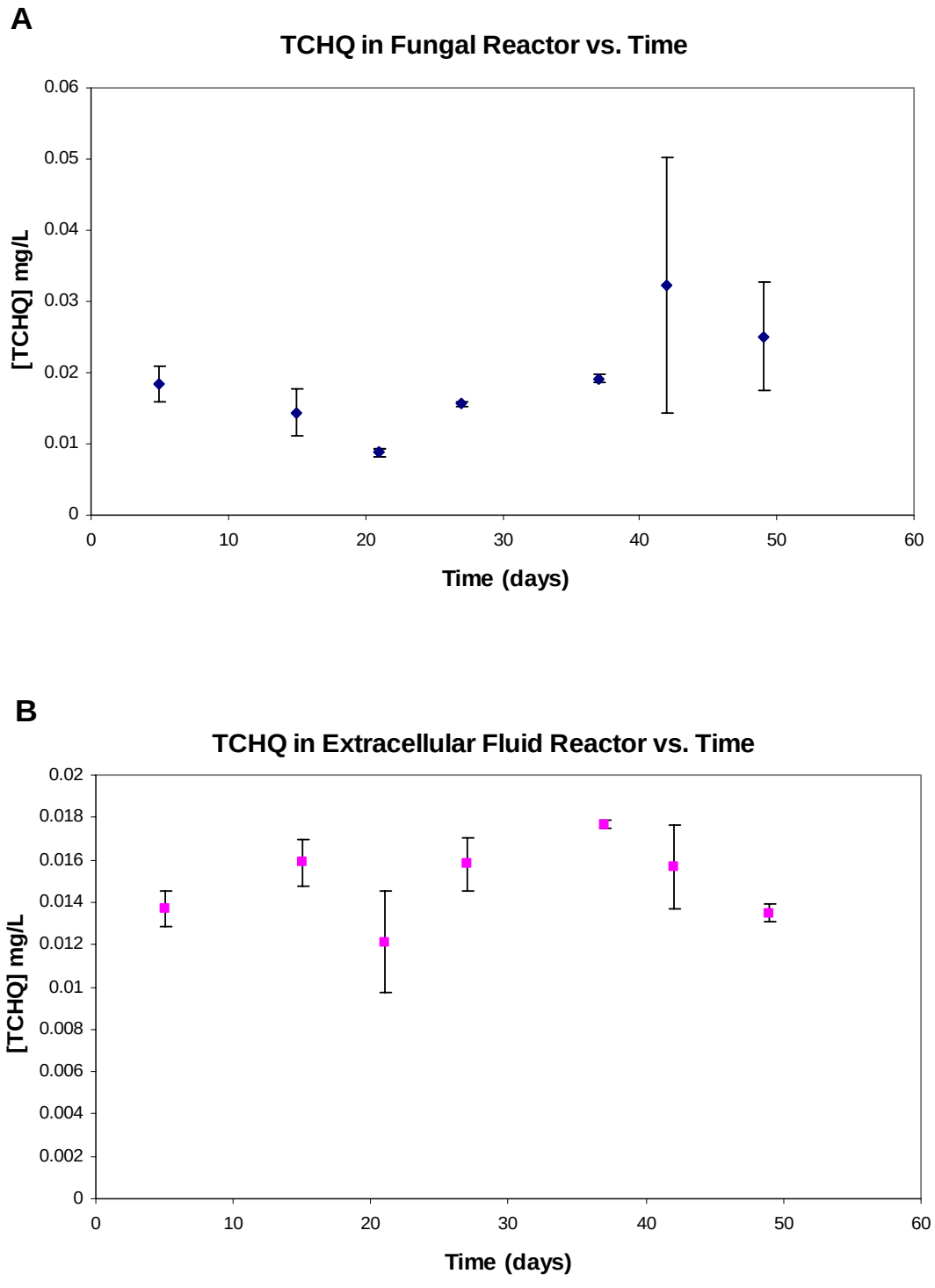


Figure 18. Concentration of TCHQ in fungal reactor (A) and concentration of TCHQ in extracellular fluid reactor (B) during reactor operation ◆-Concentration of TCHQ in fungal reactor; ■-Concentration of TCHQ in extracellular reactor

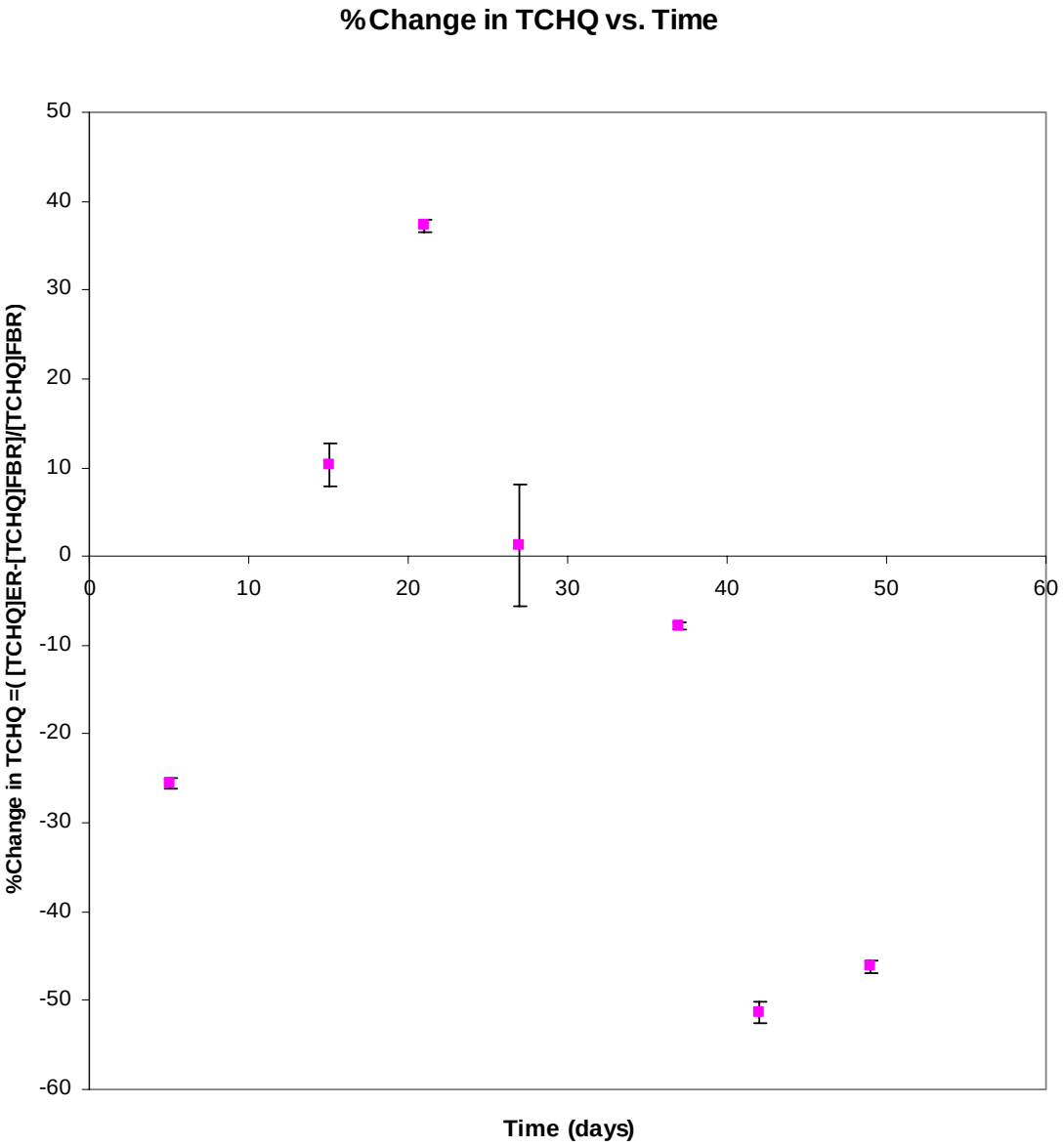
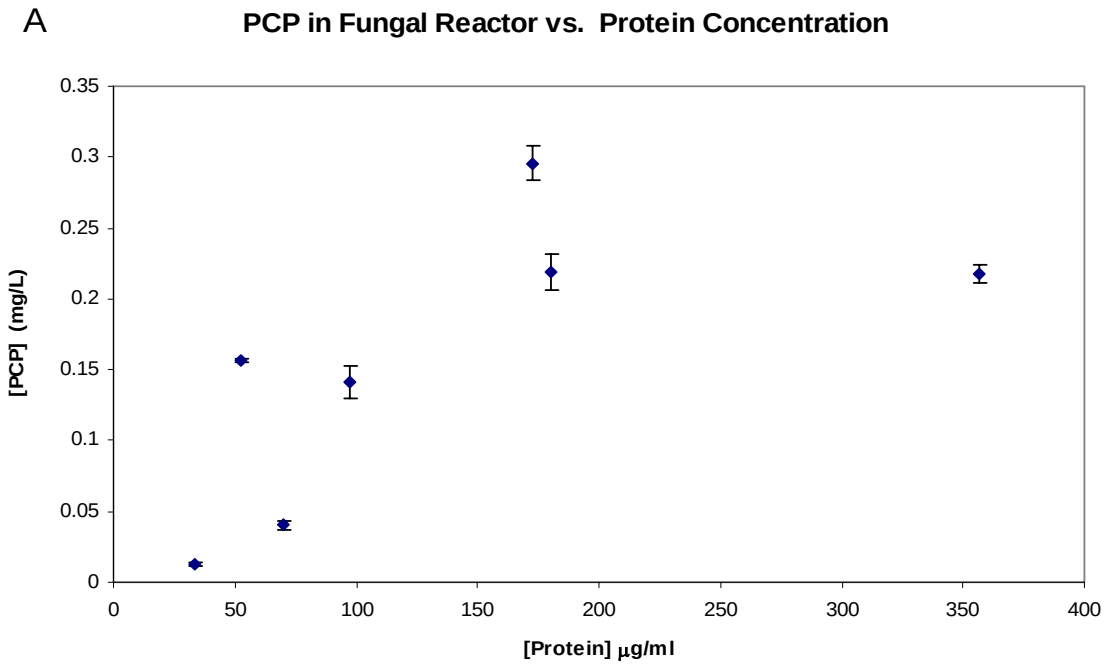


Figure 19. %Change of TCHQ in extracellular fluid reactor in relation to feed from fungal reactor; ■-%Change of TCHQ



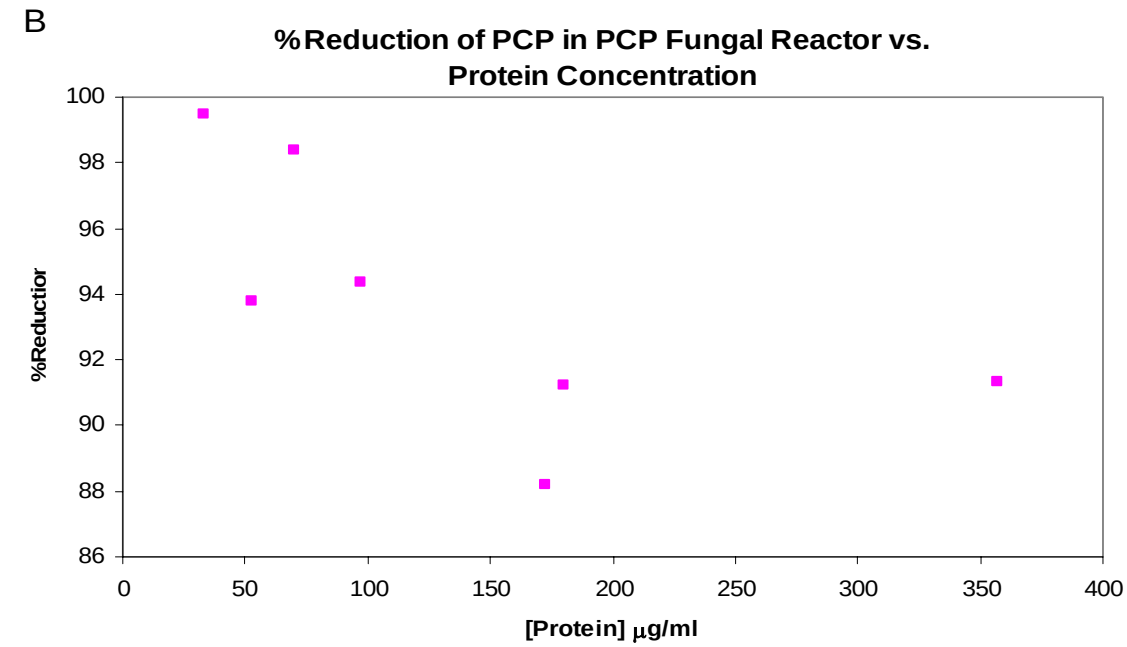


Figure 20. Concentration of PCP in FR (A) and % reduction of PCP in FR reactor (B) vs. protein concentration ◆-Concentration of PCP in FR; ■-% reduction of PCP reactor

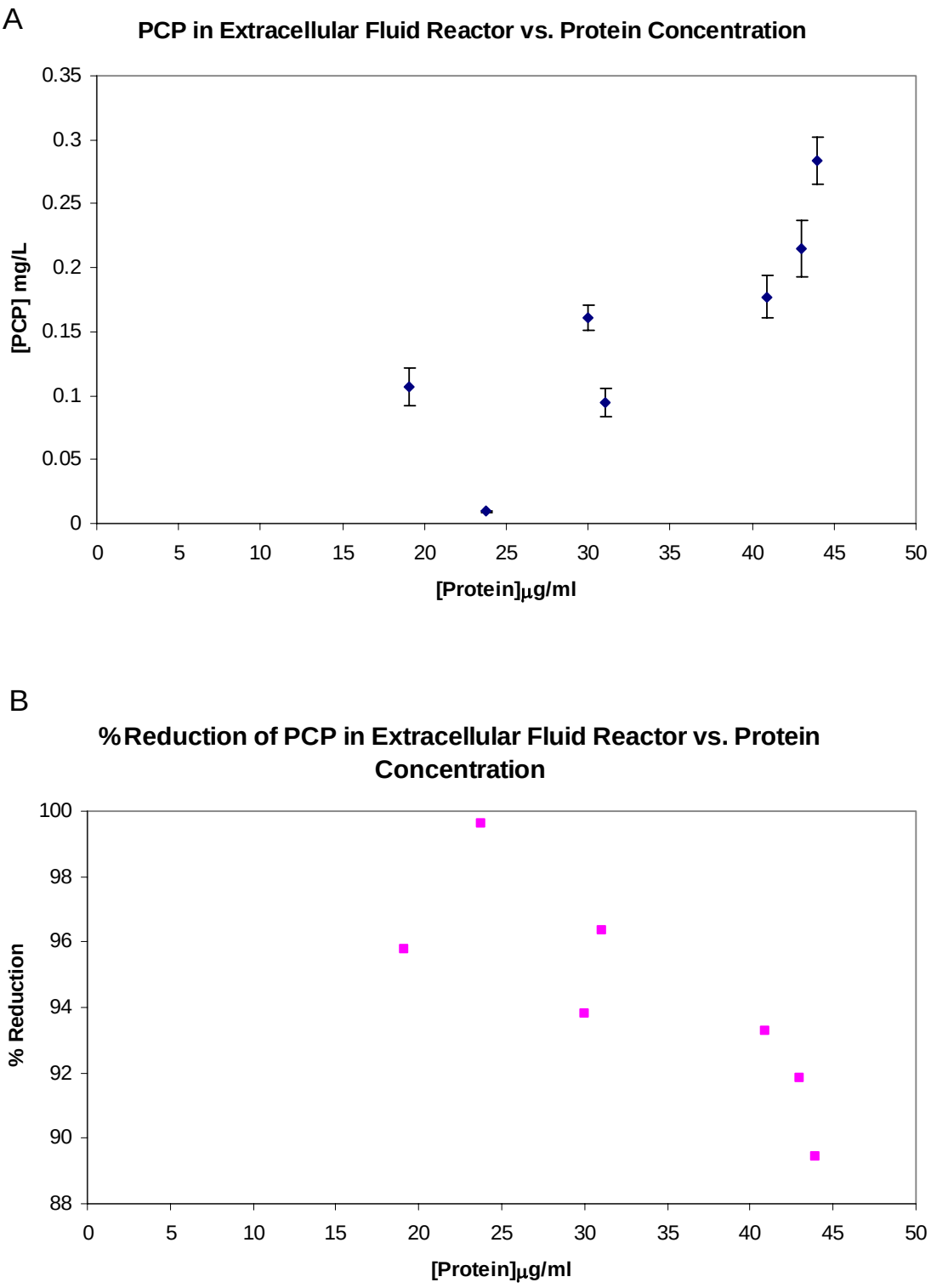


Figure 21. Concentration of PCP in CEFR (A) and % reduction of PCP in CEFR (B) vs. protein concentration ◆-Concentration of PCP in CEFR; ■-% reduction of PCP reactor

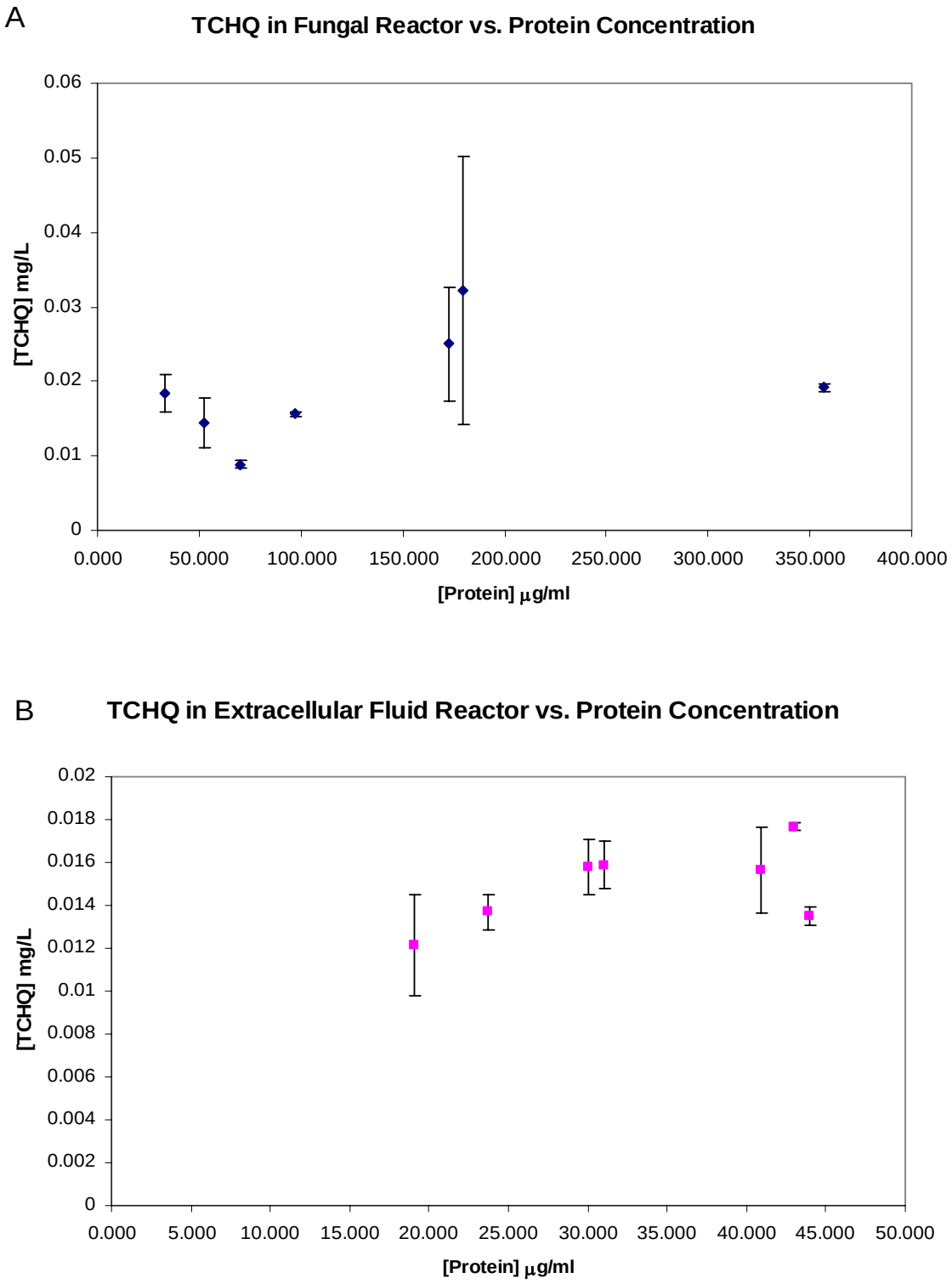


Figure 22. Concentration of TCHQ in FR (A) concentration of TCHQ in CEFR (B) vs. protein concentration ◆-Concentration of TCHQ in FR; ■- concentration of TCHQ in CEFR

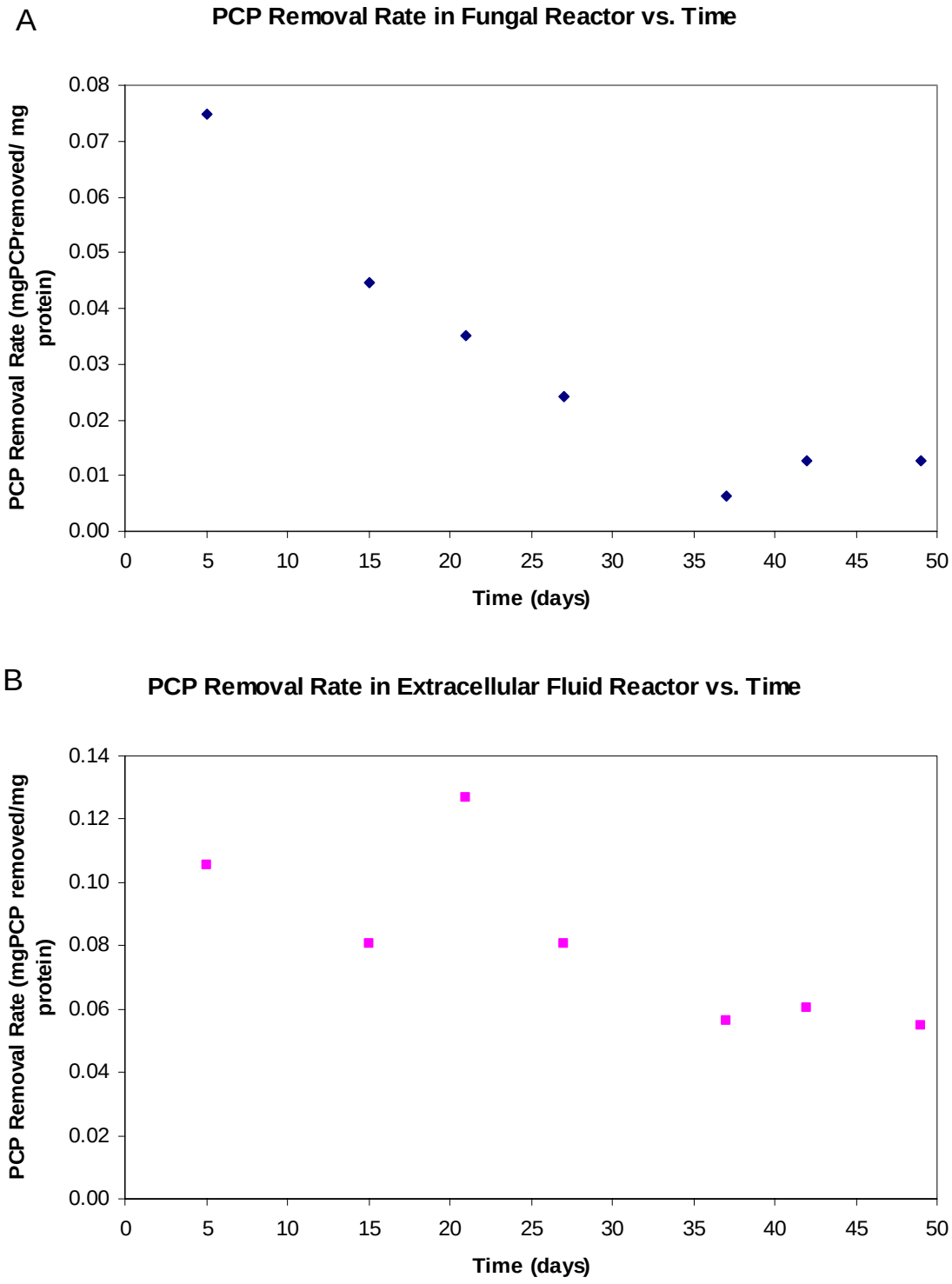


Figure 23. PCP removal in fungal reactor (A) and PCP removal in extracellular fluid reactor (B) during reactor operation. ◆-. PCP removal in fungal reactor; ■-PCP removal in extracellular fluid reactor

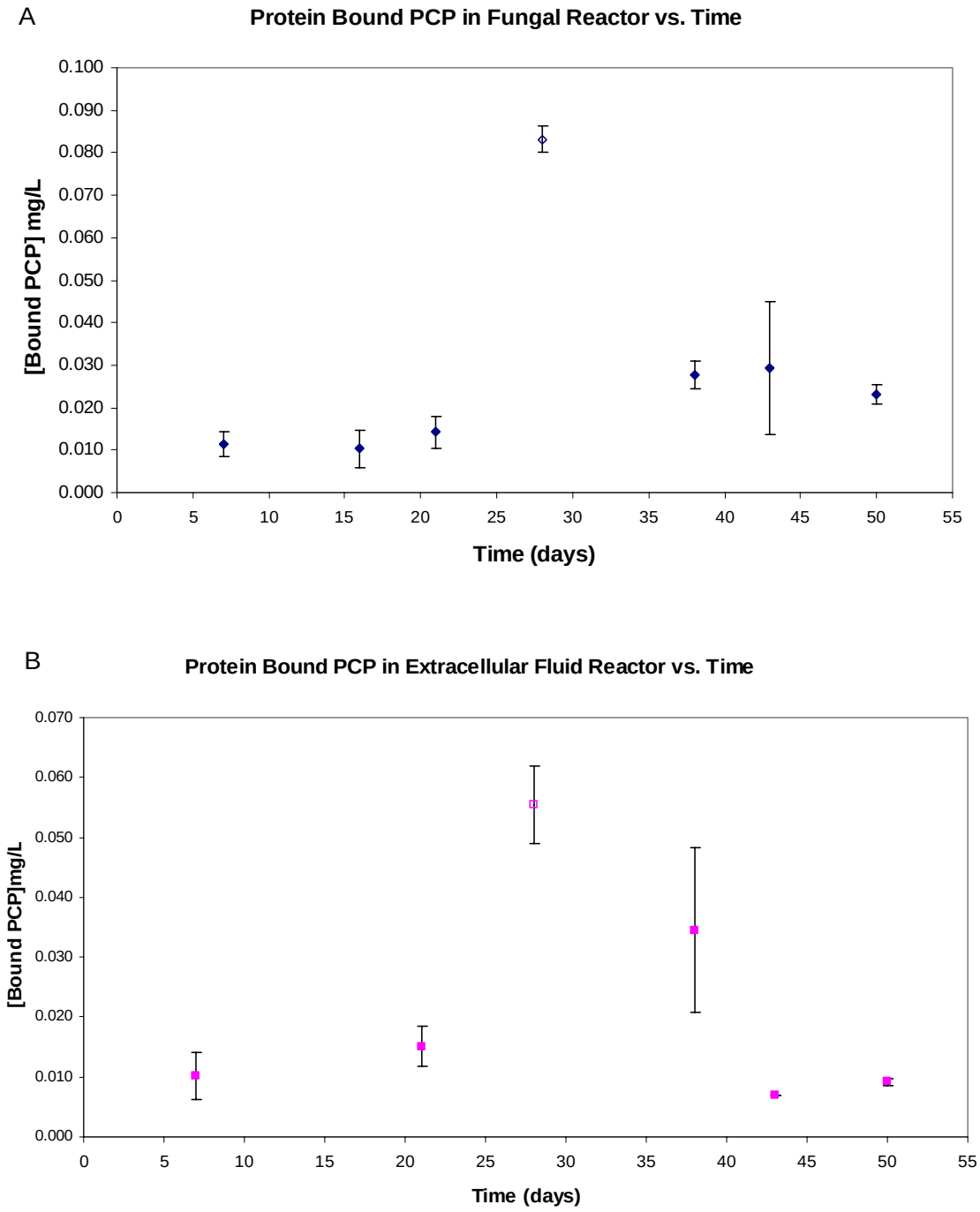


Figure 24. Concentration of apparent protein bound PCP in fungal reactor (A) and in extracellular fluid reactor (B) during reactor operation ◆-Concentration of apparent protein bound PCP in fungal reactor; ■-concentration of apparent protein bound PCP in extracellular fluid reactor

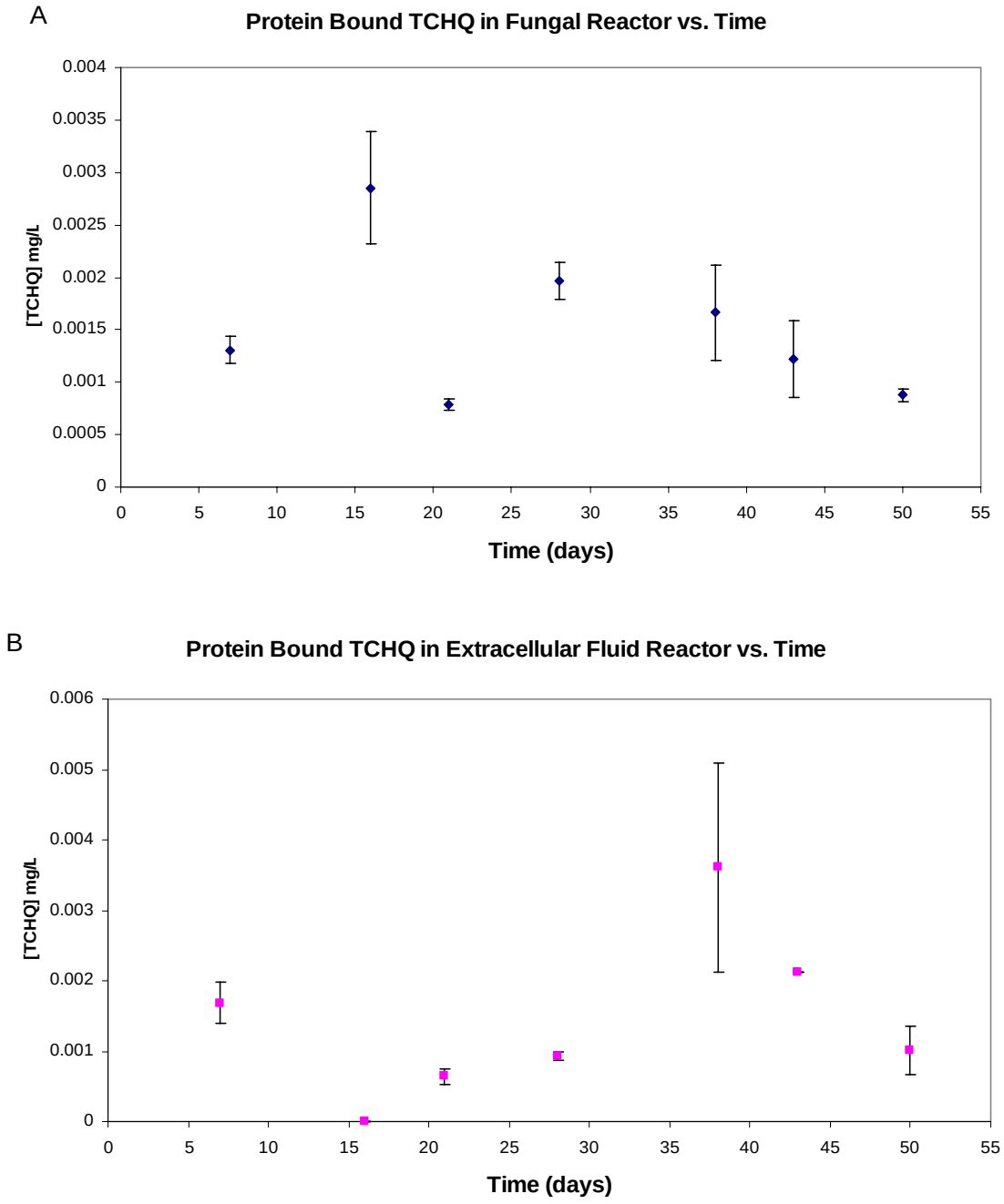


Figure 25. Concentration of apparent protein bound TCHQ in fungal reactor (A) and in extracellular reactor (B) during reactor operation ◆-Concentration of apparent protein bound TCHQ in fungal reactor; ■-concentration of apparent protein bound TCHQ in extracellular reactor

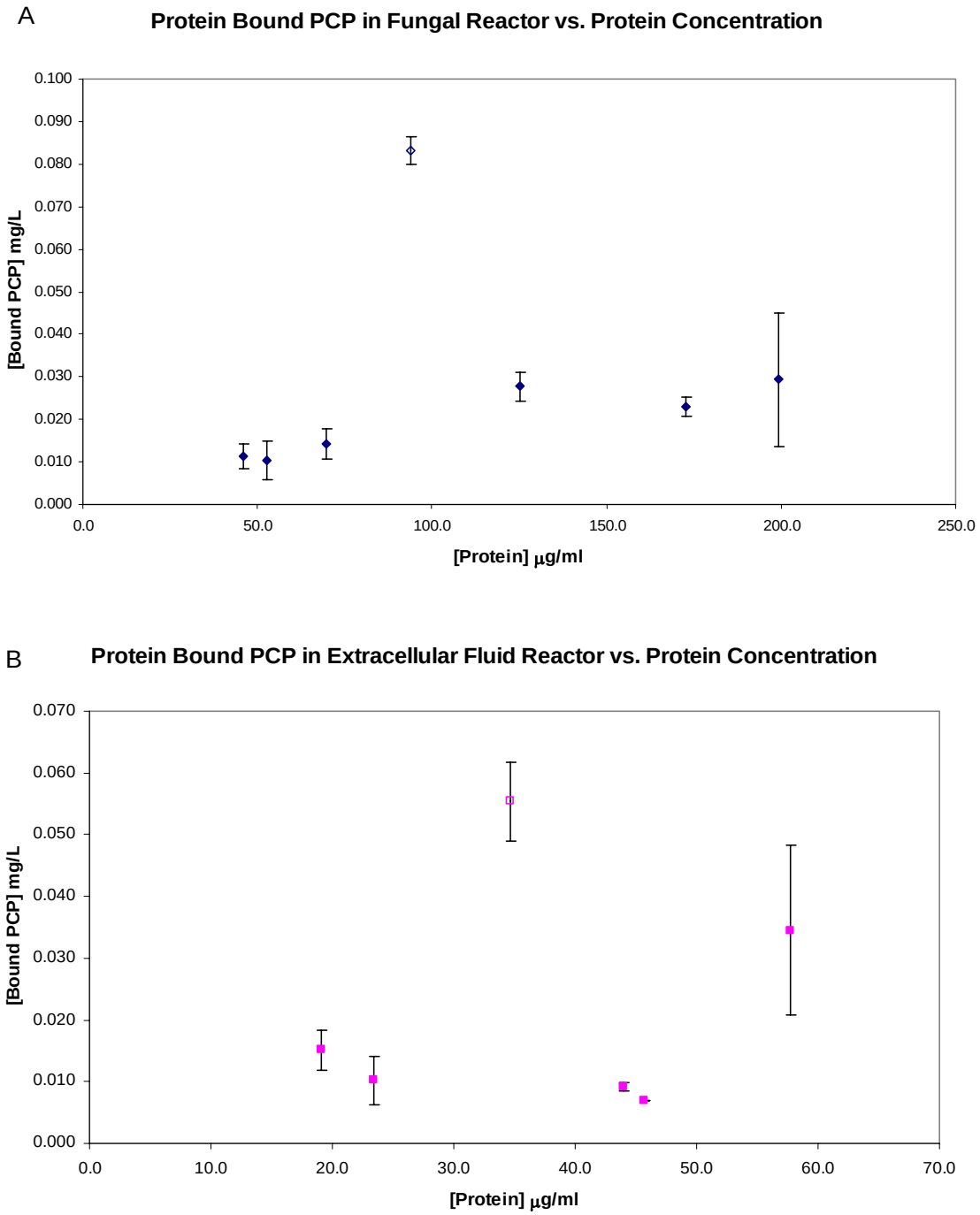


Figure 26. Concentration of protein bound PCP in fungal reactor (A) and in extracellular fluid reactor (B) vs. reactor protein concentration ◆-Concentration of PCP in fungal reactor; ■-concentration of PCP in extracellular fluid reactor

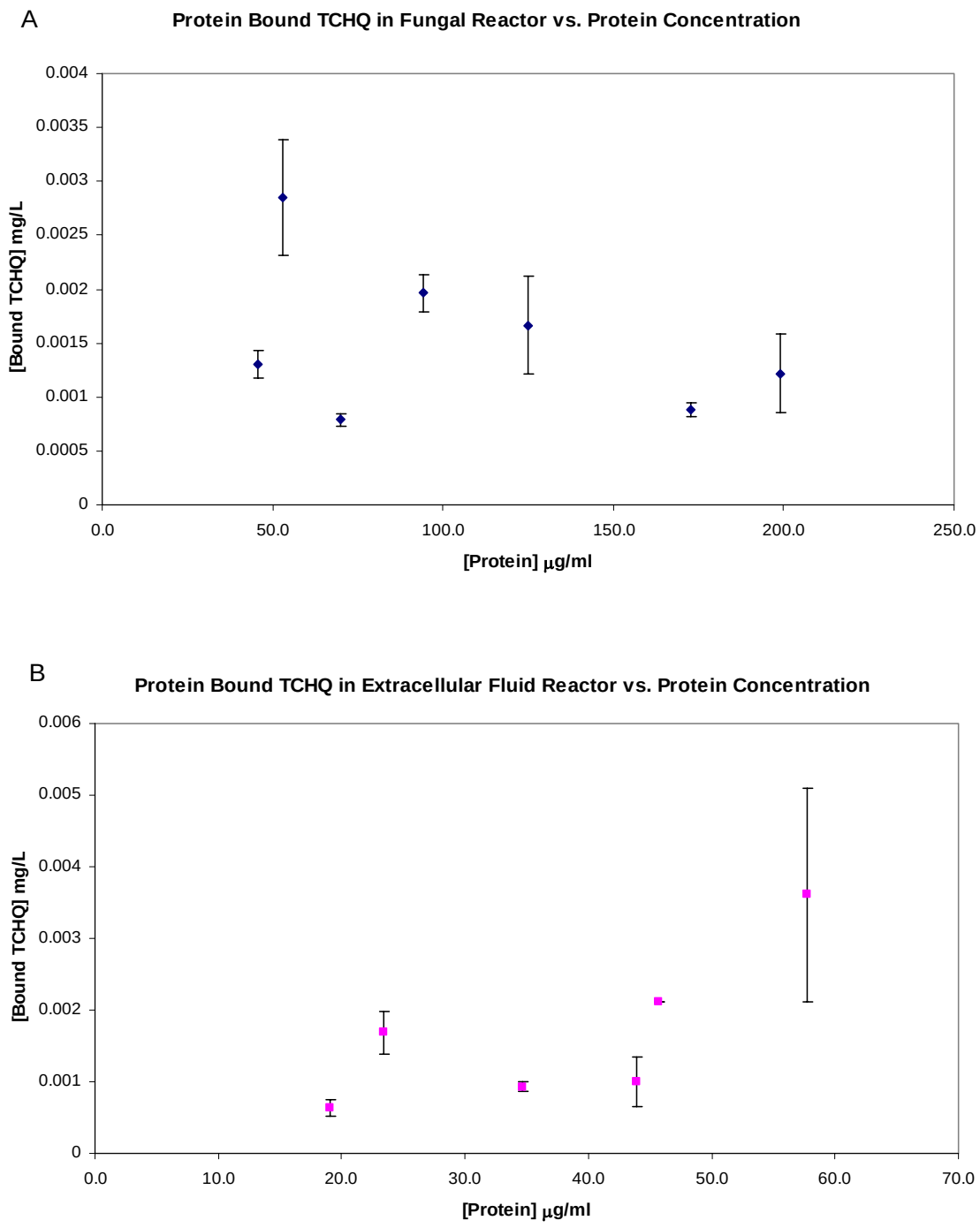


Figure 27. Concentration of protein bound TCHQ in fungal reactor (A) and in extracellular fluid reactor (B) vs. reactor protein concentration ◆-Concentration of TCHQ in fungal reactor; ■-concentration of TCHQ in extracellular fluid reactor

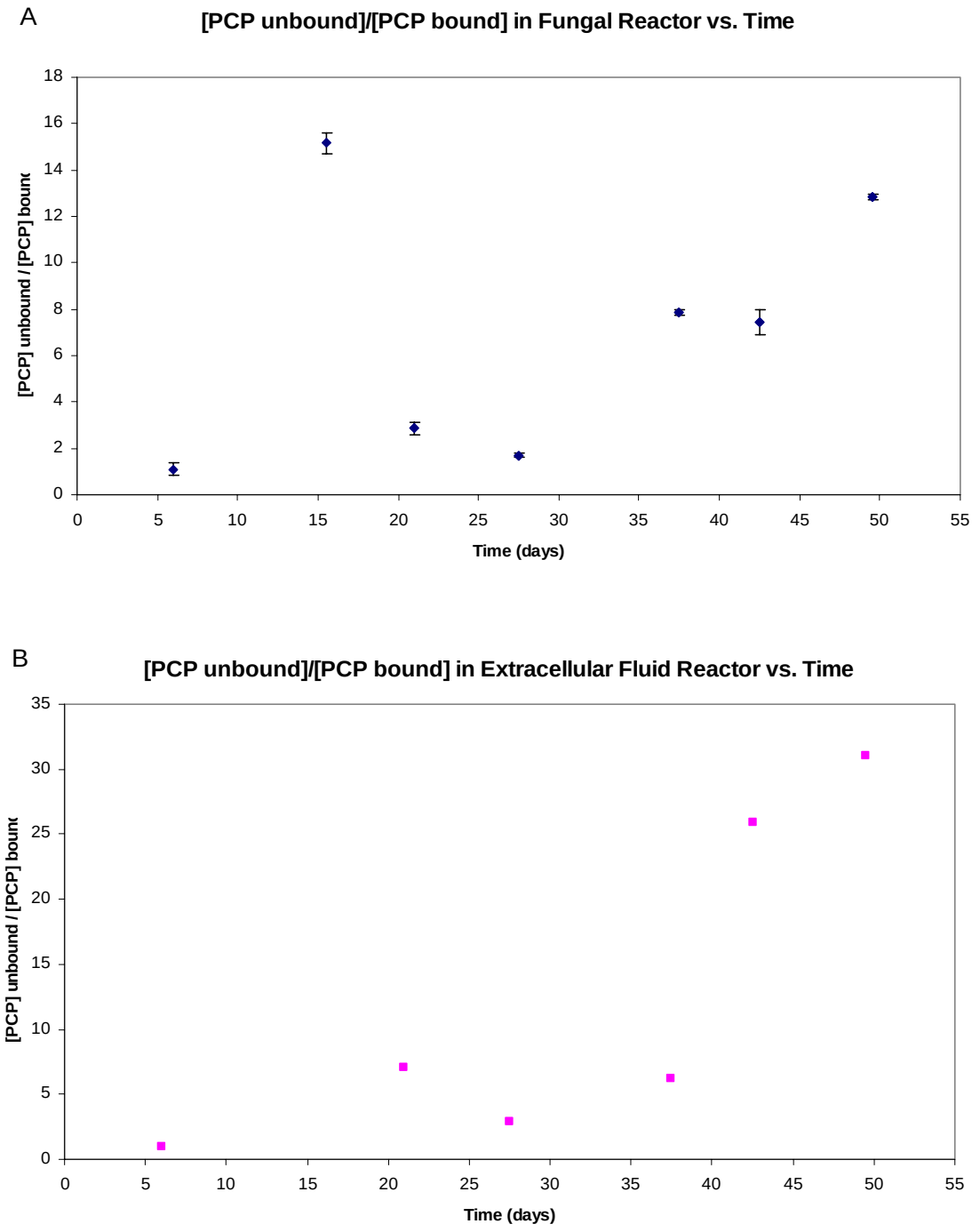


Figure 28. The ratio of the concentration of unbound PCP to protein bound PCP concentration in fungal reactor (A) and in extracellular fluid reactor (B) during reactor operation ◆-Ratio of unbound PCP to protein bound PCP in fungal reactor; ■-ratio of unbound PCP to protein bound PCP in extracellular fluid reactor

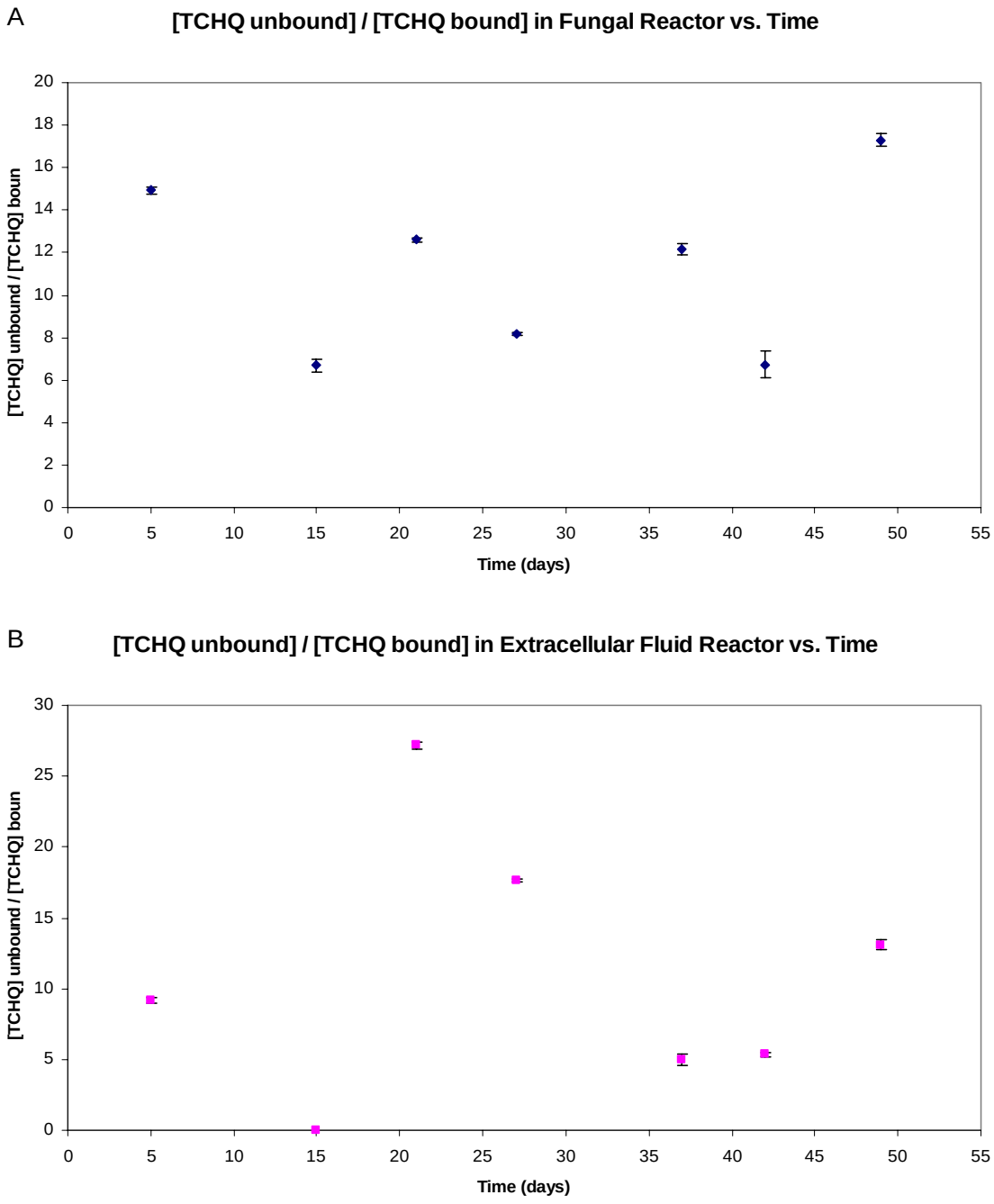


Figure 29. The ratio of the concentration of unbound TCHQ to protein bound TCHQ concentration in fungal reactor (A) and in extracellular fluid reactor (B) during reactor operation ◆-Ratio of unbound TCHQ to protein bound TCHQ in fungal reactor; ■-ratio of unbound TCHQ to protein bound TCHQ in extracellular fluid reactor

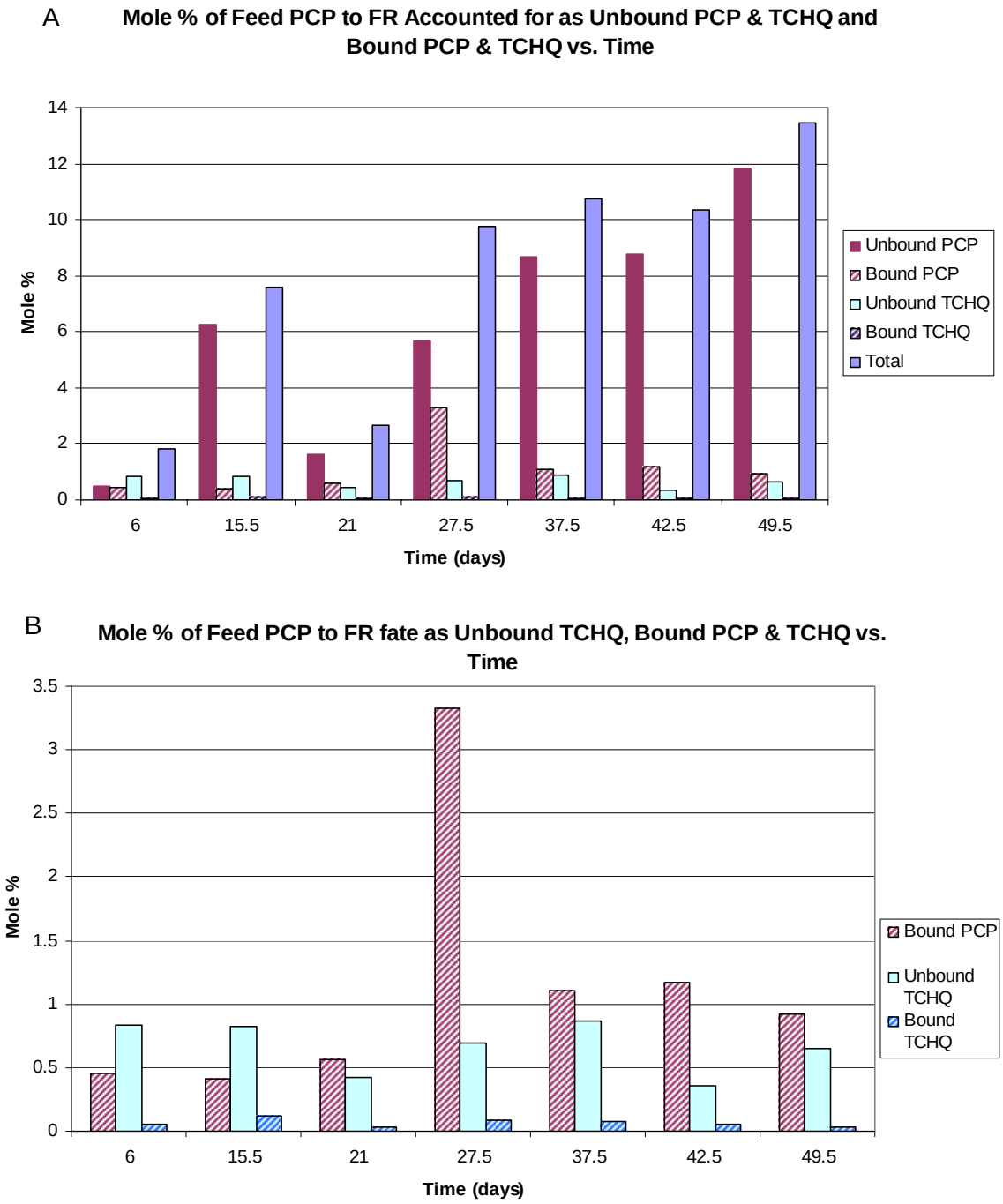
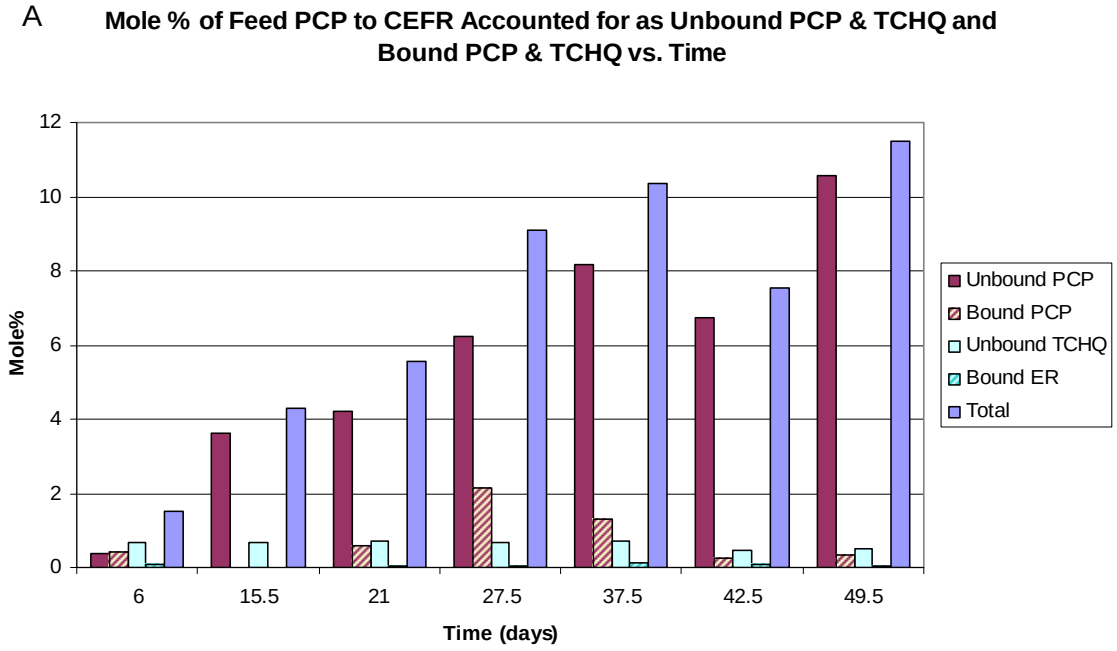


Figure 30. Mole % of feed PCP to fungal reactor accounted for as unbound PCP, unbound TCHQ, protein bound PCP, protein bound TCHQ and total (A) and mole % of feed PCP to fungal reactor accounted for as unbound TCHQ, protein bound PCP, and protein bound TCHQ (B).

A



B

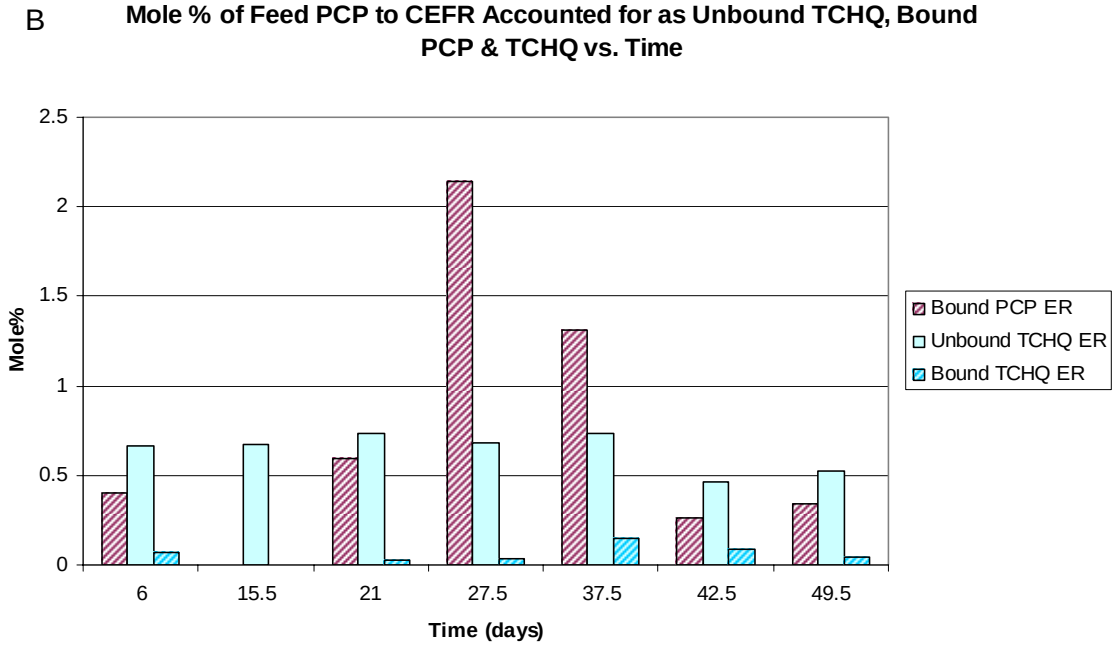


Figure 31. Mole % of feed PCP to extracellular fluid reactor accounted for as unbound PCP, unbound TCHQ, apparent protein bound PCP, apparent protein bound TCHQ and total (A) and mole % of feed PCP to extracellular fluid reactor accounted for as unbound TCHQ, apparent protein bound PCP, and apparent protein bound TCHQ (B).

CHAPTER III

TREATMENT OF BLEACH PLANT EFFLUENT BY TWO-STAGE BIOREACTOR SYSTEM

ABSTRACT

Remediation of bleach plant effluent (BPE) by a two-stage bioreactor system developed to remove PCP from aqueous was studied for the effects of treatment of BPE in terms of reducing the concentration of PCP and TCHQ and color removal. Color reduction achieved by the FR was as high as 67% and as high as 54% in the CEFR. Up to 90% of PCP was removed and up to 91% removal of TCHQ. The FR used to treat full strength BPE produced large amounts of protein and protein binding of PCP and TCHQ seemed to play a role in removal. Because treatment of full strength BPE resulted in large amounts of growth and protein production, small amounts of BPE were added to a PCP feed solution to study its effect on protein production and improved removal of PCP. Adding small concentrations of BPE increased the amount of reactor protein produced in the FR by 18% and the reactor protein in the CEFR increased 62%. The addition of BPE resulted in a more robust reactor that released laccase activity as a result of additional carbohydrates and increased microorganism stress by changes in feed stream characteristics. Protein bound TCHQ increased several fold with small amounts of BPE added to a PCP feed solution. The addition of BPE seemed to increase soluble protein

and provide a reactive component that converted PCP to TCHQ, therefore providing favorable conditions for protein binding of TCHQ.

INTRODUCTION

The pulp and paper industry is potentially a major source of water pollution. Water is used in every aspect of the paper making process, including debarking, chipping, pulping, cleaning, screening, bleaching, refining, addition of chemical treatments, paper forming, pressing and drying. Efforts have been made to minimize the amount of fresh water that is consumed as well as the amount of water effluent discharged by using extensive recycling. There still is a substantial amount of wastewater effluent being discharged by the pulp and paper industry. This effluent must be treated to meet federal and state regulations for several properties.

Water pollution is defined as any change in the condition of water which is detrimental to some beneficial use. The end use of the treated wastewater determines the amount of treated required. Humans, fish habitats and industry have different requirements for water. Humans can be adversely affected by bacterial infection, taste, and odor and in some cases foaming, radioactivity, toxic substances, or heavy metal ions. Fish habitats can be adversely affected by reduced dissolved oxygen, toxic substances and deposition of solid particles. Industry uses have specific requirements for suspended solids, organic substances, organic substances, inorganic salts, and dissolved organic substances (Smook, 1999).

In an ideal world, the effluent returned to the receiving waters would be as pure and natural as the native waters. This of course is not feasible in this industry driven world. For this reason, legislation makes its decisions on how industrial pollution affects the environment by assessing economic factors and site-specific utilization of the receiving water (Smook, 1999). Regulatory statutes specify that little or no change in dissolved oxygen, pH, toxicity, suspended solids, temperature, floatable solids, foam, taste, odor, nutrient concentration, and productivity of microorganisms be detected as an effect of effluent discharge. The major categories of water pollution that concern the pulp and paper industry are effluent solids, oxygen demand, toxicity, and color (Smook, 1999).

Pulp bleaching is a major contributor to the pollution produced by the pulp and paper industry. Bleaching of pulp requires the removal of lignin from pulp. Elemental chlorine or chlorine dioxide used during the pulp bleaching process reacts with lignin yielding chlorolignin. Chlorolignin is a dark colored, high molecular weight compound that has toxic and mutagenic properties (Rogers, 1973, Ander et al., 1977, Eriksson et al., 1979, & Kringstnad et al., 1981) and it is a significant contributor to the dark brown color of bleach plant effluent. Abatement efforts, however, focus mainly on the reduction of solids concentration and oxygen demand of leaving effluent. Current biological treatment methods are aerated lagoons and activated sludge plants, which effectively remove biological oxygen demand (BOD), reducing toxicity, but have little effect on color removal or chemical oxygen demand (COD) compared to BOD reduction. Therefore, toxicity is not a major concern after traditional biological treatment and color is only of critical concern when the dilution factor of the receiving stream is low and

when light penetration of the receiving stream is affected enough to have an impact on plant growth in the water system (Smook, 1999). However, several environmentally persistent chlorinated compounds of high and low molecular weight are present in biologically treated bleach plant effluent. Many of these low molecular weight compounds are toxic and can be incorporated in sediments as well as bioaccumulate in aquatic life in the receiving waters (Landner, 1979). It is also possible that high molecular weight compounds can be degraded over time, releasing toxic low molecular weight compounds (Martin et al., 1995). Because of the potential risks with releasing these compounds into the environment, it is beneficial to minimize their presence in effluent with additional treatment prior to discharge.

Bioremediation of BPE by use of fungal material has been shown to be effective and offers several benefits. Fungal treatment is able to remove high molecular weight chlorolignin from BPE that current treatments, such activated sludge plants and aerated lagoons, are unable to accomplish. *Trametes versicolor* is a white-rot fungus that has shown the ability to degrade native lignin and chlorolignin as well as chlorinated decomposition products, such as pentachlorophenol (PCP) by action of fungal degradation as well as enzymatic activity. Pallerla & Chambers (1996) developed a fungal reactor that effectively treated BPE. The reactor utilized polyurethane immobilized *T. versicolor* that effectively reduced AOX and color of the bleach plant effluents studied. Optimization of the reactor resulted in 88% reduction of AOX and 44% reduction of color (Samojedny, 2000). Based on the effectiveness of the fungal reactor to treat BPE and the novel extracellular fluid reactor to remediate PCP, a two-

stage bioreactor system was developed to study the effects of further treatment of the BPE with the extracellular enzymes produced by *T. versicolor*. Color reduction of BPE due to fungal action as well as enzymatic action was examined. This study investigated the presence and concentration of reactive tetrachlorobenzoquinone and tetrachlorohydroquinone in bleach plant effluent, which has not previously been determined. Degradation of the chlorinated compounds pentachlorophenol and tetrachlorohydroquine was measured. One of the unique properties of white-rot fungi is the excretion of enzymes and other extracellular protein material. Sulfhydryl protein binding of reactive compounds has previously been reported to occur in biological systems and was investigated for its contribution to remediation of pentachlorophenol and tetrachlorohydroquinone from bleach plant effluent.

BPE contains carbohydrates and a mixture of compounds that initiate fungal material to produce extracellular fluid. These attributes led to experiments to study the effects of adding small amounts of BPE to a 2.5mg/L PCP feed solution to the FR. Reactor protein concentration, enzyme activity, free PCP, free TCHQ, bound PCP and bound TCHQ were determined for several concentrations of BPE added to the 2.5mg/L PCP feed solution.

There were several objectives of this part of the study. These objectives were to determine if TCHQ was present in E_o-stage bleach plant effluent, study the effectiveness of the two-stage bioreactor system for remediation of BPE, determine the production of protein in reactors utilizing feeds of full strength BPE and and in reactors containing PCP

solutions with small amounts of BPE added, study the role of protein binding of PCP and TCHQ in the removal of these compounds from reactor solution. These studies will demonstrate the effectiveness of using fungal bioremediation to treat BPE and how adding BPE improves fungal bioremediation. Better understanding of these concepts could lead to implementation of fungal bioremediation for treating industrial real world effluents and understanding the response of the immobilized *T. versicolor* fungi to the inclusion of BPE in feed solutions to improve reactor performance.

LITERATURE REVIEW

Pulp Bleaching

The bleaching process brightens the pulp by removing the color contributing compounds from the pulp. Lignin is the main color contributor in pulp. From the start of the pulping process, lignin is removed, called delignification. The early bleaching stages are a continuation of that process. The later bleaching stages destroy residual color. Chlorine bleaching results in the formation of chlorolignins which is the color element transferred to the effluent, which if discharged, could interfere with the penetration of light into the receiving water, thus interfering with photosynthesis, which could affect all aquatic life in some way. Color is generally measured by use of spectrophotometric means.

Bleaching is done in a sequential fashion, following an alternating acid, alkaline pattern with washing between each step. The following is a list briefly describing each stage and the type of media used (Smook, 1999):

Chlorination (C): Reaction with elemental chlorine in acidic medium

Alkaline Extraction (E): Dissolution of reaction products with sodium hydroxide

Chlorine Dioxide (D): Reaction with chlorine dioxide in acidic medium

Oxygen (O): Reaction with molecular oxygen at high pressure in alkaline medium

Hypochlorite (H): Reaction with hypochlorite in alkaline medium

Peroxide (P): Reaction with peroxide in alkaline medium

Ozone (O): Reaction with ozone in acidic medium

Traditionally, the first two stages are a combination of chlorine and chlorine dioxide treatment or just chlorine dioxide and followed by second stage alkaline extraction. Most of the residual lignin is removed during these first two stages. During the chlorine/ chlorine dioxide stage, chlorine reacts with non-carbohydrate constituents, making them water or alkaline media soluble. The alkaline extraction stage, often referred to as the E1-stage, removes the chlorine/ chlorine dioxide reaction products. The chlorinated organic compounds produced have been recognized as a toxic concern to the environment (Smook, 1999) and are responsible for the dark brown color of effluent. The alkaline extraction effluent is the largest contributor of color, BOD, and COD to the total bleach plant effluent while contributing a low volume, responsible for 80% of color, 30% of BOD and 60% of COD contained the total BPE (Mehna et al., 1995). Because of the large influence of the E1-stage effluent on color and toxicity, several studies have focused on treatment of this effluent (Pallerla & Chambers, 1997, & Samojedny, 2000).

Components of Bleach Plant Effluent

Due to bleaching reactions producing chlorinated organics, bleach plant effluent contains several low molecular weight compounds, less than 1000 daltons, as well as high molecular weight material, greater than 1000 daltons (Kringstad & Lindström, 1984). A number of the low molecular weight compounds have be identified as toxic (Walden & Howard, 1977), mutagenic (Kringstnad, 1981), persistent, and bioaccumulative (Onysko, 1993). Several of the low molecular weight chlorinated organics, are severely toxic to aquatic life. Fortunately, biological treatment reduces these toxins to an acceptable level, where acute lethal affects are not experienced. However, over time, these low level

discharges contribute to the overall increase of these compounds in the environment. Traditionally, the high molecular weight chlorinated organics have been of little concern, because of their resistance to biodegradation. The majority of organically bound chlorine, approximately 70% in total bleach plant effluent, is found in compounds with molecular weights over 1000 (Raiser-Neto, 1990). Recently, it has been recognized that these high molecular weight chlorinated organics do eventually biodegrade into the low molecular weight chlorinated organic compounds, which are toxic (Smook, 1999). High molecular weight material was exposed to complete bacterial mixtures or white-rot fungi at conditions similar to that in nature and was slowly degraded into chlorinated catechols and guaiacols, which were methylated under certain conditions (Eriksson & Kolar, 1985, Eriksson et al., 1985). The toxic chlorinated compounds are of low molecular weight and methylation increases toxicity by making the compounds more lipophilic.

Up to 1983 approximately 190 compounds were identified in bleach plant effluent, which were mainly found in the chlorine and extraction stage effluents (McKague et al., 1990). Significant compounds identified were methanol, distinct hemicelluloses, several aliphatic acids, and numerous chlorinated organic compounds present in small amounts. The toxicity of the E-stage effluent is due to low molecular chlorinated compounds such as 3,4,5-trichloroguaiacol, tetrachloroguaiacol, and dichlorodehydroabietic acid (Leach & Thakore, 1975). The color of bleach plant effluent is due to the presence of water-soluble, polymeric, chlorinated, oxidized lignin degradation fragments with a low content of aromatic moieties (Bennett et al., 1971, Sundman et al., 1981, Lindstrom & Osterberg, 1984). With approximately 95% of chlorine being bound to organic material with

molecular weight over 1000 contributing to the color of BPE (Kringstad & Lindstrom, 1984), the chromophores are too large to penetrate the cell membranes of living organisms, this fraction is biologically inactive. However, the release of colored material can contribute to the temperature increase, and decrease of photosynthesis occurring in the receiving waters which decreases dissolved oxygen (Lamar et al., 1994).

In 1986, it was concluded that the production of bleached chemical pulp led to polychlorinated dioxins (referred to as 'dioxins') and dibenzofurans (referred to as 'furans') in the environment. Both dioxins and furans are powerful toxins and carcinogens. The production of these pollutants is traced to the chlorination, alkaline extraction sequence. Several other chlorinated organic compounds, known as 'adsorbable organic halides' (AOX), have been recognized as pollutants that can be attributed to the chlorination, alkaline extraction sequence (Smook, 1999). By 1989, a total of 313 low molecular weight compounds had been identified in bleached kraft mill effluent (McKague et al., 1989)

The National Council of the Paper Industry for Air and Stream Improvement (NSASI) developed gas chromatography methods to identify several chlorinated phenolic compounds present in wastewater of the pulp industry in 1986. These methods were able to detect several chlorinated compounds which included chlorophenols, dichlorophenols, trichlorophenols, tetrachlorophenol, pentachlorophenol, 4-chloroguaiacol, dichloroguaiacols, trichloroguaiacols, tetrachloroguaiacol, 4-chlorocatechol, dichlorocatechols, trichlorocatechols, tetrachlorocatachol, chlorovanillins,

5,6-dichlorovanillin, chlorosyringaldehyde, 3,5-dichloro-4-hydroxy-benzaldehyde, and trichlorosyringol. Bulter and Dal Pont (1992) developed a liquid chromatography method that was capable of separating 11 priority pollutants commonly found in BPE: 6-chlorovannillin, 2-chlorophenol, 4-chlorophenol, 4-chloro-3-methylphenol, 2,4-dichlorophenol, 4,6-dichloroguaiacol, 2,4,6-trichlorophenol, 4,5,6-trichloroguaiacol, 3,4,5-trichloroguiacol, trichlorosyringol and pentachlorophenol. These researchers determined the NCASI method to be applicable to bleach plant effluent and receiving waters.

The high molecular weight fraction of BPE is a heterogeneous mixture of small quantities of polysaccharide macromolecules and mainly of chlorinated residual lignin. Residual lignin in the pulp left after chip cooking is mainly removed during the first chlorine and caustic stages forming high molecular weight chlorinated organics while depolymerizing lignin and increasing the solubility (Martin et al, 1995). High molecular weight chlorinated organics have higher oxygen content (Sågfors & Stark, 1988) and lower levels of methoxy functional groups (Lindstrom & Osterberg, 1984, Sågfors & Stark, 1988) as a result of oxidation reactions with chlorine and other reactions occurring during bleaching. The chlorine content of the high molecular weight material is affected by the amount of elemental chlorine used during the first bleaching stage. Chlorine dioxide substitution decreases the level of chlorine in the high molecular weight fraction. When pulp is bleached with chlorine the carbon to chlorine ratio in the high molecular fraction is 9:1 compared to 45:1 with chlorine dioxide bleaching. During biological bleaching, Bourbonnais and Paice (1987) suggest that biosorption could be an important mechanism

of removal, however, little depolymerization and mineralization by microbes occurs. In receiving waters of BPE, sunlight induced minerlization is the most dominate method of degradation (Roy-Arcand & Archibald, 1993).

Martin and researchers (1995) examined the release of chlorophenolics from high molecular chlorinated organics from bleached kraft mill effluents. The high molecular weight fraction (> 400 daltons) was isolated from untreated, primary treated and secondary treated bleach plant effluent. The untreated sample was obtained from a plant that discharges untreated effluent combined from two separate bleaching lines using C_DNE_HD (N=neutralization) bleaching with 50% chlorine dioxide substitution and D_CE_ODED with 60% chlorine dioxide substitution. The treated samples were obtained from a plant bleaching hardwood pulp with bleaching sequence C_DEHD with 30% chlorine dioxide substitution and softwood pulp with bleaching sequence with D_CE_OHD with 60% chlorine dioxide substitution. Combined effluent was sampled after primary treatment with a settling chamber and after secondary treatment with a two cell aerated lagoon with 22 surface aerators. The high molecular weight chlorinated organics from the untreated BPE was incubated under abiotic conditions for 32 days and slowly released 4,5-dichloroguaiacol, 3,4,5-trichloroguaiacol, tetrachloroguaiacol, 6-chlorovanillin, and 5,6-dichlorovanillin. Under similar conditions Erikkson et al. (1985) also observed the release of chlorinated catechols. When high molecular weight chlorinated organics from the untreated BPE was alkaline treated at 50^oC a slightly higher concentration of guaiacols and substantial increase in chlorovanillins occured. In addition to the afore mentioned compounds the following compounds were released

under alkaline conditions, 4,6-dichloroguaiacol, 3,4-dichloroguaiacol, 3,4,5-trichlorocatechol and tetrachlorocatechol. Alkaline hydrolysis at 100^{o}C caused the concentration of chlorinated phenolic compounds to be increased 2 to 3.5 fold; however no additional compounds were detected. Comparisons were made between the concentrations of chlorinated phenols as free molecules and chlorinated phenols released by alkaline hydrolysis of high molecular weight fractions in primary and secondary treatment. There were 12 chlorinated phenols detected in the treated BPE samples. Secondary treatment was able to reduce the concentration of free chlorinated phenols by approximately 78% but had little to no effect on the amount released by alkaline hydrolysis. Exceptions were pentachlorophenol, which was reduced to an undetectable level and chlorovanillin and chlorosyringaldehyde released by alkaline hydrolysis increased by 44%.

Environmental Impact

The chlorinated organic compounds identified in bleach plant effluent are classified as dioxins, acidic, phenolic, and neutral compounds, which contribute to the oxygen demand, color, toxicity, mutagenicity and carcinogenicity (Bajpai & Bajpai, 1997). A number of these compounds have been found to be bioaccumulative, with over 2000ppm detected in the fish of receiving waters of BPE (Bajpai & Bajpai, 1997). Chlorinated phenolics, resin and fatty acids are the cause of the acute toxicity of BPE (Bajpai & Bajpai, 1997). Low molecular weight neutral compounds have been determined to be largely responsible for mutagenicity of BPE (Bjorseth et al., 1979).

Dioxins have been the focus of the public concerning bleach plant effluent components. Polychlorinated dibenzodioxins (PCDDs) and 2,3,7,8-tetrachlorinated dibenzodioxin (TCDD) are toxic with the later considered extremely toxic and bioaccumulative (Bajpai & Bajpai, 1997). The Canadian government recommends that fish with dioxin levels above 20ppt dioxin not be eaten, however, fish caught downstream of mills can contain up to 100ppt. Bioaccumulation in fish is a concern because it may move quickly up the food chain to humans. The EPA has reported that dioxins are a probable cause of cancer, disruption of regulatory hormones, reproductive and immune system disorders and abnormal fetal development in humans (Chem and Eng News, 1994).

The effect of the release of BPE on the receiving waters depends greatly on the amount of treatment of BPE, the dilution factor and the exchange rate of the receiving waters. Annergren et al. (1986) reported that the lethal effects on fish are limited to the area of discharge or receiving waters with poor exchange. These researchers studied several effluents utilizing different bleaching sequences for the effect of discharge on receiving waters with respect to dilution factor. Most of the effluent's lethal effects disappeared at dilution factors between 70 and 1000. However the previously commonly used bleaching sequence $(C_{95}D_{05})E_1DE_2D$ produced effluent with strong biological effects, even at high dilution factors. Even after dilution of 166-fold, low survival of fish, decrease in invertebrate density and parasitic infestation of stationary fish species was observed. These effects were observed at dilutions over 5000-fold, albeit at significantly lower levels.

A study was conducted on the effect of untreated effluent discharged into the Gulf of Bothnia, that portion of the Baltic Sea which lies between Sweden and Finland (Sodergren et al., 1987). The area studied was around a Swedish pulp mill that discharged untreated bleach plant effluent into the Gulf of Bothnia, considered to be a good representation of pulp mills in that area. Effects of untreated BPE near the discharge point were low fish biomass, change in species composition, reduction in reproduction, and disturbances in physiology. Toxicity had a marked effect 4-5 km away from the discharge point, with bioaccumulation in fish decreasing after 5 km away. Biological effects were observed in fish caught 8-10 km away from the discharge point, where dilution was 5000-fold. AOX effects on the receiving water was measured as solvent-extractable organically bound chlorine (EOCl) because this fraction of the AOX is made up of chlorinated, lipophilic, neutral compounds, primarily of low molecular weight, therefore of high environmental concern. Sediments contained high levels of EOCl 15 km away from the mill studied extensively, and 29 km away from another mill, 75 north of the studied mill. In fact, the sediment in large areas of the Gulf of Bothnia was found to have high levels of EOCl. The EOCl levels in sediment 20 to 50 km offshore from pulp mills were 10 times the levels far away from industry. Near the discharge point, fish were found to have 30 times the levels of EOCl as fish found in a reference area. These levels did not drop down to the reference levels until the mill had been shut down for 3.5 years. These extreme effects are due to the lack of treatment prior to release of BPE and a poor exchange rate in the Gulf of Bothnia, which has a residence time of several decades.

In the United States, nearly all bleached pulp mills utilize secondary treatment prior to discharge. The environmental impact of treated bleach plant effluent was studied for one year exposure at 20:1 dilution (Hall et al., 1985). While the aquatic flora and rainbow trout population increased, the productivity of benthic microinvertebrate pollution remained the same and there was no marked effect on the diversity index of histopathology of 20 different fish-tissue types. These effects are in stark contrast to the impact of untreated BPE in the Gulf of Bothnia, most likely due to increased treatment measures and better exchange in the receiving waters.

Usefulness of white-rot fungi in reducing pollution production due to bleaching of pulp

The paper making process depends on the removal of lignin from wood. Lignin is an amorphous, highly-polymerized substance responsible for cementing wood fibers together (Smook, 1992). It contributes to the rigidity and dark color of wood. Lignin removal begins in the pulping process and residual lignin is removed during bleaching. During bleaching, toxic, colored, chlorinated lignin is formed and found in effluent from bleaching plants. A class of white-rot fungus has the unique ability to depolymerize lignin. Lignolytic white-rot fungi possess a non-specific mixture of enzymes, which participate in lignin degradation. *Trametes versicolor* is a well studied white-rot fungus that has exhibited the capability to degrade lignin in nature and in several processes related to the pulp bleaching industry.

In order to understand the degradation of lignin by *Trametes versicolor* (a.k.a. *Coriolus versicolor* and *Polyporus versicolor*) Kawai and researchers (1988) studied the mechanism of lignin degradation by enzymes produced by *T. versicolor*. Purified laccase produced by *T. versicolor* catalyzed degradation of phenolic β-1 lignin substructure model compounds. It was determined that laccase catalyzed three types of reactions of the phenolic model structure via phenoxy radical molecules. The three reactions were C_α-C_β cleavage, alkyl-aryl cleavage and C_α oxidation. Laccase alone was not able to catalyze oxidation of non-phenolic lignin model substrates. However, lignin peroxidase has been shown to catalyze oxidation of non-phenolic lignin model substrates. This suggests that degradation of non-phenolic lignin by *T. versicolor* is the result of cooperation of laccase and lignin peroxidase. Further studies by Kawai and researchers (1999) showed that purified laccase in the presence of mediator compounds was capable of catalyzing the oxidation of a non-phenolic *β-O*-4 lignin model dimers. The mechanisms involved were β-ether cleavage, C_α-C_β cleavage, C_α oxidation, and aromatic ring cleavage. Bourbonnais et al. (1998) determined that while laccase alone is capable of catalyzing oxidation of lignin, the presence of mediator compounds increases the reactivity by coupling reactions involving the reactive intermediates of the mediator.

Reduction of pollution during pulping and bleaching

Use of elemental chlorine as a bleaching agent has been reduced considerably because of the high level of chlorinated organic compounds produced. The use of elemental chlorine is in direct relation to the amount of chlorinated organic compounds formed. In order to reduce the amount of toxic chlorinated compounds present in BPE one strategy currently

being used in industry is to reduce the amount of elemental chlorine used during the bleaching process. Reducing the amount of elemental chlorine used by substituting chlorine dioxide for chlorine and by using oxygen delignification are methods used to reduce the use of elemental chlorine. Graves et al. (1993) studied biological treatment, using aerated lagoons, carried out on effluents from the following sequences: C(EO), (D50+C50)EO, D(EO), OCE_o, O(D50+C50)EO, and OD(EO). It was found that oxygen delignification and substitution of chlorine with chlorine dioxide significantly reduced the COD, BOD, toxicity and color of effluent prior to treatment. Biological treatment further reduced COD, BOD, and toxicity. Stinchfield and Woods (1995) determined that complete substitution of chlorine with chlorine dioxide in the first bleaching stage reduced the concentration of chlorinated dioxins and highly chlorine-substituted chlorinated phenols to undetectable levels in the final bleach plant effluent. Currently, chloride dioxide substitution is used regularly and oxygen delignification is used where applicable.

Bleaching of pulp requires the removal of lignin and the ability of white-rot fungus to degrade lignin makes it a promising method to bleach pulp. *Trametes versicolor*, a lignolytic white-rot fungus, has shown promise in bleaching pulp. The fungal system of *T. versicolor* has shown the ability to brighten and delignify hardwood (Paice et al., 1989) and softwood kraft pulps (Reid et al., 1990, & Reid & Paice, 1994) as well as pulps produced by extended delignification and oxygen delignification (Reid & Paice, 1994). Up to two thirds of lignin was removed from softwood pulp treated with *T. versicolor* followed by alkaline extraction (Reid et al., 1990). White-rot fungi secrete several

oxidative enzymes and other mediator compounds (Bajpai & Bajpai, 1997). *Trametes versicolor* secretes a system of enzymes involved in delignification including lignin peroxidase, manganese peroxidase, laccase and cellobiose dehydrogenases (Archibald et al., 1997).

Lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase are directly involved in catalyzing lignin oxidation reactions (Bajpai & Bajpai, 1997). LiP and MnP oxidize Mn^{2+} to Mn^{3+}, the oxidizing agent which attacks lignin molecules. While LiP has been shown to have a catalytic effect in the degradation of model lignin compounds, it appears to play no role in lignin degradation while pulp bleaching (Addleman & Archibald, 1993). The peroxidase shown to be involved in pulp bleaching is MnP, evident by the strong positive correlation between MnP activity and pulp bleaching and the lack of LiP activity. Laccase performs one-electron oxidation of lignin while concurrently reducing oxygen to water (Bajpai & Bajpai, 1997). Cellobiose dehydrogenase makes lignin more bioavailable for degradation, aids in the mediation of enzyme catalyzed reactions, and prevents repolymerization of lignin released molecules (Archibald et al., 1997). The enzymes have been purified and isolated from culture media. The individual enzymes reacted with pulp and were shown to have either a lesser bleaching effect or no effect when reacted alone.

Purified laccase systems bleach pulp only when mediator compounds are added to the reaction. Laccase enzyme contains four copper atoms of copper per molecule and requires oxygen as a cosubstrate for the oxidation reaction to occur. The mediator seems

to be oxidized to a strongly oxidizing co-mediator which is the real bleaching agent (Bajpai & Bajpai, 1997). A laccase-mediator system was studied for bleaching of kraft pulp before and after oxygen delignification (Sealy & Ragauskas, 1998). Pulp was treated with the laccase-mediator system followed by alkaline extraction. Kraft pulp prior to oxygen delignification experienced 32% delignification and after oxygen delignification experienced 53%. Pulp bleaching with a similar laccase-mediator system with repeated treatment achieved 50-70% delignification (Bajpai & Bajpai, 1997).

Bleaching of softwood kraft pulps by MnP from *T. versicolor* in the presence of hydrogen peroxide and glucose was carried out (Paice et al., 1995). Delignification and demethylation of residual lignin occurred. MnP generated ortho-quinones during demethylation, which are compounds susceptible to oxidative ring opening resulting in hydrophilic soluble lignin. Manganese ions present in pulp were chelated by malonate or gluconate during the catalytic cycle, stabilizing the ions resulting in more reactivity.

The amount of chemicals used during bleaching of kraft pulp can be reduced by up to 70% with pretreatment by fungi (Reid & Paice, 1994b). The natural specificity of the fungal biological systems and the mild reaction conditions makes biological delignification a possible alternative to chemical bleaching with pressurized oxygen or ozone (Bajpai & Bajpai, 1997).

Phanerochaete chrysosporium and *Trametes versicolor* are two white-rot fungi that have been proven to have an effect on the delignification of kraft pulp. *P. chrysosporium*, after

10 days incubation, reduced the kappa number of hardwood pulp by 33%, where *T. versicolor* was able to achieve the same level of delignification after only 5 days incubation (Tran & Chambers, 1987, Kirkpatrick et al., 1989 & 1990). Brightness of hardwood pulp was increased by 6-25% with *T. versicolor* and only 0-11% with *P. chrysosporium* (Bajpai & Bajpai, 1997). Hardwood pulp is more susceptible than softwood pulp to treatment with *P. chrysosporium* and *T. versicolor*, due to the tendency of these fungi to attack hardwood over softwood in nature (Faix et al., 1985, Leisola et al., 1982, Reid et al., 1990).

Treatment of softwood pulp with *T. versicolor* achieved significant delignification without an increase in brightness. Brightness of softwood pulp and increased delignification was achieved after the fungal treated pulp was alkaline extracted (Fujita et al., 1991, Reid et al., 1990, Dawson-Andoh, et al., 1991). The first chlorination stage of the previous traditional bleaching sequence, CE_1DE_2D was replaced with a 5-day fungal (F) treatment using *T. versicolor* (Fujita et al., 1991). Hardwood pulp bleached by the FDED sequence achieved 82% comparable to the 88% brightness achieved with the previous traditional bleaching sequence. The effluent from a FCED bleaching sequence had 50% decrease in COD and 80% decrease in color compared to effluent from a previous traditional bleaching sequence.

Fungal treatment with free-cell cultures has several drawbacks. Agitated cultures increases oxygenation but often results in the formation of mycelia pellets in the pulp. Stationary cultures do not result in mycelia pellet formation in the pulp but limits the

amount of agitation and has shown to be diffucult to upscale (Bajpai & Bajpai, 1997). Pulp viscosity has shown to be negatively affected by treatment with free-cell fungal treatment due to the presence of fungal mycelia (Kirkpatrick et al., 1990). Studies with immobilized fungus (Kirkpatrick et al., 1989) and separated from pulp by filters (Archibald, 1992) have shown that direct physical contact between the fungus and pulp fibers is not necessary for bleaching to occur. Diffusional contact is required because cell-free filtrates from bleaching cultures had no effect in bleaching pulp (Archibald, 1992). Polyurethane immobilized cultures of *Trametes versicolor* bleached hardwood and softwood pulps at comparable rates and extents as free-cell cultures (Kirkpatrick et al., 1990, Reid et al., 1990). Advantages of immobilization include separation of pulp from fungal mycelia and increased pulp viscosity.

Several culture conditions must be considered to achieve delignification by white-rot fungus. *P. chrysosporium* and *T. versicolor* require growth substrate (such as glucose), nitrogen and oxygen to delignify pulp. When glucose is used as a growth substrate it serves as a carbon source to metabolize lignin and form carbon dioxide (Kirk et al., 1976) and as repressor of endo-1,4,β-gluconase, mannanase, xylanase, aryl-β-glucosidase, pectinase and cellulose which attack the carbohydrate fraction of pulp, thus protecting the pulp yield (Bajpai & Bajpai, 1997). Low nitrogen available or nitrogen depletion initiates the production of the ligninolytic enzymes of *P. chrysosporium* and *T. versicolor* (Nishida et al., 1995, Reid, 1979). *P. chrysosporium* cultures flushed with pure oxygen degraded lignin faster than cultures flushed with air, enhanced lignin degradation with increased oxygen partial pressure was also shown with *T. versicolor* cultures (Bajpai &

Bajpai, 1997). Enhanced lignin degradation by *T. versicolor* was shown with the addition of polydimethylsiloxane oxygen carriers (Ziomek et al., 1991).

Treatment of Bleach Plant Effluent

Current external treatment of effluent is done by primary treatment to remove suspended solids, secondary treatment to reduce the biological demand and acute toxicity, and in some cases tertiary treatment to remove color. Gravity sedimentation and flotation clarification are the main types of primary treatment to remove solids. Secondary treatment utilizes aerobic microorganisms to convert organic waste to carbon dioxide and water. Aerobic conditions are maintained by sufficient aeration and mixing. Secondary aerobic treatment methods include oxidation lagoons, aeration lagoons, activated sludge, or biological filtration. The type of treatment used depends upon the effluent characteristics, available area for external treatment and required BOD removal. A viable population of specially adapted microorganisms is required for all types of biological treatment. Colored compounds are often produced during secondary treatment and when necessary color must be removed by tertiary treatment. Rapid infiltration is one tertiary treatment method used, where wastewater is percolated through porous subsoil strata which remove color by adsorption and precipitation of colored compounds into the soil (Smook, 1999).

While traditional methods reduce color and acute toxicity, they have little effect on removing high molecular weight material. This high molecular weight material is important to take into account when assessing the long term effects of release of these

substances, because of its tendency to degrade over time releasing toxic compounds into the environment. Physicochemical methods as well as biological methods have been explored to deal with high molecular weight material.

Ultrafiltration is one method implemented to reduce the amount of high molecular mass compounds to be treated. Boman et al. (1991) used three different biological methods to treat BPE made up of E-stage effluent filtered using membranes with a cut-off of 8000 Dalton and (C+D)-stage effluent. Treatment with an aerated lagoon with solids recycle gave the best results with reductions of 66% in AOX, 72% in COD, and 95% in BOD. Yao et al. (1994) investigated ultrafiltration treatment of C stage and E stage effluents from a bleach plant utilizing a CEDED sequence. Membranes in series were optimized with the combination of three membranes in series with cut-offs of 10^5-$2x10^5$, $1.5x10^4$, and $6x10^3$ Dalton giving average reductions in COD of 80% and in AOX of 88%. While ultrafiltration shows promising results, the filtrate must be handled and it is a costly method. Ultrafiltration in combination with sorption/reverse osmosis (SRO) to treat bleach plant effluent was studied by Burhem and researchers (1991). Effluents treated with ultrafiltration experienced a 55-60% reduction in COD and a 65-75% reduction in AOX. These researchers found that combining the ultrafiltration and SRO techniques increased removal efficiencies of COD, AOX and chloride ions in alkaline extraction stage effluent.

Oxidation treatment methods provide insight into the degradation potential by oxidative reactions. Strong oxidizing techniques have been explored for their effects on

decolorization of bleach plant effluent. Treatment of bleach plant effluent by use of ozone revealed that there are three types of oxidizable compounds present in BPE: uncolored compounds, colored compounds that react quickly and colored compounds that react slowly (Prat et al., 1989). The colored compounds that react quickly with ozone are the majority of compounds present, make up about 90% of the color, and are considered to be easily degradable. Colored compounds that react slowly are considered to be more difficult to degrade and while decolorization does not improve significantly due to the reaction of these compounds with ozone, removal may provide some added benefit. Supercritical water oxidation (SCWO) was used by Cooper et al. (1997) to examine the decolorization and dechlorination of BPE. Water has unique abilities at temperatures above 374^{o}C and pressures above 22.1 MPa to completely dissolve most organic material and oxygen. Filtrates from DO and EOP stages were used as the BPE. The BPE was exposed to the SCWO and the resulting liquid effluent was virtually colorless and AOX was reduced by 99.47%. SCWO successfully oxidizes BPE to produce a colorless, neutral liquid with low organic values and very low AOX. Oxidation with sodium hypochlorite in conjunction with anaerobic treatment of bleach plant effluent was studied by Anderson and researchers (1994). Treatment by sodium hypochlorite oxidation reduced color of BPE by 90%, however this color reduction was accompanied by a 50% increase in AOX. Subsequent anaerobic treatment reduced the AOX back to the original level and slightly raised the color. Combined treatment resulted in 80% overall color reduction.

Catalysis driven oxidation has been examined as a technique to treat BPE. Bham and Chambers (1997) used a ferrous ion - hydrogen peroxide system, called Fenton's Reagent, to study the degradation of high molecular weight chlorinated aromatics and aliphatics in BPE. The Fenton's reaction converts Fe^{2+} to Fe^{3+} while producing reactive hydroxyl radical, with high oxidation potential, from hydrogen peroxide. Reaction of bleach plant effluent with hydrogen peroxide alone resulted in 40% color reduction and negligible reduction in AOX. Reaction of bleach plant effluent with hydrogen peroxide and the addition of Fe^{2+}, in the form of ferrous sulfate, at a H_2O_2/Fe^{2+} ratio of 50:1 resulted in 96% color reduction and 89% AOX reduction when the reaction was carried out at room temperature. When the reaction was carried out at 60^oC, the temperature of fresh effluent, the reduction of AOX was enhanced and reaction took place at a more rapid rate. One drawback of this method is that Fe^{2+} forms complexes with compounds present in BPE. At high concentration of Fe^{2+} it becomes difficult to remove these compounds. A technology called wet air oxidation (WAO) with metal catalysts was examined for its effectiveness for treatment of combined bleach plant effluents (Zhang & Chuang, 1999). Noncatalytic wet air oxidation requires long reaction times and extremely high temperatures and pressures, in the range of 473-573K and over 10MPa. Using a Pd-Pt-Ce/alumina catalyst resulted in milder reaction conditions, temperatures ranging from 403-443K at 1.5MPa. Oxidation of compounds present in bleach plant effluent depends on the catalyst loading, the initial concentration of organic carbon compounds, and reaction pH. The addition of catalyst increased total organic carbon removal to 65% compared to 10% without catalyst, and 99% color removal compared to 79% without catalyst. Even though addition of catalyst improves reaction conditions, the

high reaction temperature and pressure as well as high cost of catalysts are drawbacks of this type of treatment.

Reducing the level of chlorinated organics prior to biological treatment by chemical treatment was investigated by Francis et al. (1997). C-stage effluent from a bleaching plant utilizing C/DE$_o$DED sequence was screened and chemically treated with clarified green liquor as a source of sulphide prior to biological treatment. Biological treatment was carried out at an existing air activated sludge pilot plant. Combined chemical and biological treatment reduced AOX 53-59%, which is greater than the AOX reduction of 31-42% experienced by biological treatment alone.

Fungal treatment of bleach plant effluent

While physicochemical methods shown promising results in treating BPE, these methods are often costly due to use of expensive chemicals and in some cases extreme reaction conditions requiring expensive equipment. An additional drawback is that new treatment problems occur with these methods, such as filtrate or metal conjugates being formed. Biological treatment methods are more feasible solutions for industrial treatment of BPE. Advantages of biological treatment include mild reactions conditions and low cost. Current treatment methods such as aerated lagoons and activated sludge plants employ the use of aerobic bacteria. Aerobic treatment methods have been shown to effectively reduce COD, BOD, AOX, and toxicity. Approximately 75-95% of chlorophenolics are removed by aerobic treatment. Aerobic treatment methods do not reduce color or have an effect on the degradation of high molecular weight chlorophenolics (Bajpai & Bajpai,

1997). Anaerobic degradation has not traditionally been used; however the resistance of high molecular weight, chlorinated organic compounds to aerobic degradation has made anaerobic treatment an interesting method to degrade high molecular weight material.

Fitzsimons et al. (1990) conducted continuous studies using an anaerobic reactor seeded with anaerobic sediment bacteria obtained from a wastewater receiving basin, followed by an aerobic bioreactor. The BPE treated was obtained from a stage using chlorine plus chlorine dioxide water (CD water) and alkaline extraction water (E_1 water). The BPE was fractionated into four fractions: fraction 1 – MW > 20,000, fraction 2 – 6000 < MW < 20,000, fraction 3 – 2000 < MW < 6000, and fraction 4 – MW < 2000. Reductions of COD and AOX were measured. Fraction 1, MW > 20,000, experienced up to 56% reduction of COD and up to 14% reduction of AOX. Fraction 2, 6000 < MW < 20,000, experienced up to 69% reduction of COD and up to 34% reduction of AOX. Fraction 3, 2000 < MW < 6000, experienced up to 84% reduction of COD and up to 58% reduction of AOX. Fraction 4, MW < 2000, experienced up to 87% reduction of COD and up to 66% reduction of AOX. Anaerobic dechlorination was not very effective on the chlorolignins of highest molecular weight. These researchers earlier postulated that anaerobic dechlorination of chlorolignins is due to a combination of energy metabolism, growth, chemical hydrolysis and adsorption and/or solubilization (Fitzsimons et al., 1989).

Anearobic treatment has also been used to investigate the degradation of specific chlorinated phenols, guaiacols, catechols and vanillins. Parker et al. (1993) obtained BPE

from a bleach plant using the bleaching sequence of $C_DE_ODE_PD$. An anaerobic reactor, utilizing a modified sludge blanket was used to treat the BPE. The biomass was obtained from a pilot-scale up flow anaerobic sludge blanket unit located at the Wastewater Technology Centre. This study targeted 30 different compounds, of which only 20 were present in the BPE studied. Treatment was found to have an average removal efficiency of 92.7% of these specified compounds.

Even though anaerobic treatment of bleach plant effluent has an effect on the degradation of high molecular weight compounds, the anaerobic nature makes the required environment a drawback. Another biological treatment method examined for treatment of BPE is the use of fungal material. White-rot fungi specifically are effective at degradation of lignin, lignincellulose, and chlorinated phenolic compounds. Lignin degradation by white-rot fungi in nature occurs during secondary metabolism and under nutrient limited conditions with a carbon co-substrate and oxygen available to the fungi. Lignolytic enzymes are secreted by white-rot fungi under these conditions, even when lignin is not present. Degradation of other compounds occurs under conditions similar to conditions required for lignin degradation. Fungal treatment offers several advantages such as mild reaction conditions and low cost.

A well studied white-rot fungi is *Phanerochaete chrysosporium*. Eaton and Chang (1980) found that cultures of *P. chrysosporium* removed 60% of color from E1-stage effluent after four days. Color removal was not observed until 2 days after inoculation, indicating that decolorization takes place after the growth phase. Prasad & Joyce (1993)

studied the treatment of E1 stage effluent from a bleach plant utilizing a CE1DE2D sequence, by comparing a rotating biological contactor, inoculated with *Phanaerochaete chrysosporium*, to an aerated lagoon, both followed by an anaerobic digester. It was found that the fungal-anaerobic treatment underwent higher reduction of AOX compared to the treatment by the aerobic / anaerobic system (65% vs. 49%). High molecular weight chlorinated organic material is biodegradable by the white-rot fungus. A separate study by Jaspers & Penninckx (1996), examined *P. chrysosporium* for decolorization and degradation of chlorinated compounds. E1-stage effluent was treated by being inoculated with preformed pellets of *P. chrysosporium*. The pellets were shown to strongly absorb color and AOX from the effluent. *P. chrysosporium* has been determined to be capable of degrading and mineralizing low molecular weight chlorinated compounds and degrading high molecular weight chlorolignins derivatives present in E1-stage effluent. COD is reduced by this fungus by degrading chlorolignins to CO_2, chloride and color is reduced by degrading the color bodies and chromophoric structures, and total organic chlorine is converted to inorganic chloride (Bajpai & Bajpai, 1997). Lignin peroxidase and manganese peroxidase are secreted by *P. chrysosporium*, with manganese peroxidase having a more important role in bleach plant effluent (Micheal et al., 1991).

Trametes versicolor is another white-rot fungus that has been studied for treatment of BPE. Several researchers have found *T. versicolor* to outperform other white-rot fungi when experiments are conducted comparing white-rot fungi for their effectiveness for treatment of bleach plant effluent (Livernoche et al., 1983; Bajpai et al., 1993). Livernoche and researchers (1983) determined *T. versicolor* removed color from BPE

more rapidly than *P. chrysosporium,* within 2 days of incubation compared to 4 days. They also observed the mycelium of *T. versicolor* did not adsorb chromophoric compounds, indicated by little change in mycelium color, whereas the mycelium of *P. chrysosporium* has been shown to strongly adsorb chromophoric compounds. Studies to determine if mycelium of *T. versicolor* absorbed color or aromatic substances were carried out by Marton et al. (1969). They found that about half of the color removed and about one-quarter of removed aromatic substances were alkaline extracted from fungal cells. These observations indicate that decolorization by *T. versicolor* is not limited to secondary metabolism and decolorization by *T. versicolor* is due to biochemical action as well as physical adsorption. Another advantage *T. versicolor* has over *P. chrysosporium* is that a substantial amount of laccase is secreted by *T. versicolor* which is not observed with *P. chrysosporium* (Archibald et al., 1990). Similar to *P. chrysosporium*, *T. versicolor* requires oxygen and a carbon source to decolorize BPE. Oxygen serves several purposes, it is precursor for active oxygen species involved in oxidation reactions and it acts as a substrate for phenol or sugar oxidases and for the mitochondrial respiration chain (Archibald et al., 1990). Carbon sources that result in the most efficient color removal by *T. versicolor* are mono- and disaccharides. Glucose has been determined be the sugar that results in the most efficient color removal (Bajpai et al., 1993) and high decolorization was obtained with industrial sugar sources final molasses and brewery cake (Archibald et al., 1990).

Decolorization of BPE by *T. versicolor* is a result of the fungus's ability to degrade and mineralize high molecular weight chlorolignins compounds as well as low molecular

weight chlorinated organics. Livernoche et al. (1983) found that within 6 days of incubation of kraft pulp BPE with mycelium pellets of *T. versicolor*, in the presence of sucrose, 60% color removal was achieved. Archibald et al. (1990) and Bajpai et al. (1993) found pellets of *T. versicolor* incubated with kraft pulp BPE achieved 80% color removal within 3 days of in the presence of glucose. In addition to destruction of visible chromophoric compounds, Archibald et al. (1990) observed substantial mineralization of high molecular weight organic chlorine by *T. versicolor*. These researchers also found decolorization to be unaffected by the presence of a number of compounds reported to suppress or enhance the secretion of the lignolytic enzymes. This suggests that decolorization is accomplished intracellularly by the fungal material. At optimum conditions, at 30^{o}C and pH 4.5-4.5, Bajpai et al. (1993) observed 93% color reduction and 35% COD reduction in kraft pulp BPE incubated with pellets of *T. versicolor*. Mycelium pellets of *T. versicolor* in batch studies with kraft pulp BPE lost activity rapidly, while mycelium pellets of *T. versicolor* with kraft pulp BPE in continuous studies did not lose activity for over 30 days of decolorization (Royer et al., 1991; Bajpai et al., 1993).

Effluents resulting from industrial processes other than kraft sulphate pulping and bleaching, such as from sulphite pulping, thermomechanical pulping or soda pulping of materials other than hardwood and softwood, such as wheat straw and rice straw, have been studied for decolorization by mycelia pellets of *T. versicolor*. Bergbauer et al. (1991) inoculated combined bleach plant effluent from sulphite pulping with pellets of *T. versicolor* following the degradation of chlorolignins with color and AOX reduction.

Sulphite pulping effluents contain more recalcitrant colored compounds than effluents from kraft sulphate pulp. They found that with 90% reduction in color and 45% reduction in AOX there was over 50% degradation of total aromatic compounds and depolymerization of high molecular weight compounds. Martin and Manzanares (1994) evaluated effluent resulting from soda pulping of wheat straw for treatment by *T. versicolor*. Wheat straw is a surplus cereal product and pulping in an alkaline environment at mild conditions resulting in high quality pulp. The resulting effluent cannot be recycled because of its high silica content. The effluent was collected after treatment by aerobic and anaerobic methods, which have little effect on reducing effluent color. Mycelia pellets of *T. versicolor* removed over 50% of color in 8 days. Effluent from a pulp mill utilizing agriresidues, wheat straw and rice straw with *Saccharum munja*, was treated with mycelia pellets of *T. versicolor* (Mehna, et al., 1995). At optimum pH 4.5, mycelia pellets of *T. versicolor* incubation with E1-stage reduced effluent color by 88% after 4 days and up to 92% color reduction by 7 days with 69% reduction in COD and 68% reduction in BOD. Effluent from a bagasse based pulp-paper mill was inoculated by mycelia pellets of *T. versicolor* and evaluated by Modi et al. (1998). This effluent was supplemented with ammonium nitrate and within two days a 40% reduction in color was observed due to the native microflora. When inoculated with *T. versicolor* and supplemented with glucose and ammonium nitrate the effluent experienced 60% reduction in color within four days. Cai et al. (1998) compared 19 species of white-rot and brown rot fungi for treatment of whitewater from a Canadian thermomechanical pulp mill for newsprint. *T. versicolor* grew well in the whitewater and reduced the total organic carbon and lipophilic extractives. These findings show that *T.*

versicolor has characteristics that make it well-suited to effectively treat a number of waste effluents.

Immobilization is a valuable biotechnology used with enzymes, microbial cells, plants cells, cellular organelles and animal cells (Samojedny, 2000). Advantages of immobilized cell systems over free cell systems include the prevention of biomass washout, complete separation of product from fungal material, improved activity, and enhanced robustness to operational conditions. *T. versicolor* was used in a rotating biological contactor (RBC) to treat alkaline extraction stage effluent of a bleach plant using sulfite dissolved pulp (Van Drissel & Christov, 2001). The RBC was run at a retention time of 18hrs. Within 23 hrs of reactor operation, *T. versicolor* removed 61% of color from BPE. Removal of color remained between 53% and 61%, while 40% removal of AOX and 38% reduction in toxicity was observed. Reactor biomass was found to absorb 45% of the color removed. When *T. versicolor* was immobilized in beads of calcium-alginate gel, 80% color removal was achieved within three days of incubation compared to 60% removal in six days with free cells (Livernoche et al., 1983). Recycled immobilized beads of *T. versicolor* were effective at color removal. During decolorization of BPE by immobilized beads of *T. versicolor*, the reactor pH decreased from 5.7 to 3.6 due to the formation of organic acids, however decolorization occurs in a wide range of pH environments, with optimum decolorization occurring between pH 4.5-5.5 (Livernoche et al., 1983; Bajpai et al., 1993). Martin & Mananares (1994) found plastic immobilized *T. versicolor* removed over 90% after 14 days compared to 50% after 8 days with free cells. These immobilized cells were recyclable and removed up to 80% of color when re-used.

During decolorization, these researchers also observed the reactor pH decrease from 5 to 3.5. Adsorption of color to fungal material was found to account for 50% of initial color when 80% of initial color was removed; therefore 30% of the initial color was biochemically degraded. The content of alkaline soluble lignin was decreased by 25%. Immobilization of *T.versicolor* allows for improved activity over longer periods of time and makes the fungal material robust to environmental fluctuations and recyclable. Industrial applications of immobilized *T.versicolor* are feasible due to the ability of this technology to treat a wide range of wastewaters and treated product can be easily separated from mycelium.

Pallerla and Chambers (1996, 1997a, 1997b) studied the immobilization of *T. versicolor* to treat caustic bleach plant effluents in a fluidized bed reactor. These researchers examined the effectiveness of *T. versicolor* immobilized in calcium-alginate gel beads and polyurethane foam cubes to decolorize and detoxify kraft bleach plant effluent in a continuous fluidized bed reactor. The bioreactor consisted of a glass cylinder with a working volume of 600ml that was continuously aerated using an air dispersion tube. The feed solution was continuously fed to the reactor and was composed of E_o stage (alkaline extraction stage enriched with oxygen) effluent, glucose, ammonium nitrate, potassium phosphates, vitamins and minerals, and adjusted to pH 5. Product was continuously withdrawn from the reactor. The inlet feed solution and the inlet air, prior to reactor entry, were sterilized by passing through an in-line micro filter and an air purifying membrane filter, respectively. The bioreactor performance was evaluated by determining the effects of several parameters on decolorization and detoxification. Some

of these parameters include immobilization technique, residence time, feed glucose, fluidizing media and effluent concentration.

Using calcium-alginate gel bead immobilized *T. versicolor* to treat E_o stage effluent in the fluidized bed reactor effective color and AOX removal was achieved (Pallerla & Chambers, 1996). At 24 hour residence time, 72% reduction in color and 49% reduction in AOX was achieved. The reactor was found to also perform effectively at shorter retention times. However at residence time of 10 hours color removal decreased to 61% and AOX removal increased slightly to 54%. Effluent color concentration had a marked effect on color removal. Treatment of effluent of higher color concentration resulted in higher color removal rates and effluents of lower color concentration resulted in lower color removal rates, however removal of AOX increased. At low concentrations, chromophoric material, such as chlorolignins, may be less available as a substrate for the fungal material and extracellular activity. Examination of the effect of reactor pH on decolorization of BPE indicated that the optimum pH for growth was different from the optimum pH for decolorization. The former was achieved by the formation of acidic products and removal was most effective when the pH environment was allowed to come to a natural pH as opposed to being pH controlled at the optimum pH or higher. Evaluation of fungal growth in the calcium-alginate beads determined that fungal growth was restricted to a limited thickness at the outer surface area of the bead and extended out of the polymer bead. Growth did not occur into the interior of beads, possibly due to diffusional limitations or depletion of oxygen or other nutrients in the inner layers of the beads. The beads were determined to lose 0.8% of activity a day.

Polyurethane foam immobilization was another technique used by Pallerla and Chambers (1996, 1997b) to study decolorization and detoxification of caustic stage effluent by *T. versicolor*. This technique could be an improvement upon calcium-alginate gel immobilization which is susceptible to degradation and diffusional limitations resulting in localization of mycelia growth of limited thickness on the outer surface area of the bead. Several characteristics make polyurethane foam immobilization a suitable support for cell entrapment. The open celled structure of the foam allows for non-diffusional limitations of fungal growth and the mechanical strength makes the foam entrapped mycelium stable against pH and media variations, therefore well suited for use in bioreactors. Batch studies using cubes of urethane immobilized *T. versicolor* to treat E_o stage effluent resulted in 77% color reduction in 7 days, however cell-free cubes resulted in 70% color reduction in 7 days. After 7 days, however, the cubes with *T. versicolor* continued to effectively decolorize effluent and the cell free cubes had little effect on color removal. These findings indicate that initially, absorption into the polymer matrix accounts for a major amount of color removal, then over time, the polymer matrix is saturated with color bodies and the fungus becomes acclimated to the effluent and biochemical degradation plays the largest role in color removal.

Polyurethane immobilized *T. versicolor* was utilized in the continuous fluidized bed bioreactor described previously to treat E_o stage effluent (Pallerla & Chambers, 1996, 1997b). The continuous fluidized reactor with foam immobilized *T. versicolor* was operated for 32 days, at which time no decrease in activity was observed. Ca-alginate beads were found to lose activity by day 22 of reactor operation. Color removal was

determined to follow first order kinetics and was a strong function of feed glucose concentration up to 0.8wt%. At residence time of 24 hours, the effluent color was reduced by 69% and 53% reduction in AOX, when the feed color was 2476CU. Shorter residence times were evaluated for decolorization and detoxification of effluent with feed color of 2626CU. Little decolorization or detoxification occurred at residence time of 6 hours (36% reduction in color and 31% reduction in AOX). Increasing the residence time to 9 hours significantly improved treatment with 65% reduction in color and 57% reduction in AOX observed. At residence time of 20.5 hours color removal increased to 76% and removal of AOX did not change significantly. Treatment of concentrated feed effluent was studied for the effect on decolorization and detoxification. Effluents with feed color of 2626CU, 3143CU and 3756CU were treated by the continuous fluidized bed reactor with polyurethane immobilized *T. versicolor*. Removal of AOX decreased with increasing feed color concentration. Color removal efficiency increased from 63% to 67% when feed color increased from 2626CU to 3143CU, then fell drastically when the feed color was at 3756CU. The different trends of color removal and AOX removal with increased effluent concentration are likely due to the different components of color and AOX therefore, different mechanisms of removal by the bioreactor system. Color is due to the presence of water-soluble, polymeric, chlorinated, oxidized lignin degradation fragments, mostly of high molecular weight compounds with conjugated double bonds. AOX consists of a complex mixture of chlorinated phenols, anisols and other chloro-organics. The highest color concentration seemed to have an inhibitory effect on the bioreactor system, probably due to one or more compounds present in kraft liquor and/or the presence of large non-metabolized molecules. Pure oxygen and air were compared

for use as the fluidizing media. When pure oxygen was used as the fluidizing media 81% color removal and 68% AOX removal was observed, compared to 76% color removal and 59% AOX removal when air was used. Fluidization with oxygen also allowed for more effective treatment of highly concentrated effluents. When the feed color was 5025CU, 73% color removal and 81% AOX removal was observed. These results show that biocatalyst stability and activity increased when utilizing pure oxygen as the fluidizing media. However, due to the high cost of pure oxygen its use is not economical and using air as the fluidizing media provides effective treatment.

Growth of *T. versicolor* in foam cubes of polyurethane was evaluated. The foam was found to develop an interconnected pocket structure and fungal growth developed well within the pocket structure. This type of growth allows for excellent mycelia retention and discourages the mycelia mat formation on outside of the cubes. However, when pure oxygen was used as the fluidized media, growth occurred inside as well as outside of the cubes. Growth within the foam continued until attaining a steady-state level, after which little growth occurred. During this lag/growth phase, adsorption is believed to play a major role in removing color, after the fungus reached a steady-state growth level biodegradation plays the largest role in color reduction. Growth of cubes in growth media prior to exposure to BPE was suggested as a method to reduce lag time and increase biodegradation.

Samojedny (2000) evaluated the fluidized bed reactor utilizing polyurethane immobilized *T. versicolor* for the effects of feed pH and feed glucose on decolorization and

detoxification of bleach plant effluent. This bioreactor system was also studied for extended continuous operation and use of regenerated biomass in the reactor. The range of feed pH assessed was between 1.5 and 5. At all pH values studied decolorization was observed with highest decolorization achieved at pH 2 and pH 2.5. The percentage of AOX removal ranged from 35%-47% at the pH values studied except at pH 1.5, where no AOX was removed from the effluent. Based on the combined decolorization and detoxification effectiveness, optimum feed pH 3 was chosen for further studies. The effect of feed glucose concentration on decolorization and detoxification was evaluated by ranging the glucose levels from 1g/L to 8 g/L. At feed pH 3, decolorization increased with increasing glucose concentration from 1 g/L to 5 g/L, after which increasing glucose concentration, decolorization remained constant. Maximum decolorization of 77% was achieved at feed pH 3 and feed glucose 5 g/L. Maximum AOX removal occurred at glucose 8g/L.

Extended operation and regeneration experiments were carried out at feed pH 5 and feed glucose 8 g/L. Extended operation studies continued for 76 days, with consistent decolorization throughout operation and AOX removal became sporadic at day 27. When the reactor reached a severe state of instability this signified the end of productive degradation by the bioreactor system. At this time, the fungal cubes were regenerated by removing the cubes from the reactor and rinsing them with fresh effluent, prior to replacing them in a clean reactor. The reactor was operated under batch growth conditions until the fungal material was revived and steady-state conditions were achieved. Use of the regenerated fungal cubes actually increased color removal from

66% to 80%, and AOX removal was 42%-46%. During the extended continuous reactor operation the fungal cubes were regenerated twice.

Enzymatic treatment of bleach plant effluents has been evaluated using purified enzymes from white-rot fungus. Laccase from *T. versicolor* was used to study dechlorination of chlorophenolic compounds in pulp and paper mill effluents by Arcand and Archibald (1991). These researchers found most of the laccase secreted by *T. versicolor* could partially dechlorinate a number of chlorophenolics and dechlorination was accompanied by extensive polymerization of the substrate. Fungal peroxidase from *P. chrysosporium* was found to degrade several chlorinated organics present in bleach plant effluent, except PCP. Apparently, enzymatic action oxidizes chromophoric compounds, therefore removing the color from bleach plant effluent. Aeration in the presence of laccase reduced 75%-99% of chlorophenolics in bleach plant effluent depending on the source of effluent and mixture of chlorophenolics present (Forss et al., 1987). Bajpai and Bajpai (1997) report that a patented process was developed to treat effluent from pulp and paper mills using laccase from *C. versicolor*, with the addition of phenolic and nonphenolic compounds, with the continuous addition of oxidizing species such as air, pure oxygen or hydrogen peroxide. The lignin present in the waste effluent was almost all polymerized, with 70%-90% of lignin formed into insoluble compounds that can be removed by flocculation or filtration.

MATERIALS AND METHODS

A fungal treatment process has been shown to decolorize and detoxify caustic extraction stage bleach plant effluent (BPE). This treatment process utilizes polyurethane immobilized *T. versicolor* in a single stage bioreactor. Using PCP as a model compound, a two-stage bioreactor was developed that is able to convert PCP into less toxic compounds. The first stage fungal reactor, which uses polyurethane immobilized *T. versicolor* to decolorize and detoxify caustic extraction stage bleach plant effluent (BPE), is a fluidized bed reactor. Untreated BPE and the supernatant from the fungal reactor are continuously fed to the second stage reactor, a continuous extracellular fluid reactor, where product is continuously removed. Both stages of the bioreactor system are aerated to facilitate fungal growth, enzymatic reactions and provide mixing.

The bioreactor system is a two-stage system consisting of two multiphase bioreactors. The first stage reactor was a three phase fungal reactor with a working volume of 600 ml which utilizes polyurethane immobilized *T. versicolor*. The fungal reactor was aerated and fluidized by flowing compressed breathing air (BOC Gases) through an in-line air sterilizing membrane filter (pore size 0.2μm, Millex-FG_{50}, Millipore) and a glass dispersion tube (Ace Glass). A constant flow of feed solution was fed through an in-line micro-filter (pore size 0.2μm, Dominick) to ensure the feed solution was free of contaminates. The reactor solution was constantly withdrawn from the fungal reactor and passed through a hold-up basin, a 250 ml polyethylene sample container with 100 ml working volume, where biomass and high molecular weight compounds precipitate out of

the extracellular fluid prior to being fed to the second stage reactor. The second stage reactor was continuous extracellular fluid reactor (CEFR) with a working volume of 1000ml. The CEFR was constantly stirred and aerated by flowing compressed breathing air (BOC Gases) through an in-line air sterilizing membrane filter (pore size 0.2μm, Millex-FG_{50}, Millipore) and a glass dispersion tube (Kimble) to provide the necessary oxygen for the enzymatic reactions. Fresh make-up feed to the CEFR was fed through an in-line micro-filter (pore size 0.2μm, Dominick) to ensure the feed solution was free of contaminants. CEFR product was continuously withdrawn and held in a 1gallon storage container until full, then stored at 4^{o}C until processed and analyzed. Both reactors' designs were modified by adding an air venting system, consisting of a glass tube, silicon tubing and a sterile air venting filter (pore size 0.2μm, Millex-FG_{50}, Millipore), to prevent contamination of the reactors. All materials used in the reactor systems were autoclaved at 121^{o}C for 20 min prior to use. The first stage fluidized bed fungal reactor system has been successfully used in a number of studies in bleach plant effluent detoxification and decolorization by Pallerla and Chambers (1996,1997ab, 1998) and Samojedny (2000).

Feed Composition

Caustic E_o stage filtrate was obtained from the filtrate line at an integrated paper mill. For the reactor set up for treatment of full strength BPE the feed to fungal reactor was a solution of filtered E_o stage filtrate with 0.6% dextrose, 0.16% $(NH_4)_2SO_4$, 0.3% K_2HPO_4, and 0.2% KH_2PO_4 (w/v) to provide carbon, nitrogen and phosphorus (Bergbauer et. al., 1991). A vitamin supplement final concentration was 0.2 mg/L each of D-pantothenic acid, riboflavin, folic acid, pyridoxal, thiamine-HCl, nicotinic acid and

biotin was added to fungal feed solutions. Similarly, a mineral salt supplement was added to the final concentrations of 0.1 μM $NiCl_2$, 5μM $CoCl_2$, 20 μM $MnCl_2$, 5μM $ZnCl_2$, 1 μM $CuSO_4$, 40 μM Na-citrate, 20 μM $FeSO_4$, and 0.5 μM $(NH_4)_6Mo_7O_{24}$. The pH of the fungal feed reactor solution was adjusted to pH 5 with HCl. The make-up BPE feed to the continuous extracellular fluid reactor was a solution of filtered E_o stage filtrate with 0.16% $(NH_4)_2SO_4$, 0.3% K_2HPO_4, and 0.2% KH_2PO_4 (w/v). The pH of the makeup feed solution was adjusted to pH 3.5 with HCl.

For the reactor set up to study the effects of small amounts of BPE added to a PCP solution on protein production the feed to the fungal reactor contained either 1% v/v, 2.5% v/v, 5% v/v or 10% v/v BPE with distilled water and PCP stock solution added to final concentration of 2.5mg/L assuming the BPE adds a negligible amount of PCP. The solution was supplemented as described above. The make up feed to CEFR was identical to the make up feed described in Chapter II.

Color Measurement

The color of bleach plant effluent and treated bleach plant effluent samples was determined by means of the NCASI Method Color 71.01: Color measurement in pulp mill wastewaters by spectrophotometry. Platinum cobalt color standard (500 PCU) was diluted to obtain standard solutions of 25, 50, 100 and 250 color units (PCU). These standard solutions were stored in amber glass bottles at room temperature. The color standard curve was obtained by measuring the absorbance of each standard sample at 465nm and plotting the absorbance (nm) versus color units (PCU).

The color of bleach plant effluent samples was determined by adding approximately 110mg buffer powder (pH 7) to a 10 ml sample and adjusting the sample pH to 7.6 ± 0.05 with 1 N NaOH. The samples were then filtered through a pre-wetted 0.8micron filter using vacuum filtration. The filtered sample was placed in 2 cuvettes and absorbance was measured at 465nm. The color units were determined from the standard curve. All other methods and analytical procedures were detailed in Chapter II.

Laccase Assay

Laccase activity was measured by the change in absorbance when 100µl reactor solution was reacted with 100µl 0.1mg/ml ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6'sulfonic acid) (Sigma) in phosphate-citrate buffer with sodium perborate solution at pH 5.0. A KC4 spectrophotometer was used to measure the change in absorbance at 420nm at 30^{o}C for 30min. Laccase activity was determined by comparing the change in absorbance per minute achieved by a standard solution of purified laccase from *T. versicolor* (Fluka)

Experimental Approach

Products formed by *T. versicolor* fungal and extracellular fluid reactor treatment of Eo-stage effluent were studied by running the fungal and extracellular fluid reactor continuously at 18 hr retention times for 43 days. Samples were removed from the reactors periodically and the amount of protein bound and unbound PCP and TCHQ/TCBQ were identified by GC/ECD. The reactor pH, protein concentration and color concentration was measured for each sample.

The effect of BPE on protein production by *T. versicolor* was studied by adding 1%, 2.5%, 5% or 10% BPE to a 2.5mg/L PCP feed solution to the FR. The reactor was allowed to stabilize at each concentration of BPE for 5 days before samples were taken. Samples from the FR and CEFR were measured for the concentration of unbound PCP, unbound TCHQ, protein bound PCP and protein bound TCHQ. Each sample was measured for laccase activity and protein concentration. A reactor set-up without BPE added to the 2.5mg/L PCP feed solution to the FR was running simultaneously at the same conditions as the reactor set-up with BPE to the feed solution to compare the protein concentrations and enzyme activities.

RESULTS AND DISCUSSION

Bioremediation of bleach plant effluent was studied in the continuous fungal and extracellular fluid bioreactors by determining the concentrations of unbound and bound PCP and TCHQ. Reactor pH, protein concentration, and color concentration were measured for each sample taken.

Reactor conditions

On the fourth day of reactor operation, the first samples were taken and the reactor ran for 43 days, on which day the last sample was taken. Table 22 gives details of reactor conditions for each sample taken from the fungal reactor (FR) and the continuous extracellular fluid reactor (CEFR). During the third week of FR operator the pH stabilized and remained relatively constant, between 3.51 and 3.61 throughout reactor

operation. The FR pH during the reactor operation is shown in Figure 32A. The CEFR reactor pH stabilized during week 3 and remained between 3.77 and 3.81 until week 7, when the pH increased to 4.04. The CEFR pH during the reactor operation is shown in Figure 32B.

Protein concentration in the BPE fungal reactor varied with time between average low concentration of 7.0 mg/ml and an average high concentration of 17.6 mg/ml, shown in Table 22 and Figure 33A. The reactor was often subjected to low flow of feed to the fungal reactor, allowing protein to build up in the reactor. Low flow also resulted in low levels of nutrients to the reactor which may result in cell death and release of biomass protein. When flow was restored to normal flow conditions this reduced reactor protein to lower protein concentrations. The effluent was sterilized prior to preparation as the feed solution to the fungal reactor; however, microorganisms were able to grow in the feed solution. Also, the feed solution contained insoluble material including inorganics and cellulosic fines. Microorganisms and other insoluble material were filtered from the feed solution prior to entering the fungal reactor, this resulting in clogging of the filter which slowed and stopped the flow of feed to the fungal reactor. During these periods of time, the concentration of protein built up in the reactor to approximately 18 mg/ml. When flow was resumed for several days the concentration of reactor protein returned to levels achieved during the first week of operation, about 7 mg/ml. Because the feed to the extracellular reactor did not contain glucose, microorganism growth did not occur and the make-up fresh feed to the extracellular fluid reactor remained constant. Protein concentration in the CEFR remained relatively stable with an average concentration 0.71

mg/ml, except on day 11 and 39 of operation with concentrations of 1.14mg/ml and 1.43 mg/ml respectively, shown in Figure 33B. It is expected that the source of protein in the CEFR is the extracellular fluid produced in the fungal reactor. Before the fungal extracellular fluid is fed to the CEFR it passes through a settling basin where insoluble protein precipitated out of the fluid. It is expected that most residual biomass, including cellulosic fines and dead cell material from the FR, would precipitate out, therefore it is suspected that the extracellular fluid contains much less residual biomass.

Decolorization of the effluent was determined by measuring the color of each bleach plant effluent reactor sample. The untreated E_o-stage effluent feed solutions had a color intensity of 1626 color units. Color reductions in the FR and CEFR were based on the color of the feed solution. Figure 34A and Table 22 exhibits the reduction of color during fungal reactor operation. During operation, color reduction in the fungal reactor was between 40% and 67%. During fungal reactor operation the average removal of color was 54%. Pallerla and Chambers (1996) achieved 69% color removal when the feed color was 2476CU and the product color was 772CU. These researchers found that as the color intensity of the feed solution decreased the efficiency of color removal also decreased (Pallerla & Chambers, 1997b). Figure 3B exhibits the reduction of color during CEFR operation. During CEFR operation the average removal of color was 43%, ranging between 30% and 54%. Dilution of make-up feed with extracellular fluid from the fungal reactor has a role in reduction in color. At ideal flow conditions the flow rate ratio of extracellular fluid feed to fresh make up feed is 2 to 1. However due to the flow control problems with the filtration of feed to the fungal reactor, the flow rate ratio was

skewed towards 1 to 1 or less than 1. Because of this, it is difficult to determine how much dilution has an effect on color reduction compared to enzymatic or other biochemical activity.

Removal of PCP, TCHQ and color from bleach plant effluent

Table 23 and Figure 35A show the removal performance of PCP from bleach plant effluent in the FR. During reactor operation, PCP removal varied, but remained between 79% and 90%. Removal performance of TCHQ from bleach plant effluent in the FR is shown in Table 34 and Figure 35B. Removal of TCHQ remained between 77% and 91% in the FR. Because the protein concentration in the FR varied between about 7 mg/ml and 18.4 mg/ml, data was summarized at these two separate conditions, shown in Table 35. At the low protein concentrations, where ideal flow conditions prevail, removal of PCP was 89%, removal of TCHQ was 77% and reduction of color was 58%. At high protein concentrations, where low flow conditions are assumed, removal of PCP was 83%, removal of TCHQ was 89% and removal of color was 48%. Throughout reactor operation the removal of PCP was 86%, the removal of TCHQ was 85% and removal of color was 53%. Normal conditions improved PCP removal slightly and color removal by 10%. High protein concentration at low flow conditions seems to improve TCHQ removal slightly. Table 26 summarizes removal of color, PCP and TCHQ in the CEFR during reactor operation. Compared to concentrations in untreated feed effluent, PCP was removed by 90%, TCHQ was removed by 81% and color was reduced by 40%.
In the FR, high reduction of color was accompanied by low reduction of TCHQ, shown in Figure 37A. At the low TCHQ removal of 77%, the removal of color was 57%. At the

high TCHQ removal of 91%, color removal was only 40%. A similar trend was seen in the CEFR, shown in Figure 37B. In general, the removal of color decreased with increasing removal of TCHQ. At reduction in TCHQ of 80% the color removal was 48%. When the reduction of TCHQ was 87% the color was reduced by only 37%. From this data, the concentration of TCHQ free in solution does not contribute to color and removal mechanisms may contribute to color. Smook (1999) reports that traditional secondary biological treatment methods often increase color requiring tertiary treatment for color removal. Anderson et al. (1994) found that anaerobic treatment of a previously chemically treated effluent increased the color of the treated effluent. Color removal by chemical treatment alone was 90%, but after treatment with anaerobic organisms total removal was only 80%. While fungal treatment has been shown to be unique for its color removal abilities, Archibald (1992) determined that fungal bleaching of pulp required the presence of the fungal material and the cell culture media alone had no effect on bleaching of pulp.

Dependence of removal of PCP, TCHQ and color on reactor protein concentration

The removal rates of PCP and TCHQ give an indication of how much of an effect that protein concentration has on removing these compounds from solution. In the FR, the protein concentration varied between high and low concentrations depending on the feed flow rate. Since removal rate is dependent on protein concentration, the removal of PCP and TCHQ per gram of protein increased to high values when protein concentration was low and decreased to low values when protein concentration was high, shown in Table 23 and Table 24. In the extracellular fluid reactor, removal of PCP averaged 0.0076mg

PCP/g protein during normal flow conditions (low protein concentration) and 0.0026mg PCP/g protein during low flow conditions (high protein concentration). Removal of TCHQ in the extracellular fluid reactor averaged 0.013mg TCHQ/g protein during normal flow conditions (low protein concentration) and 0.0068mg TCHQ/g protein during low flow conditions (high protein concentration). In the extracellular fluid reactor average PCP removal was 0.069 mg PCP/g protein and average TCHQ removal was 0.015 TCHQ/ g protein.

In the FR removal of PCP and TCHQ exhibited a positive relationship with color removal. PCP removal in the FR seemed to increase with increasing color removal, shown in Figure 39A. At the highest color removal of 68% the PCP removal was at the high level of 0.0084mg PCP /g protein and at the lowest color removal of 40% the PCP removal was at the lowest level of 0.0026mg PCP/ g protein. The removal of TCHQ, in the FR, increased with increasing color removal, shown in Figure 39B. With a TCHQ removal of 0.0035 mg TCHQ/g protein, the reduction in color was 46%. When the TCHQ removal was 0.013mg TCHQ / g protein the color removal was 57%. Based on the inverse relationship between color removal and TCHQ removal, the positive relationship between TCHQ removal and color removal is not expected. However, protein seems to play a role in color removal, since more color was removed when the FR was operated at low protein conditions than at high concentration of proteins. This may indicate that at low protein levels, enzymatic activity is higher, or at high protein levels portions of the protein do not contribute to decolorization. The high reactor protein concentration was due to low flow rate of the feed solution which led to non-optimum

reactor condition and the starvation of the fungal cells due to the absence of nutrients. Therefore, the excess protein is most likely due to dead cells which sloughed off of the polyurethane fungal cubes.

Effect of incubation temperature on PCP and TCHQ removal from bleach plant effluent

Incubation temperature of the fungal malt agar plates had some effect on removal of PCP and TCHQ, shown in Table 27. In the FR, the concentration of PCP was slightly higher when the plates were incubated at 85^{o}F compared to when plates were incubated at 75^{o}F. However the concentration of PCP in the CEFR and the concentration of TCHQ in both the FR and CEFR were lower when the plates were incubated at the higher temperature. Since PCP in the FR was only slightly higher when incubated at a higher temperature and the other compounds measured were appreciably lower the higher incubation temperature appears to have a positive effect on bioremediation.

Remediation of BPE compared to remediation of PCP solution by fungal and extracellular fluid treatment

Protein concentration, PCP concentration and TCHQ concentration in the reactors used to treat BPE were compared to the concentrations in the reactors used to treat PCP solution. The initial concentration of PCP in the BPE was a fraction of that in the PCP solution. BPE, however, contains a mixture of several organic compounds that are subject to remediation and participate in enzyme and protein induction. Large amounts of protein were produced in the BPE FR compared that produced in the PCP FR, shown in Table

25. The percent removal of PCP was higher in the PCP FR. The average concentrations of TCHQ in the PCP FR and the BPE FR were essentially the same.

In the CEFR's the concentration of protein was also much higher in the BPE CEFR compared to the concentration in the PCP CEFR, shown in Table 26. Removal of PCP was only slightly higher in the PCP CEFR and the concentration of TCHQ was lower in the PCP CEFR compared to in the BPE CEFR. In the BPE reactors TCHQ is removed from solution by a combination of fungal activity and action of the extracellular fluid whereas in the PCP reactors, TCHQ is generated by a combination of fungal activity and action of the extracellular fluid. Dilution of fresh BPE feed to the CEFR by the extracellular fluid from the FR also contributes to the reduction of TCHQ in the CEFR. The reactor concentrations of TCHQ were however in the same magnitude in all reactors, suggesting TCHQ is quite reactive and remains in the quinone form for a short time prior to being involved in other reactions.

Protein binding of PCP and TCHQ

In the previous chapter, degradation of PCP by the same bioreactor system was studied. Protein binding of PCP and TCHQ was determined to occur with PCP binding being more dependent on protein concentration than TCHQ binding. Protein binding of PCP and TCHQ in the FR and CEFR was examined for the role it has on removal of PCP and TCHQ from solution and the role of protein binding on decolorization.

During reactor operation the concentration of protein bound PCP increased dramatically and by the end of reactor operation it had increased by 650%, shown in Table 28 and Figure 40A. Overall, the concentration of protein bound PCP in the FR increased about 10% per day. The change in the concentration of protein bound TCHQ followed a similar trend as that for the change in the concentration of protein bound PCP, shown in Table 29 and Figure 40B. During FR operation, the amount of protein bound TCHQ in bleach plant effluent increased about 6% per day. By the end of reactor operation the amount of protein bound TCHQ increased 881%. During CEFR operation, the concentration of protein bound PCP in bleach plant effluent increased as much as 517%, shown in Table 28 and Figure 41A. In the CEFR, the concentration of protein bound TCHQ, shown in Table 29 and Figure 41B, in BPE increased up to 467%. Protein binding of PCP was 50% higher when the reactor was operated at high protein concentrations than at low protein concentrations. Protein binding of TCHQ was about 13% higher when the reactor was operated at high protein concentrations than at low protein concentrations. Protein concentration in the FR clearly had an effect on protein binding of PCP and TCHQ, with more binding occurring at high protein concentrations. This is likely due to more protein being available for binding of PCP and TCHQ.

The binding of PCP or TCHQ is relationship of the concentration of protein bound PCP or TCHQ to reactor protein concentration. Binding gives an indication of how effective the protein present in the reactor is at forming conjugates with PCP or TCHQ. The binding of PCP varied during FR operation; remaining between 0.0002 mg bound PCP/ g protein and 0.017 mg bound PCP/ g protein, averaging 0.006, shown in Table 28 and

Figure 42A. The binding of TCHQ during FR operation is shown in Table 29 and Figure 42B. Apparent binding of TCHQ occurred at a much lower rate than PCP in the FR. During FR operation the apparent binding of TCHQ remained between 0.00009mg bound TCHQ/ g protein and 0.0019 mg bound TCHQ / g protein, averaging 0.0007 mg bound TCHQ/ g protein. It is interesting that even though the reactor protein concentration varied significantly during operation the apparent binding of PCP and TCHQ increased at a steady pace until day 39. This indicates that the FR steadily produces more active protein, protein available for binding or catalytically active, over time. The amount of active protein is a fraction of the total reactor protein concentration. When apparent binding dropped this signaled that the FR was no longer producing active protein.

Binding of PCP in the CEFR during reactor operation is shown in Table 28 and Figure 33A. During the CEFR operation, binding of PCP remained between 0.014 mg bound PCP / g protein and 0.15mg bound PCP / g protein, averaging 0.07mg bound PCP / g protein. The change in the relationship of protein bound PCP to reactor protein concentration followed an increasing trend with reactor operation, with the exception of the sharp increase on day 25. Table 29 and Figure 33B show the binding rate of TCHQ in the CEFR during reactor operation. The binding of TCHQ remained between 0.0005 mg bound TCHQ / g protein and 0.01mg bound TCHQ / g protein, averaging 0.006 mg bound TCHQ / g protein during reactor operation. Binding of PCP and TCHQ was about ten times higher in the CEFR than in the FR. This is relevant because a large fraction of protein present in the extracellular fluid produced in the FR is lost in the settling basin.

The high binding relationships suggest that even though there is a smaller amount of protein in the CEFR, a larger fraction of this protein is active.

Table 30 reveals what fractions of PCP and TCHQ in the feed solutions are measured as protein bound PCP and TCHQ in the fungal reactor solution. The ratio of the amount of PCP bound to protein in the fungal reactor effluent to the amount of PCP in feed was 0.30 during the first two weeks, but averaged 1.8 during the duration of reactor operation. Protein bound components may accumulate over time in the fungal reactor due to absorption on the fungal cubes and precipitation due to limited solubility resulting in the amount of bound PCP almost twice that of the amount of PCP in the feed. In a previous work, Pallerla (1997) found that transient absorption of chlorinated organics in the BPE in the polyurethane fungal cubes occurred during the first two weeks until the polyurethane foam was fully saturated. Binding of PCP to protein seems to be a major mechanism for removal of PCP. The ratios of TCHQ in the feed to protein bound TCHQ in the reactor effluent were much lower than those for PCP. However the TCHQ ratio followed a similar trend, with the ratio around 0.01 during the first two weeks of operation and increased significantly during operation up to 0.10 by the end of operation. Based on these results it appears that binding of TCHQ increases over time and may be accumulating over time or binding may be increasing over time, however binding of TCHQ does not seem to be a major mechanism for removal of TCHQ from full strength BPE. TCHQ is likely involved in other reactions such as forming addition and combination products.

Protein binding of PCP and TCHQ in the bioreactor system followed an interesting trend, increasing significantly over time. The most significant factor for protein binding is the large amount of protein produced in the FR. Because of the dramatic increase in protein bound PCP and TCHQ over time, especially as reactor protein increased it is evident that protein binding of PCP and TCHQ in the FR is an important mechanism for removal of these compounds from solution and depends heavily on the amount of protein present for binding. Even though the reactor protein concentration varied significantly during operation the binding of PCP and TCHQ increased at a steady pace until day 39. This indicates that the FR steadily produces more active protein available for binding over time. BPE is an excellent effluent to treat using this fungal technique due to the inherent reactivity of BPE, along with the natural enzymatic catalytic activity, and protein produced by *T. versicolor*, resulting in several avenues of removal of chlorinated organics from BPE. One such mechanism is protein binding of chlorinated organics.

Effect of incubation temperature on apparent binding of PCP and TCHQ

Incubation temperature of the fungal plates had a marked effect on the concentration of protein bound PCP and protein bound TCHQ in the FR, shown in Table 31. The concentration of protein bound PCP was four times higher when the fungal plates were incubated at 85^{o}F compared to 75^{o}F and the concentration of protein bound TCHQ was twice as high. These effects suggest that incubation at higher temperatures results in fungal material that produces more protein, which increases in amount of binding which occurs in the fungal reactor.

Occurrences of protein binding in BPE compared to PCP solution

Comparing the amount of reactor protein, initial PCP and TCHQ concentration and protein bound PCP and TCHQ in the BPE reactors and the PCP reactors provides interesting information on the binding nature of PCP and TCHQ. The amount of protein present during the operation of the BPE FR was eighty-one times that present in the PCP FR. The initial concentration of PCP free in BPE was about 2% of the initial concentration of PCP in the PCP solution. However the binding of PCP in the BPE FR was twice that in the PCP FR. The untreated BPE contained TCHQ and bound TCHQ therefore the concentration of bound TCHQ was significantly higher in the BPE FR compared to the PCP FR, but did not change markedly from the initial concentration of bound TCHQ in untreated BPE. Similar comparisons can be made with the BPE CEFR and PCP CEFR. These findings support the hypotheses that reactor protein plays a major role in binding since the concentration of available PCP is so much lower in BPE than in the PCP solution, but the high concentration of protein in the BPE reactors allows for more binding. The PCP reactors, however, seem to produce protein that is more efficient at binding, but binding is likely limited by the amount of protein present, whereas binding in the BPE reactor may be limited by available PCP competing with a number of other possible similar organics for the available protein binding sites. Protein binding of TCHQ followed similar trends in the BPE and the PCP reactors, in that the concentrations did not change very much during operation. An almost constant level of apparent binding of TCHQ occurs in these different solutions, and seems to reach equilibrium quickly and is not affected by reactor protein, rather the nature of the solution

and the amount of TCHQ present for binding. It is possible that TCHQ has a higher affinity for protein binding, therefore TCHQ is more competitive for binding.

Ratios of PCP to protein bound PCP and TCHQ to protein bound TCHQ

The ratio of free PCP or TCHQ to protein bound PCP or TCHQ gives an indication of how large a role protein binding has in removing these compounds from solution. These ratios during FR and CEFR operation are shown in Table 33. In the FR, the ratio of the concentration of free PCP to the concentration of protein bound PCP was highest during week two of reactor operation at 0.31, shown in Figure 44A. This ratio decreased steadily to 0.059 by the end of reactor operation. The ratio of the concentration of free TCHQ to the concentration of protein bound TCHQ, shown in Figure 44B, averaged 12 during FR operation, but by the end of reactor operation the ratio of the concentration of free TCHQ to the concentration of protein bound TCHQ was 0.94. The magnitudes of the ratios of free PCP or TCHQ to protein bound PCP or TCHQ in the CEFR were similar to those in the FR. In the CEFR, the ratio of free PCP to protein bound PCP was highest during week two of reactor operation at 0.37 and decreased to 0.032 by the end of reactor operation, averaging 0.14, shown in Figure 45A. During CEFR operation, the ratio of the concentration of unbound TCHQ to the concentration of protein bound TCHQ generally was between 2.7 and 9, shown in Figure 45B. A high ratio was obtained on day 19, due to a very low measurement of protein bound TCHQ.

Overall, in both the FR and CEFR this ratio was about 0.14. In the FR the ratio of free PCP to protein bound PCP was twice as high during low protein operation than during

high protein operation, indicating more binding occurring when more protein is available. The ratios of free PCP to protein bound PCP in the reactors were significantly less than one indicating that protein binding of PCP is an important mechanism of PCP removal from solution. The ratios of free TCHQ to protein bound TCHQ in the FR only dropped below one at the end of FR operation, but were high, around 17, at the beginning of operation. In the CEFR the ratio of free TCHQ to protein bound TCHQ remained between 2.7 and 9 with the exception of the ratio of 71. These high ratios suggest that TCHQ tends to remain in solution rather than binding to protein.

Comparisons between ratios in the BPE reactors and the PCP reactors

The free to protein bound ratios of PCP in the BPE reactors were significantly lower than in PCP reactors. This reiterates the fact that binding of PCP occurred at a higher level in the BPE reactors compared to the PCP reactors. While the concentration of PCP was appreciably higher in the PCP solution and reactors, the concentration of protein was significantly higher in the BPE reactors. Protein concentration limits the amount of binding, which occurs; therefore binding of PCP occurs at a higher rate in the BPE reactors. Theoretically, if more protein were available in the PCP reactors, then the ratio of free to protein bound PCP would be much lower. The ratios of free to protein bound TCHQ in the BPE reactors and the PCP reactors were in the same magnitude. Binding of TCHQ does not seem to be as important as binding of PCP in these bioremediation systems and does not appear to be affected by protein concentration.

Fraction of Actively Bound Protein

The fractions of reactor protein involved in binding of PCP and TCHQ were determined using the following relationships:

$$\phi_{PCP} = PCP_{bound,app} / \text{Reactor Protein} \quad (1)$$

$$\phi_{TCHQ} = TCHQ_{bound,app} / \text{Reactor Protein} \quad (2)$$

$$\phi_{Total} = (PCP_{bound,app} + TCHQ_{bound,app} + \text{Other Organics}_{bound}) / \text{Reactor Protein} \quad (3)$$

where:

ϕ_{PCP} = fraction of reactor protein bound to PCP

ϕ_{TCHQ}=fraction of reactor protein bound to TCHQ

ϕ_{Total}= fraction of reactor protein bound to PCP and TCHQ

$PCP_{bound,app}$ = molar concentration of protein bound PCP

$TCHQ_{bound,app}$ = molar concentration of protein bound TCHQ

$\text{Other Organics}_{bound}$ = molar concentration of bound organics

Reactor Protein = molar concentration of reactor protein assuming protein molecular weight of 100,000.

In the BPE fungal reactor, interruptions in the flow of feed resulted in higher reactor protein concentrations as well as higher concentrations of bound PCP and TCHQ, however, the fraction of protein bound to PCP and TCHQ were higher during ideal flow conditions, shown in Table 34. Fractions of protein bound to PCP and TCHQ in the CEFR were actually higher during low flow conditions, when more reactor protein was

present. The low flow conditions in the FR resulted in higher reactor protein concentrations with a lower fraction of active protein. However, the higher level of reactor protein in the CEFR also contained a higher fraction of active binding protein, suggesting that inactive protein released in the FR was removed in the settling basin and that the higher level of protein released in the FR also contained a higher level of active protein that was fed into the CEFR. A large portion of the excess protein released during low flow conditions could be attributed to dead fungal cells, which could have interfered with the active binding portion of the protein. When these dead cells were allowed to settle out of the extracellular fluid, active binding protein became more available in the CEFR. Therefore, in addition to the overall increase in reactor protein there seems to be an increase in the production of active binding protein.

Effects of adding BPE to PCP feed solution to the FR on reactor pH, protein concentration and laccase activity in the FR and CEFR

Protein production in the bioreactor system treating full strength BPE produced extremely large amounts of protein compared to that produced in the bioreactor system treating 2.5mg/L PCP solution. This increased protein production is likely due to a mixture of inducers at low concentrations, such to induce protein production but not high enough concentrations to inhibit excess growth, and an additional amount of carbohydrates present in the BPE to facilitate excess growth. By adding small amounts of BPE to a 2.5mg/L PCP, the effects of the BPE on increasing protein production and the response of laccase activity to a changing environment could be determined.

Table 35 and Table 36 show the reactor pH, the concentration of reactor protein and laccase activity in the FR and CEFR used to treat 2.5mg/L PCP solution with BPE added and without BPE added. The reactors were allowed to stabilize for 9 to 10 days after the feed to the FR was started. Samples were taken from each reactor and measured for reactor pH, protein concentration and laccase enzyme activity. Initially, the pH of both fungal reactors' were around 4.5, however the FR used to treat the PCP solution with BPE added had pH values were slightly lower than the FR used to treat the PCP solution without BPE. Lower reactor pH has been shown to produce better results in removing PCP from solution. A similar trend was observed in the CEFRs. Both reactor set-ups showed instability in the amount of protein present in the FRs, with the FR used to treat the PCP solution with added BPE showing more instability due to changing the concentration of BPE in the feed to the FR. However the overall average protein produced in the FR used to treat PCP solution with BPE added was about 18% higher than in the reactor used to treat PCP solution without BPE, shown in Table 37. The reactor protein in the CEFR of the reactor set up used to treat the PCP solution was much more constant than in the CEFR used to treat PCP solution with BPE added to the feed to the FR. However, the overall average protein concentration in the CEFR with BPE added to feed to the FR had 62% more protein than the CEFR with no BPE added to the feed to the FR. Even though the overall average reactor protein produced in the FR used with BPE added to the feed solution was only 18% higher than in the FR used without BPE added to the feed solution, a larger fraction of the protein remained in the extracellular fluid that ended up in the CEFR. This suggests that the additional protein produced by

adding BPE was more soluble and less likely to precipitate out of solution in the settling chamber, it may be more active and of lower molecular weight.

At the specific concentrations of BPE the reactor pH and reactor protein concentrations in the FR and CEFR varied significantly, shown in Table 38. Interestingly, the highest and lowest concentrations of BPE added to the feed resulted in lower average reactor protein concentrations in both the FR and CEFR. The highest amount of average reactor protein in the FR was measured when the feed solution contained 5%, while the highest amount of average reactor protein in the CEFR occurred when 2.5% BPE was in the feed solution. Because the reactor was fed 5% BPE during start-up, some of the excess protein could be due to active growth of the fungal cubes. The conditions of the reactor at 2.5% BPE were such that the fungal cubes experienced a period of starvation (explained later in detail) prior to being fed 2.5%BPE, therefore it is possible that some of the excess protein in the CEFR could be due to washout of starved cells from the FR.

During the course of the experiment the concentration of BPE in the feed to the FR was initially 5%, and then increased to 10%, after which was decreased to 2.5% and finally decreased to 1%. The reactor was run for at least 5 days at each concentration before samples were taken, and samples were taken two to three days apart at each condition. On the last day samples were taken at a specific condition, the feed to the FR was changed to the next BPE concentration. Changing the feed composition caused the reactor to respond differently after each change. The continuous nature of the experiment

means that the immobilized fungus responds to the reactor environment as well as the changing environment.

Initially the feed to the FR contained 5% BPE. The reactor protein concentrations in both the FR and the CEFR were relatively high; however no laccase activity was detected. Additional inducers and carbohydrates present in the BPE likely caused the immobilized fungus to have an extended growth period, producing more fungal material and secreting more protein. As the fungus became acclimated to the environment, growth likely reached a plateau and the amount of protein secreted dropped dramatically. Tomati et al. (1991) stated that some types of protein are secreted in large amounts by fungus during growth are preparing better conditions for the growing organisms, and after a few days of growth, secretion of these proteins rapidly decreases. Similarly, the reactor set-up that used PCP solution without BPE in the feed only showed enzyme activity in the first sample taken, however the protein concentration was not significantly high.

When the concentration of BPE in the feed to the FR was increased to 10%, the protein concentration in the FR increased only slightly and remained relatively constant, whereas the concentration of protein in the CEFR increased initially. As the reactor was run with 10% BPE in the feed to the FR, the amount of growth in the cubes increased and there was more growth in the feed line, similar to the growth present in the reactor when it was run with full strength BPE. Laccase activity was detected in the CEFR after the reactor was run with 10% for 7 days. It is interesting that laccase was not detected in the FR at

the same time. As the feed lines became full of growth and the flow of feed solution was hampered. Lower flow rate of feed to the FR would have caused a lower amount of carbohydrates being fed to the FR. This would lead to a state of starvation to the increased amount of growth in cubes in the FR. At the point where the cells were starved for carbohydrates it was also deprived of the inducing compounds. Froehner & Eriksson (1974) noted that non-induced cells that experienced autolysis secreted small amount of laccase. Gomez-Alarcon et al. (1989) found that when cells were in media with depleted carbon source, a state of starvation, they underwent autolysis and secreted laccase. Because the CEFR contains the extracellular fluid produced in the FR, the laccase in the CEFR was a result of washout from the FR and the reactor solution in the FR no longer contained the laccase produced by autolysis when the sample was taken. After the last sample was taken with the feed solution containing 10% BPE, the feed was stopped, the feed lines were rinsed out to clean out the growth, a new filter was installed and the feed concentration of BPE was changed to 2.5%. During this time the cells were deprived of nutrition and inducers. After the feed was restored to the FR at 2.5% BPE, laccase was detected in the FR and the CEFR. Froehner & Eriksson (1974) found that when cells were starved they produced more laccase when they were exposed to the inducer. From this point forward during reactor operation the fungal cubes were continuously exposed to the feed solution, and they no longer experienced starvation and were continuously exposed to the inducer. Therefore, the laccase activity dropped to undetectable levels for the duration of reactor operation. Lack of enzyme activity during normal operation of the reactor is expected, because the fungal cubes were no longer in the growth phase, did not experience starvation and even though they were exposed to a high concentration of PCP

in the feed solution, the reactor environment contained much less due to action of the fungal material. Laccase is secreted during the growth period or suddenly in response to a stressful environment, such as starvation causing cell lysis or sudden exposure to inducer, then after the fungal cells become acclimated to the new environment laccase activity decreases suddenly to undetectable levels (McLellan et al., 1970; Froehner & Eriksson, 1974; Gigi et al., 1980; Gomez-Alarcon et al., 1989; Tomati et al., 1991; Rogalski & Leonowicz, 1992). Marbach et al. (1983) suggest that the ability of fungi to respond to changing environments enables fungi to adjust its secretory properties to the host plants signals and conditions.

Effects of adding BPE to PCP feed solution to the FR on free PCP and protein bound PCP in the FR and CEFR

The addition of BPE to the feed solution adds only a small amount of PCP to the feed since untreated BPE was shown to contain 0.059mg/L. The 10% BPE added to the feed solution only contributes 0.01 mg/L to the 2.5mg/L of PCP added to the feed as stock solution. One percent BPE added to the feed solution adds a mere 0.001mg/L to the feed solution. Therefore the concentration of PCP is attributed to action of reactor bioremediation performance and not the increased contribution of adding BPE to the reactor feed solution. Reactor PCP solution at each feed condition is shown in Table 39. The FR performed very well at most of the BPE feed conditions with the reactor concentrations of PCP within 0.016mg/L when the feed to the FR contained between 1% and 5% BPE. The FR achieved 92.1% reduction of PCP at 1% BPE and 91.5% reduction of PCP at 5% BPE, shown in Table 41. Adding 10% to the feed to the FR resulted in

only 88% reduction in PCP. However, the removal rates of PCP in the reactors, shown in Table 40, were greatest when the reactor had the lowest amount of reactor protein, at 10% and 2.5% BPE, when removal rate of PCP was 0.0273, highest at 2.5% BPE. At 2.5% BPE added to the feed, the reactor achieved high reduction and high removal rate, while exhibiting the lowest pH and reactor concentration on the sample date. Optimum pH for this reactor set-up has been found to be between 3.5 and 4.0, the feed with 2.5% BPE maintained pH values well within that range.

Comparing the effects of adding BPE to the PCP solutions, it should be noted that the reactor without BPE added to the feed achieved slightly higher reduction of PCP after 10 days of operation at 96% reduction of PCP in the FR and 89.6% reduction of PCP in the CEFR. However, after 25days of operation, the reactors in the set-up used to treated BPE added feed solution achieved over 90% removal in the FR and CEFR, the reactor without BPE added to the feed only removed 83.4% of PCP from the feed to the FR and 73.0% of PCP in the feed to the CEFR. While addition of BPE only slightly decreased reduction performance initially, it cultivated a fungal bioreactor into one that was more robust and more effective over time at removing PCP.

The average concentrations of protein bound PCP in the reactors treating feed with BPE added, shown in Table 41 during operation were about 0.03 mg/L in both the FR and the CEFR and which were about the same as the reactors used to treat feed without BPE added, approximately 0.026mg/L in the FR and 0.037mg/L in the CEFR. When the reactor was used to treat full strength BPE, it contained much more protein bound PCP at

0.105mg/L in the FR and 0.058mg/L in the CEFR, even though there was much less PCP in the full strength BPE than in the PCP reactor feed, with or without BPE added. The concentration of reactor protein in the set up used to treat full strength BPE was several times higher than set-ups using the other feeds however the concentration of PCP in the full strength feed was quite low, much of the PCP present in that reactor was bound to protein, while only a fraction of the PCP was bound to protein with the other feeds. Increased protein production did not appear to play a significant role in binding when comparing the reactors used to treat PCP solution with or without BPE added. However, when comparing reactors used to treat full strength BPE or PCP solution with BPE added, PCP binding values were significantly higher, shown in Table 28 and Table 42. The increase in PCP binding indicates that the protein produced as a result of adding BPE is more available and active for binding then the protein produced when treating full strength BPE.

Effects of adding BPE to PCP feed solution to the FR on free TCHQ protein bound TCHQ in the FR and CEFR

The average concentration of TCHQ in the FR during treatment of feed containing BPE was 0.009 mg/L and 0.017mg/L in the CEFR, shown in Table 43. The average in the FR was lower than values seen when treating full strength BPE and PCP solution without BPE; however the average in the CEFR was approximately the same as seen with those other feeds. The amount of protein bound TCHQ was surprisingly high in the reactors used to treat feed with BPE, many times higher than when the feed was PCP solution without BPE or full strength BPE, shown in Table 44. In the reactor that was used to

treat full strength BPE it is highly likely that due to the extent of protein precipitation in the FR that some of the protein bound TCHQ precipitated and was not soluble during sampling. It is possible that the additional protein produced in the FR due that the addition of small amounts of BPE provides is more soluble, active and available for binding of TCHQ. The small addition of BPE, which has been shown to contain TCHQ, may provide enough reactive compounds to cause more conversion of PCP to TCHQ to make more available for binding. Experiments detailed in Chapter IV provide evidence that even a small amount of additional protein has a dramatic effect on the amount of TCHQ that is apparently bound to protein, and as the protein concentration reaches a level where solubility decreases apparent binding of TCHQ actually decreases. Table 45 shows the apparent binding of TCHQ. When comparing reactors used to treat full strength BPE or PCP solution with BPE added, the TCHQ binding ratios were significantly higher, shown in Table 29 and Table 43. The increase in binding ratios indicates that the protein produced as a result of adding BPE is more available and active for binding then the protein produced when treating full strength BPE. With the added amount protein produced a small amount of reactive component in the feed that adding the BPE to the feed may provide enough beneficial action to increase TCHQ binding to very high amounts. By increasing reactivity of PCP conversion to TCHQ and having the right amount of active, available protein for binding large amounts of PCP is removed from solution by way of TCHQ conversion and binding of protein.

Effects of adding BPE to feed to the FR on ratios of free PCP or TCHQ to bound PCP or TCHQ

Adding BPE to a PCP feed solution had a significant effect on the ratios of free PCP to bound PCP and free TCHQ to bound TCHQ, shown in Table 46. In the FR the ratios of free PCP to bound PCP ranged between 4.8 and 13.9 whereas the ratios of free TCHQ to bound TCHQ averaged 0.027. In the CEFR the ratios of free PCP to bound PCP averaged 11.4 and the ratios of free TCHQ to bound TCHQ averaged 0.027. The addition of BPE seemed to provide characteristics that produced favorable conditions for binding of TCHQ. The presence of a small amount of free radicals can be enough it initiate degradation reactions which have been shown to be an important component of PCP bioremediation. Adding BPE to the feed produced a slightly more diverse feed with more carbohydrates and initiating compounds which provided more energy for growth, more compounds for fungal response and compounds that could initiate PCP oxidation. The type of protein produced in response to a more diverse solution could be more apt to remain soluble and be more reactive for binding.

CONCLUSIONS

In conclusion, this fungal bioreactor utilizing foam immobilized *T. versicolor* is effective at removing low molecular weight chlorinated compounds and color from bleach plant effluent. Treatment of effluent by extracellular fluid produced by *T. versicolor* has an effect on the removal of color, though not to the extent of direct fungal treatment by the immobilized *T. versicolor* cubes alone, and is effective at removing low molecular weight chlorinated compounds.

Several chlorinated organics have been identified by researchers and pollution regulatory agencies. Chlorinated quinone compounds, however, have not previously been verified to be present in bleach plant effluent. These compounds are inherently reactive and traditional identification methods have not been able to separate quinones from BPE. This research has identified and quantified the presence of TCHQ in BPE. The concentration of TCHQ present in untreated Eo-stage effluent is about 2.5 times that of PCP. Identification of these compounds indicates that the pulp bleaching process results in the formation of several chlorinated compounds as well as the formation of reactive chlorinated quinones, therefore bleach plant effluents possess reactive properties.

Low molecular weight PCP and TCHQ were followed for degradation by this bioremediation method to study degradation tendencies and mechanisms. Up to 90% of free PCP was removed by the fungal reactor with average removal of 86% during operation. Maximum removal of free TCHQ in the fungal reactor was 91%, averaging

85% during reactor operation. In the continuous extracellular fluid reactor, free PCP was reduced by up to 95%, averaging 90% during reactor operation, while free TCHQ was reduced by up to 87%, averaging 81% during reactor operation.

Color removal was achieved in both the FR and the CEFR. In the FR up to 68% of color was removed, with average removal during operation being 53%. The CEFR was able to remove up to 54% of color, averaging 40% removal during reactor operation. Color removal may not appear to be as effective as studies performed with this reactor and other effluents, however, the effluent used in these studies possess less color than the previous effluent studied, about 1000CU less. Pallerla and Chambers (1997b) found color removal to be less efficient when the effluent was of lower color intensity.

Compared to the PCP solution used in the PCP model bioreactor, bleach plant effluent contains several chlorinated and non-chlorinated compounds that generally inhibit microorganisms, including fungi. However, these compounds do not inhibit *T. versicolor*; instead these compounds initiate the production of large amounts of extracellular protein by the fungus. Even at low protein concentration conditions in the FR treating BPE, the amount of protein present in the FR treating BPE was 67 times that present in FR treating the PCP solution. The presence of protein provides a mechanism for removal of chlorinated compounds by protein binding of chlorinated compounds. Even though TCHQ is a more reactive compound than PCP, protein binding of PCP occurred more frequently than with TCHQ. The reactive nature of TCHQ may lead to reactions with other chlorinated compounds instead of with protein. Protein binding of

PCP depended on the amount of protein present in the reactor. During reactor operation, binding of TCHQ and PCP increased dramatically over time. In the FR, the concentration of protein bound PCP increased by 650% by the end of reactor operation and the amount of protein bound TCHQ increased 881%. Protein binding in the CEFR of PCP increased by as much as 517% and binding of TCHQ increased 467%. Comparing the performance of the reactors used to treat BPE to the reactors used to treat PCP solution, supports the finding that reactor protein plays a major role in protein binding of PCP.

Incubation temperature of the *T. versicolor* fungal plates was shown to have some effect on removal of PCP and TCHQ and a significant effect on the protein binding of PCP and TCHQ. Improved removal and increased protein binding suggests that a higher incubation temperature results in a fungal colony that produces larger amounts of protein, resulting in more protein binding and more effective remediation.

Based on these studies it is evident that this fungal bioremediation system is a unique and promising method to treat bleach plant effluents. Effective color removal and reduction of low molecular weight compounds can be achieved by this system. These studies show that this system uniquely produces large amounts of protein, providing removal of chlorinated compounds by protein binding.

Adding a small amount to BPE to a 2.5mg/L PCP feed solution to the FR had several effects on the remediation of PCP. BPE was added to the 2.5mg/L PCP at 1%, 2.5%, 5%

and 10% concentrations. The addition of small amounts of BPE to the 2.5mg/L PCP feed increased protein production in the FR by 18% and the amount of reactor protein in the CEFR was 62% higher than with the feed containing no BPE. Higher increase of protein in the CEFR suggests that the additional protein produced in the FR may be more soluble in the extracellular fluid and less likely to precipitate out of solution before being fed into the CEFR. Changing the conditions of the feed resulted in a reactor that was more robust and effective over time by increasing the stress on the fungal material causing the fungal material to respond to more stimuli. The changing environment led the reactor to produce enzyme activity several weeks into reactor operation as opposed to only in the initial stage as in the reactor used to treat PCP solution without added BPE. While adding BPE to the 2.5% PCP feed solution had little effect on the concentrations of free PCP and protein bound PCP, the amount of protein bound TCHQ was increased several fold. This effect could be due to the addition of small amount of reactive compound present in the BPE could lead to more conversion of PCP to TCHQ. The additional protein produced by the reactor containing BPE seems to be more soluble and active for binding TCHQ.

Table 22: Reactor characteristics during operation of the FR and the CEFR

	Day of Reactor operation	Reactor pH	Reactor Protein (mg/ml)	Color Intensity (CU)	%Color Removal
FR					
	4	3.31	5.8	529	67.7
	11	4.04	15.4	864	46.9
	12	3.90	16.9	885	45.6
	19	3.59	8.4	705	56.6
	25	3.59	18.5	697	57.1
	39	3.51	6.2	801	50.7
	43	3.61	19.6	969	40.4
CEFR					
	4	3.37	0.777	747	54.1
	11	3.61	1.143	801	50.7
	12	3.61	0.696	1007	38.1
	19	3.81	0.655	982	39.6
	25	3.78	0.656	848	47.9
	39	3.77	1.485	1120	31.1
	43	4.04	0.751	1140	30.0

Note: Data points presented are an average of three sample measurements

Table 23: PCP removal performance in the FR and the CEFR

	Day of Reactor operation	Reactor PCP (mg/L)	Reactor Protein (mg/ml)	PCP Removal (mg PCP/g Protein)	%Reduction of PCP
FR					
	4	0.0101	5.8	0.008	83%
	12	N/A	16.9	N/A	N/A
	19	0.0056	8.45	0.0063	90%
	25	0.0127	18.5	0.0025	79%
	39	0.0064	6.2	0.0085	89%
	43	0.0074	19.6	0.0026	87%
CEFR					
	4	0.0081	0.777	0.0657	86%
	12	N/A	0.696	N/A	N/A
	19	0.0036	0.655	0.0849	94%
	25	0.0030	0.656	0.0856	95%
	39	0.0088	1.485	0.0339	85%
	43	0.0025	0.751	0.0754	90%

Note: 1. Data points presented are an average of three sample measurements

2. PCP Removal = (Concentration of PCP_{free} in feed – Concentration of PCP_{free} in reactor)/ Concentration of reactor protein

Table 24: TCHQ removal performance in the FR and the CEFR

	Day of Reactor operation	Reactor TCHQ (mg/L)	Reactor Protein (mg/ml)	TCHQ Removal (mg TCHQ/g Protein)	%Reduction of TCHQ
FR					
	4	N/A	5.8	N/A	N/A
	12	0.0185	16.9	0.0035	87%
	19	0.0321	8.45	0.0130	77%
	25	N/A	18.5	N/A	N/A
	39	N/A	6.2	N/A	N/A
	43	0.0131	19.6	0.0066	91%
CEFR					
	4	N/A	0.777	N/A	N/A
	12	0.0206	0.696	0.175	86%
	19	0.0252	0.655	0.179	82%
	25	0.0286	0.656	0.173	80%
	39	0.0389	1.485	0.0696	73%
	43	0.0187	0.751	0.165	87%

Note: 1. Data points presented are an average of three sample measurements

2. TCHQ Removal = (Concentration of $TCHQ_{free}$ in feed – Concentration of $TCHQ_{free}$ in reactor)/ Concentration of reactor protein

Table 25: Summary of data in BPE FR compared to PCP FR

	Protein (mg/ml)	PCP_{free} (mg/L)	% Reduction in PCP_{free}	$TCHQ_{free}$ (mg/L)	%Reduction in $TCHQ_{free}$	Color (CU)	% Reduction in Color
BPE							
Untreated BPE Feed Solution	0.549	0.059		0.142		1626	
Ideal Flow Conditions in the FR	6.8	0.0074	89%	0.032	77%	679	58%
Low Flow Conditions in the FR	18.4	0.01	83%	0.016	89%	850	48%
Average During FR Operation	12.6	0.008	86%	0.021	85%	765	53%
PCP							
Untreated PCP Feed Solution	0	2.5		0			
Before Flow Disturbance in the FR	0.0732	0.112	96%	0.013			
After Flow Disturbance in the FR	0.237	0.244	90%	0.025			
Average During FR Operation	0.155	0.178	93%	0.019			

Table 26: Summary of data in BPE CEFR compared to PCP CEFR

	Protein (mg/ml)	PCP_{free} (mg/L)	% Reduction in PCP_{free}	$TCHQ_{free}$ (mg/L)	%Reduction in $TCHQ_{free}$	Color (CU)	% Reduction in Color
BPE							
Untreated BPE Feed Solution[1]	0.549	0.059		0.142		1626	
Average During CEFR Operation	0.836	0.005	90%	0.026	81%	973	40%
PCP							
Untreated PCP Feed Solution[2]		2.61		0.019			
Average During CEFR Operation	0.0347	0.173	93%	0.015	9.4%		

Note: 1. Autoclaved BPE feed solution data. Feed to the FR
2. Data calculated by a material balance of the concentration of compounds in extracellular fluid and that in the make-up feed solution. Feed to CEFR

Table 27: Effect of Incubation Temperature on Concentration of PCP and TCHQ in FR and CEFR

Incubation Temperature	Average Day of Reactor Operation	PCP(mg/L)		TCHQ(mg/L)		Fungal Growth on Fungal Cubes
		FR	CEFR	FR	CEFR	FR
75°F	7	0.0079	0.0136	0.0213	0.0429	Low
85°F	8	0.010	0.0081	0.0185	0.0206	High

Table 28: Protein binding of PCP in treated BPE in the FR and the CEFR

Day of Reactor operation	Reactor PCP_{bound} (mg/L)	Reactor Protein (mg/ml)	Binding (mgPCP/g Protein)	%Increase of PCP_{bound}
FR				
11	0.017	16.9	0.0011	-
19	0.018	8.45	0.002	+11%
25	0.095	18.5	0.005	+472%
39	0.105	6.2	0.017	+536%
43	0.125	19.6	0.006	+650%
CEFR				
11	0.016	0.696	0.014	-
19	0.010	0.655	0.015	-39%
25	0.097	0.656	0.148	+517%
39	0.088	1.485	0.059	+460%
43	0.079	0.751	0.106	+407%

Note: 1. Data points presented are an average of three sample measurements

2. Binding= Concentration of $PCP_{bound,}$/ Concentration of Reactor Protein

3. % Increase $PCP_{bound} = 100 * (PCP_{\text{bound treated BPE}} - PCP_{\text{bound treated BPEat day 11}}) / PCP_{\text{bound treated BPE at day11}}$

Table 29: Protein binding of TCHQ in the FR and the CEFR

	Day of Reactor operation	Reactor $TCHQ_{bound}$ (mg/L)	Reactor Protein (mg/ml)	Binding (mg TCHQ/g Protein)	%Increase of $TCHQ_{bound}$
FR					
	11	0.0014	16.9	0.00009	-
	19	0.0015	8.5	0.00018	+8%
	25	0.0074	18.5	0.0004	+423%
	39	0.012	6.2	0.0019	+743%
	43	0.014	19.6	0.0007	+881%
CEFR					
	11	0.0023	0.696	0.002	-
	19	0.0003	0.655	0.0005	-85%
	25	0.007	0.656	0.0102	+192%
	39	0.013	1.485	0.0087	+467%
	43	0.007	0.751	0.0072	+207%

Note: 1. Data points presented are an average of three sample measurements

2. binding= Concentration of $TCHQ_{bound, app}$/ Concentration of Reactor Protein

3. %Increase in $TCHQ_{bound} = 100 * (TCHQ_{bound\ treated\ BPE} - TCHQ_{bound\ treated\ BPE\ at\ day\ 11}) / TCHQ_{bound\ treated\ BPE\ at\ day\ 11}$

Table 30: Fraction of Protein Bound PCP in FR to Free PCP in Feed to FR

	Day of Reactor operation	$\frac{PCP_{bound\ reactor}}{PCP_{free\ feed}}$	$\frac{TCHQ_{bound\ reactor}}{TCHQ_{free\ feed}}$
FR Solution			
	11	0.30	0.01
	19	0.29	0.01
	25	1.6	0.05
	39	1.8	0.08
	43	2.0	0.10

Note: $PCP_{free\ feed}$ = 0.059 mg/L

Table 31: Effect of Incubation Temperature on Concentration of Protein bound TCHQ in FR and CEFR

Incubation Temperature	Average Day of Reactor Operation	PCP_{bound} mg/L FR	$TCHQ_{bound}$ mg/L FR
75°F	18.5	0.0105	0.0015
85°F	18.3	0.044	0.003

Table 32: Ratio of free PCP or free TCHQ to protein bound PCP or TCHQ in the FR and the CEFR

Day of Reactor operation	$\frac{PCP_{free}}{PCP_{bound}}$	$\frac{TCHQ_{free}}{TCHQ_{bound}}$
FR		
11	N/A	13
19	0.31	21
25	0.13	N/A
39	0.06	N/A
43	0.06	0.94
CEFR		
11	N/A	9.0
19	0.37	71
25	0.03	4.3
39	0.10	3.0
43	0.03	2.7

Note: Data points presented are an average of three sample measurements

Table 33: Summary of the ratios of free PCP or free TCHQ to protein bound PCP or TCHQ in the FR and the CEFR in the BPE and PCP reactors

	$\frac{PCP_{free}}{PCP_{bound}}$	$\frac{TCHQ_{free}}{TCHQ_{bound}}$
BPE		
FR		
Overall Operation	0.14	12
Ideal Flow Conditions	0.18	0.94
Low Flow Conditions	0.096	17
CEFR		
Overall Operation	0.13	4.74*
PCP		
FR		
Overall Operation	6.9	14.9
CEFR		
Overall Operation	12.3	11.6

Note: *Excludes the high $[TCHQ]_{free}/[TCHQ]_{bound}$=71 on day 19. The average including this point is 18.

Table 34: Fraction of protein bound to PCP and TCHQ in the FR and the CEFR

	ϕ_{PCP}	ϕ_{TCHQ}	$\phi_{PCP} + \phi_{TCHQ}$
FR			
Ideal Flow Conditions	0.0036	0.0004	0.0040
Low Flow Conditions	0.0022	0.0002	0.0024
CEFR			
Ideal Flow Conditions	0.014	0.0019	0.016
Low Flow Conditions	0.048	0.0040	0.051

Table 35: Protein and enzyme concentration in reactors with BPE added to feed to FR during operation

Day of Reactor Operation	Percentage of BPE in Feed to FR	Reactor pH	Reactor Protein (μg/ml)	Relative Enzyme Activity (U/μl reactor solution)	Specific Enzyme Activity (U/μg reactor protein)
FR					
9	5%	4.60	217	0	0
11	5%	4.50	91	0	0
16	10%	4.45	102	0	0
19	10%	4.48	110	0	0
25	2.5%	3.93	84	1.0	12
27	2.5%	3.90	135	0	0
32	1%	4.18	201	0	0
34	1%	4.16	61	0	0
CEFR					
9	5%	3.85	92	0	0
11	5%	3.85	44	0	0
16	10%	3.88	63	0	0
19	10%	3.85	36	2.1	58
25	2.5%	3.72	46	0.5	10
27	2.5%	3.71	46	0	0
32	1%	3.74	131	0	0
34	1%	3.78	44	0	0

Note: 1 Unit of enzyme activity is defined as the amount that oxidizes 1μmol of ABTS / min

Table 36: Protein and enzyme concentration in reactors without BPE added to feed to FR during operation

Day of Reactor Operation	Percentage of BPE in Feed to FR	Reactor pH	Reactor Protein (μg/ml)	Relative Enzyme Activity (U/μl reactor solution)	Specific Enzyme Activity (U/μg reactor protein)
FR					
10	0%	4.54	101	0.31	3.1
13	0%	4.68	97	0	0
21	0%	4.46	133	0	0
24	0%	5.02	79	0	0
26	0%	4.33	107	0	0
30	0%	4.37	62	0	0
33	0%	4.32	102	0	0
CEFR					
10	0%	3.82	36	0	0
13	0%	4.00	39	0	0
21	0%	3.93	38	0	0
23	0%	4.02	37	0	0
26	0%	4.07	38	0	0
28	0%	3.92	46	0	0
33	0%	3.87	43	0	0

Table 37: Overall average reactor protein in the FR and CEFR with percentages of BPE added to feed to FR compared to overall average reactor protein reactors with no BPE in feed

	Overall Average Reactor Protein During Operation (μg/ml)
FR w/o BPE in feed to FR during operation	106
FR w/ BPE in feed to FR during operation	125
Increase in average overall protein in FR due to BPE	**18%**
CEFR w/o BPE in feed to FR during operation	39
CEFR w/ BPE in feed to FR during operation	63
Increase in average overall protein in CEFR due to BPE	**62%**

Table 38: Average reactor pH and reactor protein in the FR and CEFR at different percentages of BPE in feed to FR

	Percentage of BPE in feed to FR	Average Reactor pH	Average Reactor Protein (μg/ml)
FR	1%	4.17	110
	2.5%	3.91	131
	5%	4.55	154
	10%	4.46	106
CEFR			
	1%	3.76	46
	2.5%	3.71	88
	5%	3.85	68
	10%	3.87	50

Table 39: Concentration of free PCP and reactor pH at different percentages of BPE in feed to FR

	Day of Reactor Operation	Percentage of BPE in feed to FR	Reactor pH	PCP_{free} (mg/L)
FR	9	5%	4.60	0.213
	16	10%	4.45	0.281
	25	2.5%	3.93	0.209
	32	1%	4.18	0.197
CEFR	9	5%	3.85	0.295
	16	10%	3.89	0.259
	25	2.5%	3.72	0.242
	32	1%	3.74	0.387

Table 40: PCP removal performance in the FR and the CEFR

Day of Reactor operation	Percentage of BPE in feed to FR	Reactor PCP (mg/L)	Reactor Protein (μg/ml)	PCP Removal (mg PCP removed/mg Protein)	%Reduction of PCP
FR					
9	5%	0.21	217	0.011	92
11	10%	0.28	102	0.022	89
25	2.5%	0.21	84	0.027	92
32	1%	0.20	201	0.012	92
CEFR					
9	5%	0.29	92	0.025	89
11	10%	0.26	63	0.038	90
25	2.5%	0.24	46	0.052	91
32	1%	0.39	131	0.017	85

Note: 1. PCP Removal = (PCP feed – PCP Reactor)/ Reactor Protein; 2. % PCP reduction of PCP in reactor run simultaneously with no BPE added to feed: Day 10 FR = 96.0%, CEFR= 89.6%; Day 26 FR = 83.4%; CEFR=73%

Table 41: Concentration of protein bound PCP and reactor pH at different percentages of BPE in feed to FR

	Day of Reactor Operation	Percentage of BPE in feed to FR	Reactor pH	PCP_{bound} (mg/L)
FR	11	5%	4.50	0.015
	19	10%	4.48	0.029
	27	2.5%	3.90	0.037
	34	1%	4.16	0.042
CEFR	11	5%	3.85	0.032
	19	10%	3.85	0.017
	27	2.5%	3.71	0.046
	34	1%	3.78	0.024

Table 42: Protein binding of PCP in the FR and the CEFR at different percentages of BPE in the feed to the FR

	Day of Reactor operation	Percentage of BPE in feed to FR	Reactor $PCP_{bound,}$ (mg/L)	Reactor Protein (μg/ml)	PCP Binding ($mgPCP_{bound,}$ /g Protein)
FR	11	5%	0.015	91	0.16
	19	10%	0.029	110	0.26
	27	2.5%	0.037	135	0.27
	34	1%	0.042	61	0.69
CEFR	11	5%	0.032	44	0.73
	19	10%	0.017	36	0.47
	27	2.5%	0.046	46	1.0
	34	1%	0.024	44	0.55

Table 43: Concentration of free TCHQ and reactor pH at different percentages of BPE in feed to FR

	Day of Reactor Operation	Percentage of BPE in feed to FR	Reactor pH	$TCHQ_{free}$ (mg/L)
FR	9	5%	4.60	nd
	16	10%	4.45	0.004
	25	2.5%	3.93	0.004
	32	1%	4.18	0.021
CEFR	9	5%	3.85	0.005
	16	10%	3.89	nd
	25	2.5%	3.72	nd
	32	1%	3.74	0.029

Table 44: Concentration of protein bound TCHQ and reactor pH at different percentages of BPE in feed to FR

	Day of Reactor Operation	Percentage of BPE in feed to FR	Reactor pH	$TCHQ_{bound}$ (mg/L)
FR	11	5%	4.50	0.44
	19	10%	4.48	0.15
	27	2.5%	3.90	0.61
	34	1%	4.16	0.48
CEFR	11	5%	3.85	0.42
	19	10%	3.85	0.10
	27	2.5%	3.71	0.35
	34	1%	3.78	0.49

Table 45: Protein binding of TCHQ in the FR and the CEFR at different percentages of BPE in the feed to the FR

	Day of Reactor operation	Percentage of BPE in feed to FR	Reactor $TCHQ_{bound}$ (mg/L)	Reactor Protein (μg/ml)	TCHQ Binding (mg $TCHQ_{bound}$ /g Protein)
FR	11	5%	0.44	91	4.8
	19	10%	0.15	110	1.4
	27	2.5%	0.61	135	4.5
	34	1%	0.48	61	7.9
CEFR	11	5%	0.42	44	9.8
	19	10%	0.10	36	2.8
	27	2.5%	0.35	46	7.6
	34	1%	0.49	44	1.1

Table 46: Ratio of free PCP or free TCHQ to protein bound PCP or TCHQ in the FR and the CEFR at different percentages of BPE in feed to FR

	Percentage of BPE in feed to fungal reactor	$\frac{PCP_{free}}{PCP_{bound}}$	$\frac{TCHQ_{free}}{TCHQ_{bound}}$
FR	1%	4.8	0.044
	2.5%	5.6	0.0069
	5%	13.9	N/A
	10%	9.6	0.029
CEFR	1%	16.2	0.060
	2.5%	5.3	N/A
	5%	9.1	0.012
	10%	15.1	N/A

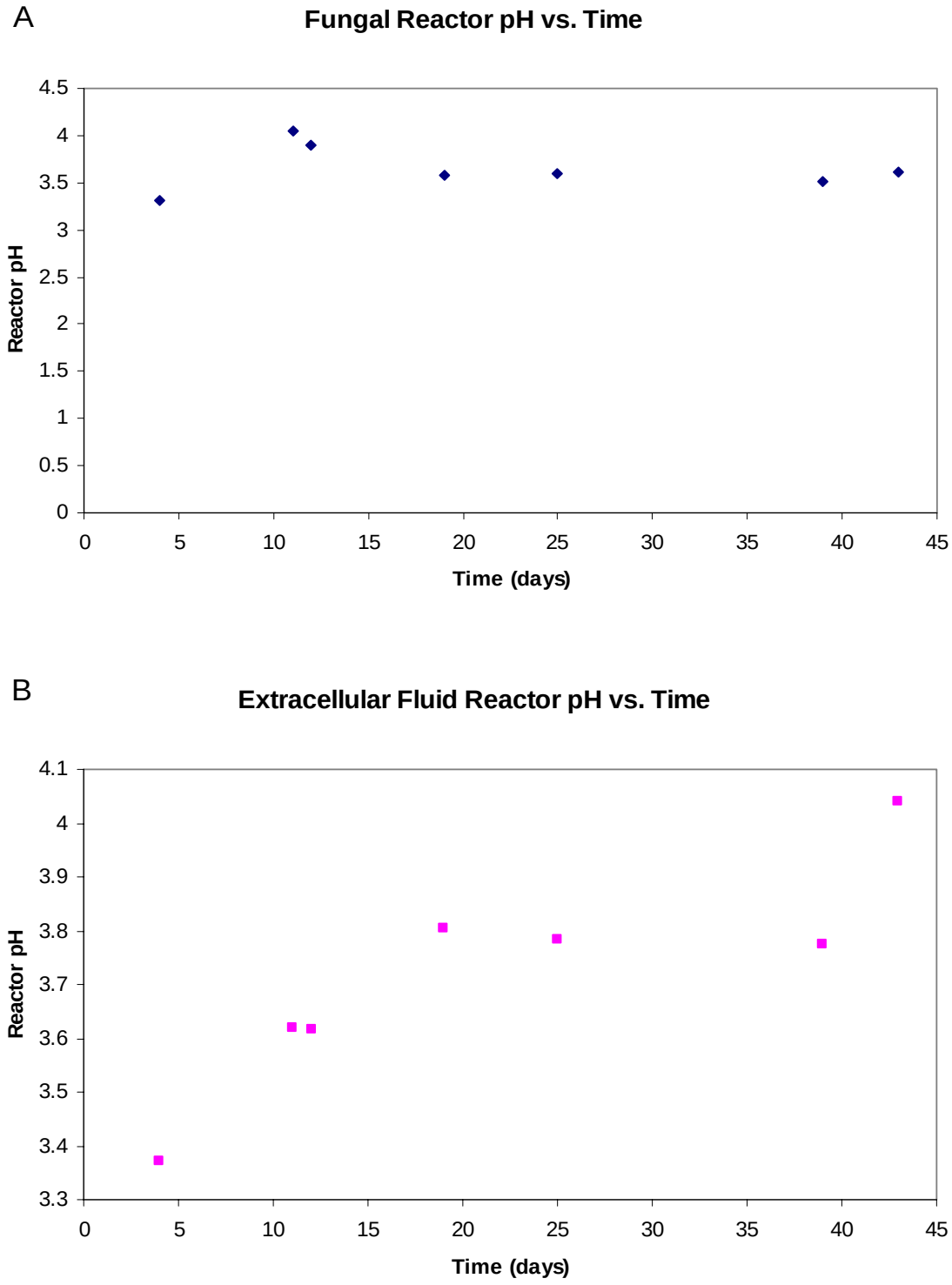


Figure 32. Reactor pH in fungal reactor (A) and reactor pH in extracellular fluid reactor (B) during reactor operation ◆-Reactor pH in fungal reactor; ■-Reactor pH in extracellular reactor

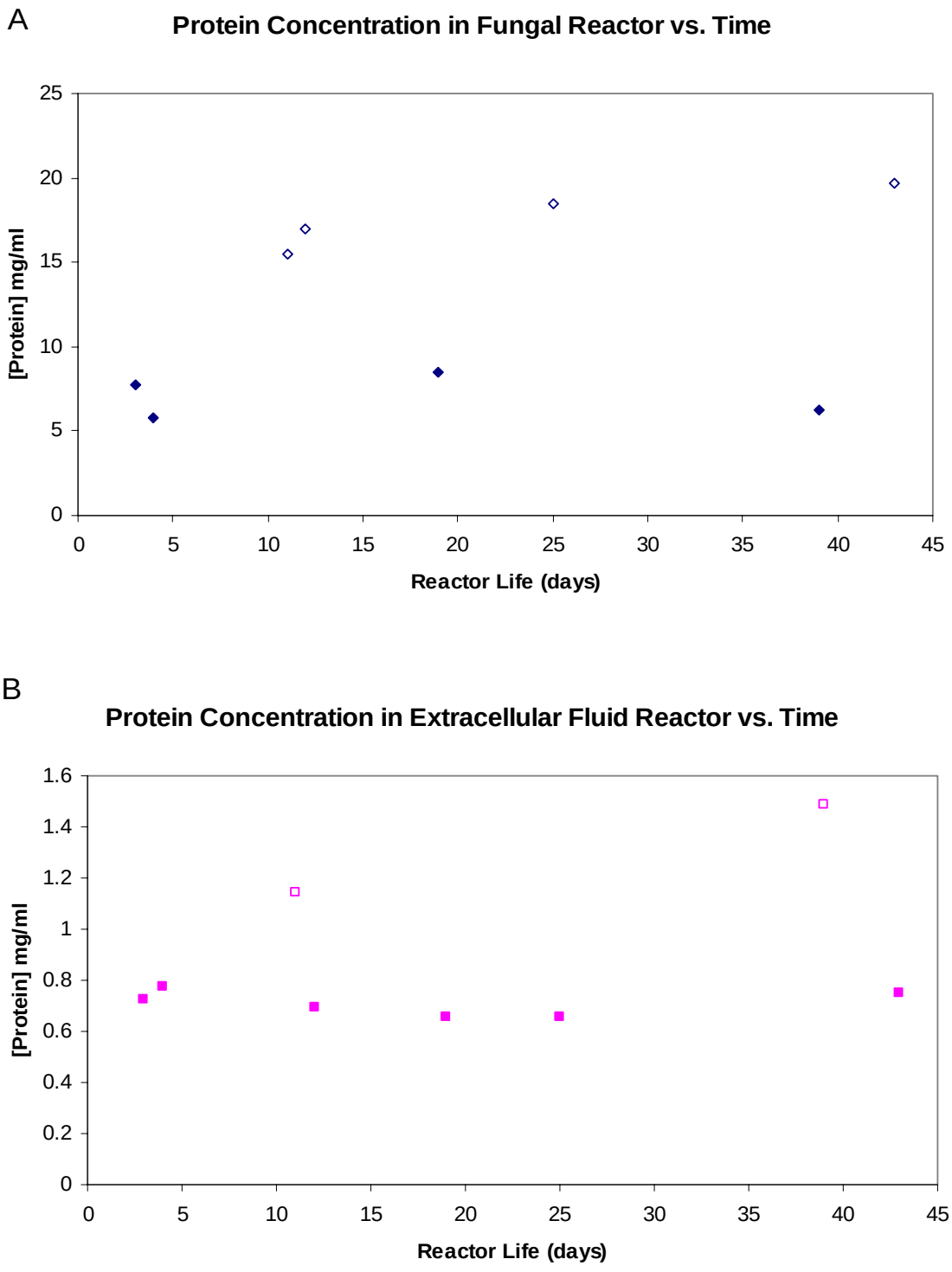


Figure 33. Protein concentration in fungal reactor (A) and protein concentration in extracellular fluid reactor (B) during reactor operation ◆-Protein concentration in fungal reactor; ■-Reactor concentration in extracellular fluid reactor

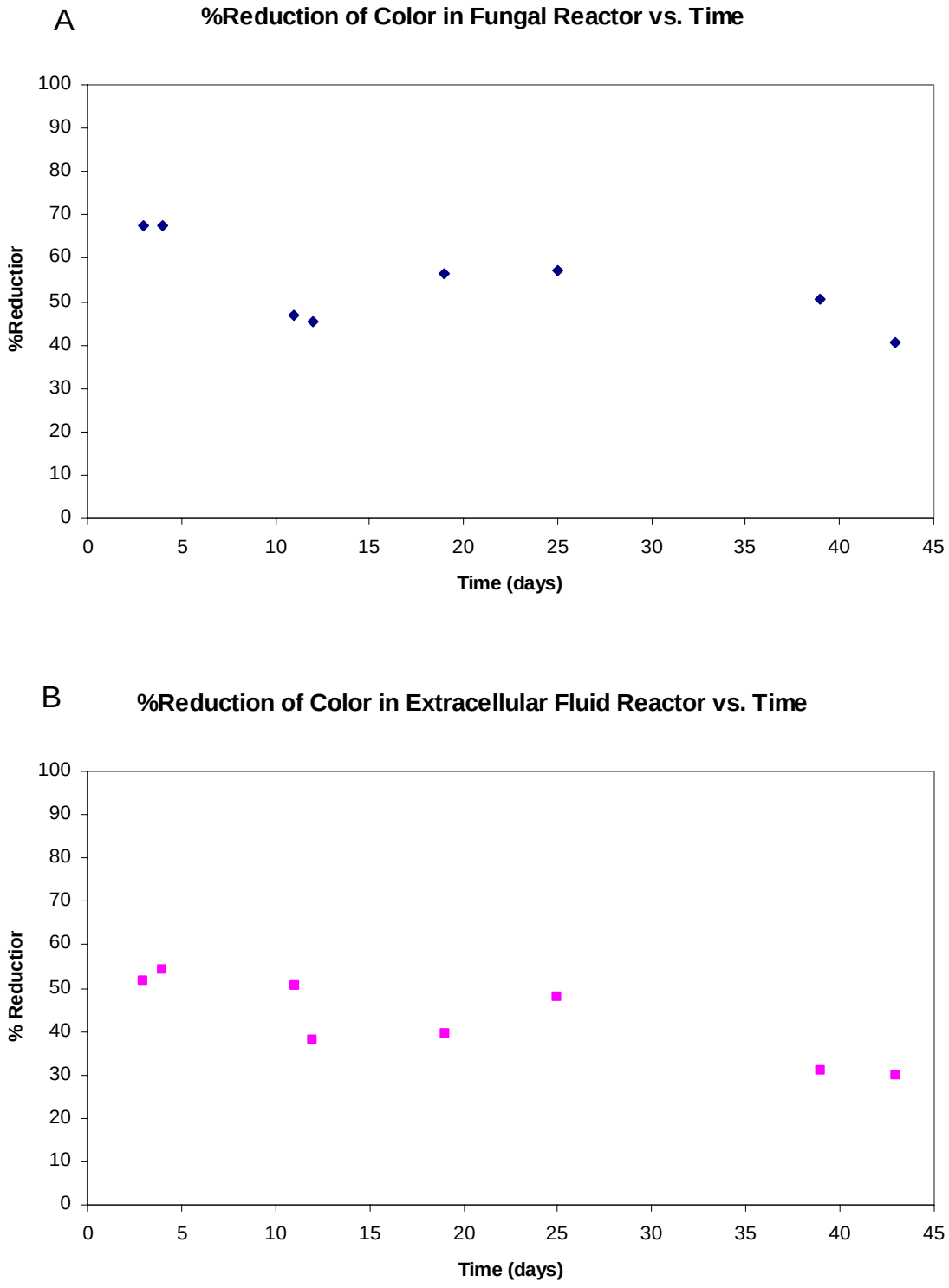


Figure 34. %Reduction in color in fungal reactor (A) and % reduction in color in extracellular fluid reactor (B) during reactor operation ◆-% color reduction in fungal reactor; ■-% color reduction in extracellular fluid reactor

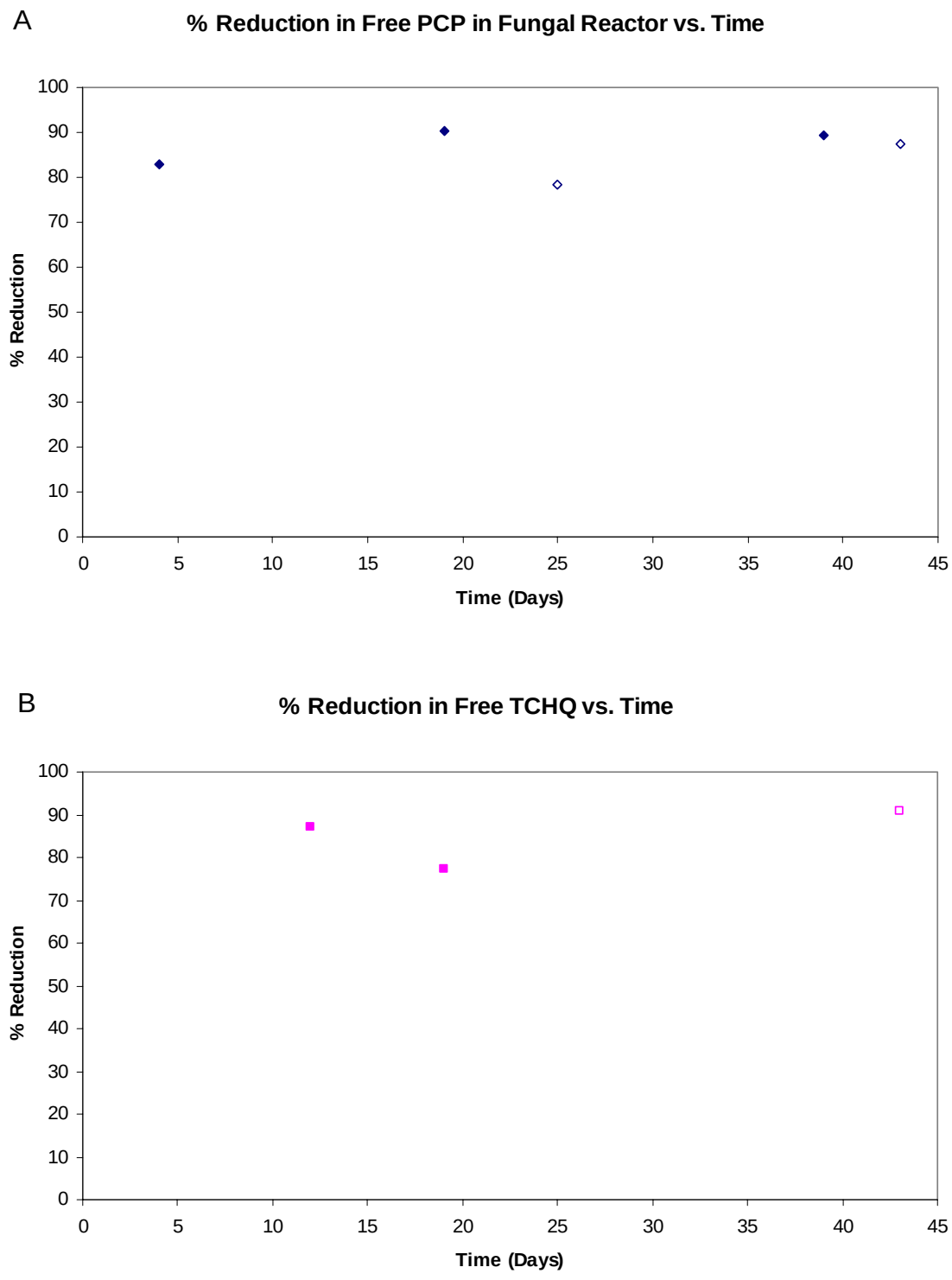


Figure 35. %Reduction in free PCP in fungal reactor (A) and % reduction in free TCHQ in fungal reactor (B) during reactor operation ◆-% Reduction of free PCP in fungal reactor; ■- %Reduction of free TCHQ in fungal reactor.

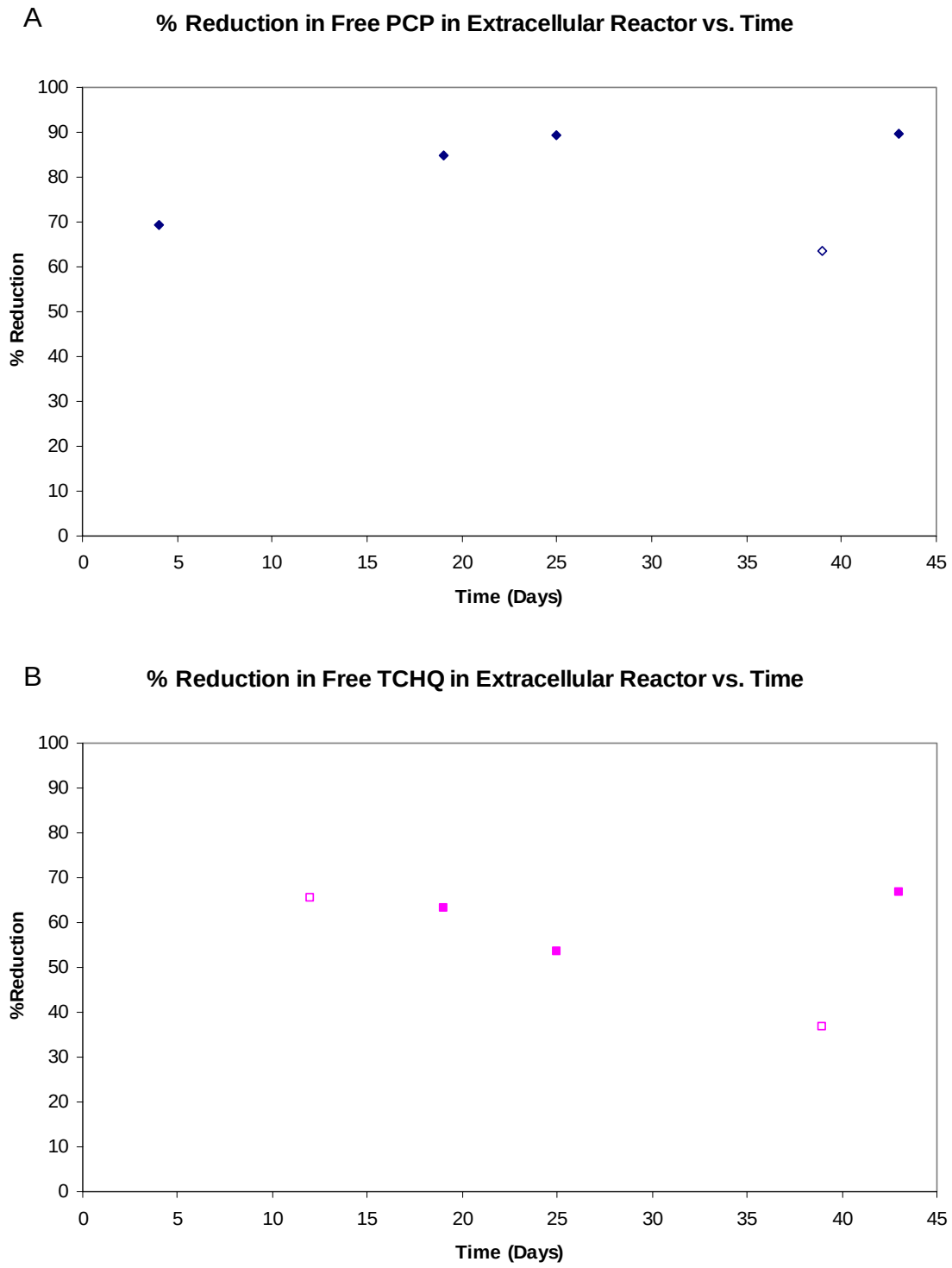


Figure 36. %Reduction in free PCP in CEFR (A) and % reduction in free TCHQ in CEFR (B) during reactor operation ◆-% Reduction of free PCP in CEFR; ■- %Reduction of free TCHQ in CEFR.

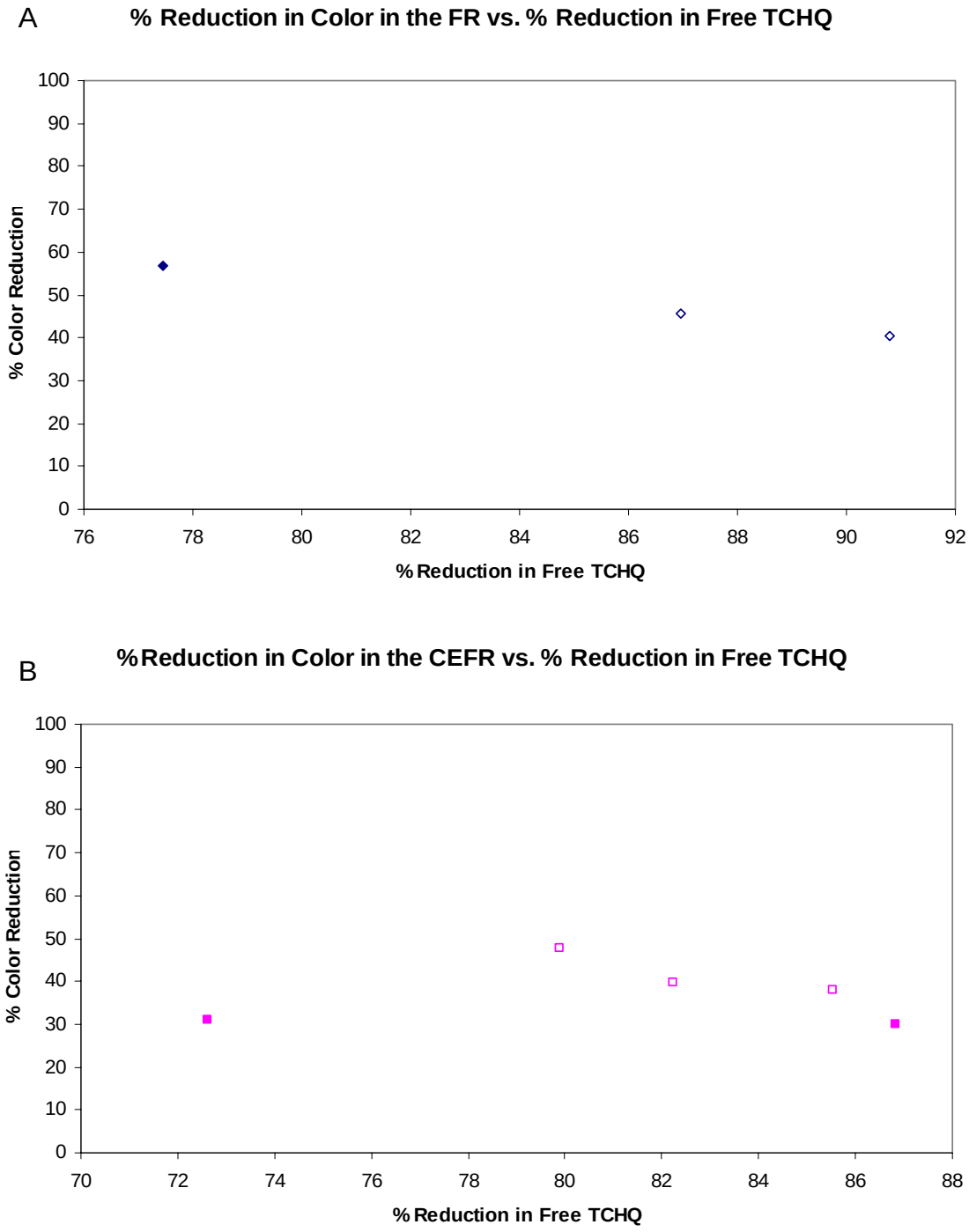


Figure 37. %Reduction in color in FR (A) and % reduction in color in CEFR (B) versus the % reduction in free TCHQ ◆-% Reduction of color in FR; ■- %Reduction of color in CEFR.

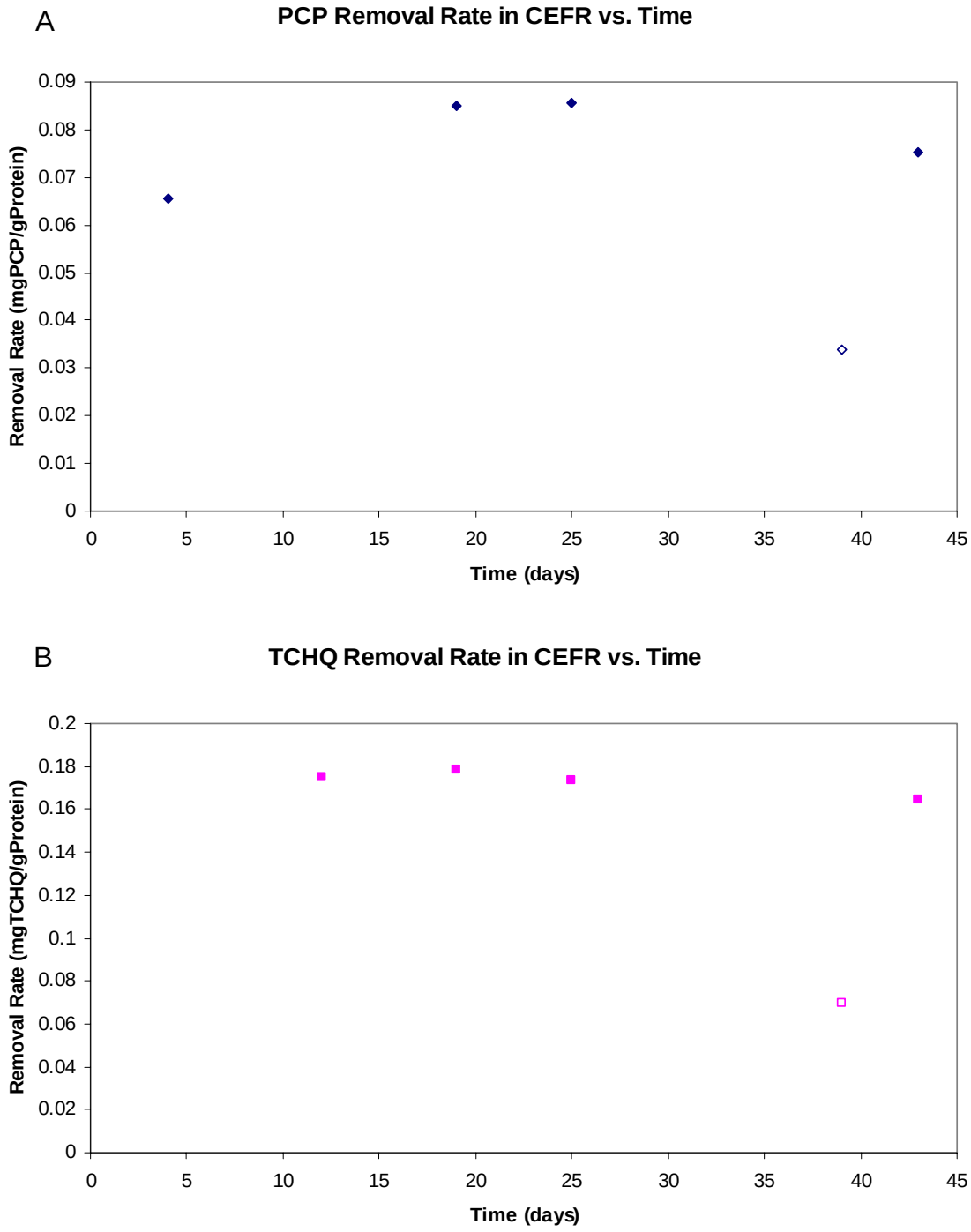
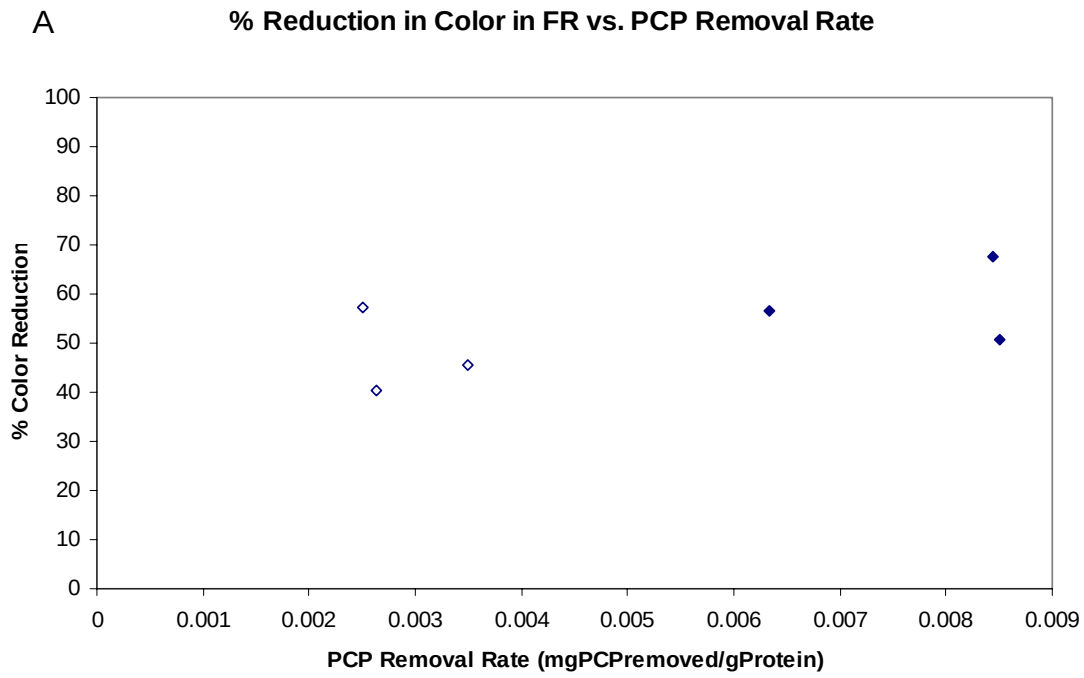


Figure 38. Removal of PCP in CEFR (A) and removal TCHQ in CEFR (B) during reactor operation ◆- Removal of PCP in CEFR; ■- Removal of TCHQ in CEFR.



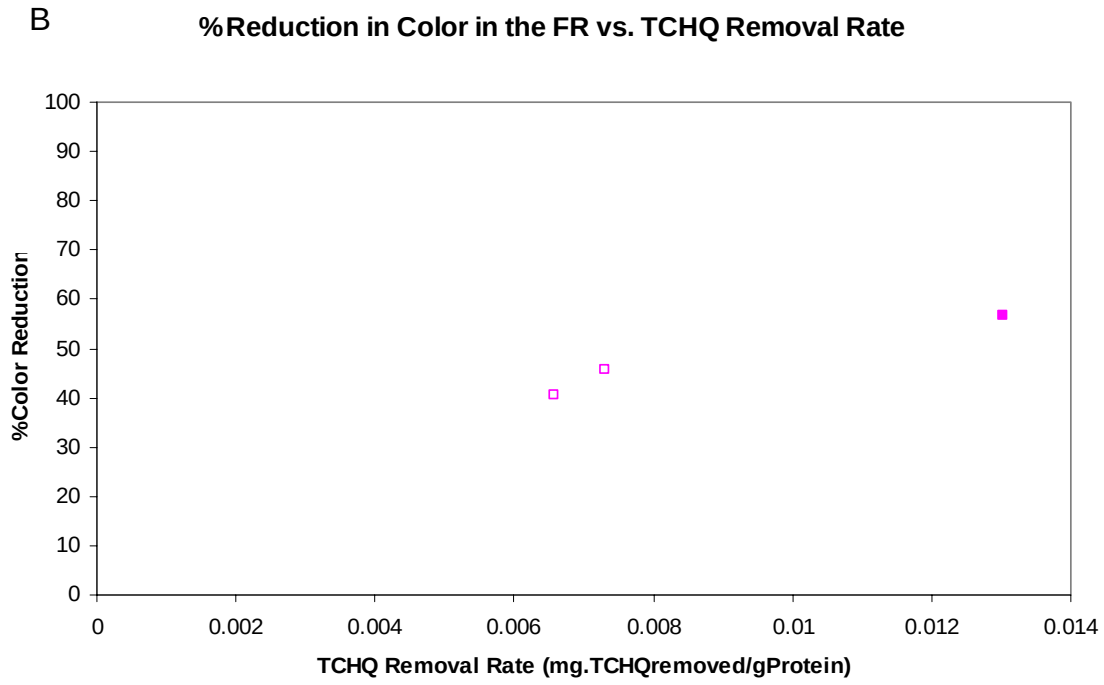
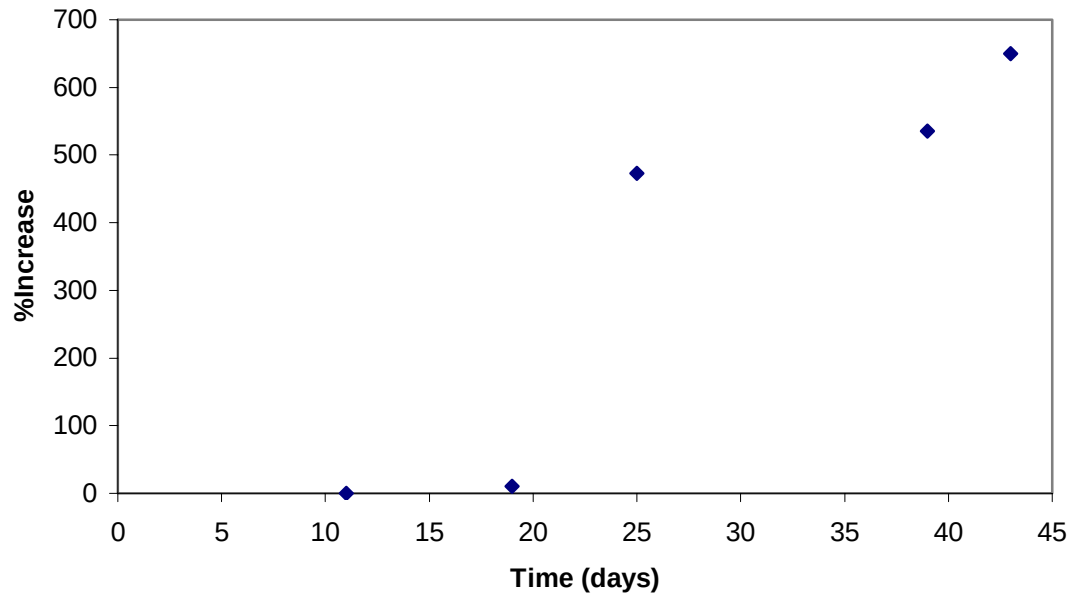


Figure 39. %Reduction in color in FR with respect to PCP removal (A) and % reduction in color in FR with respect to TCHQ removal(B). ◆-% Reduction of color in FR; ■-%Reduction of color in FR.

A



B

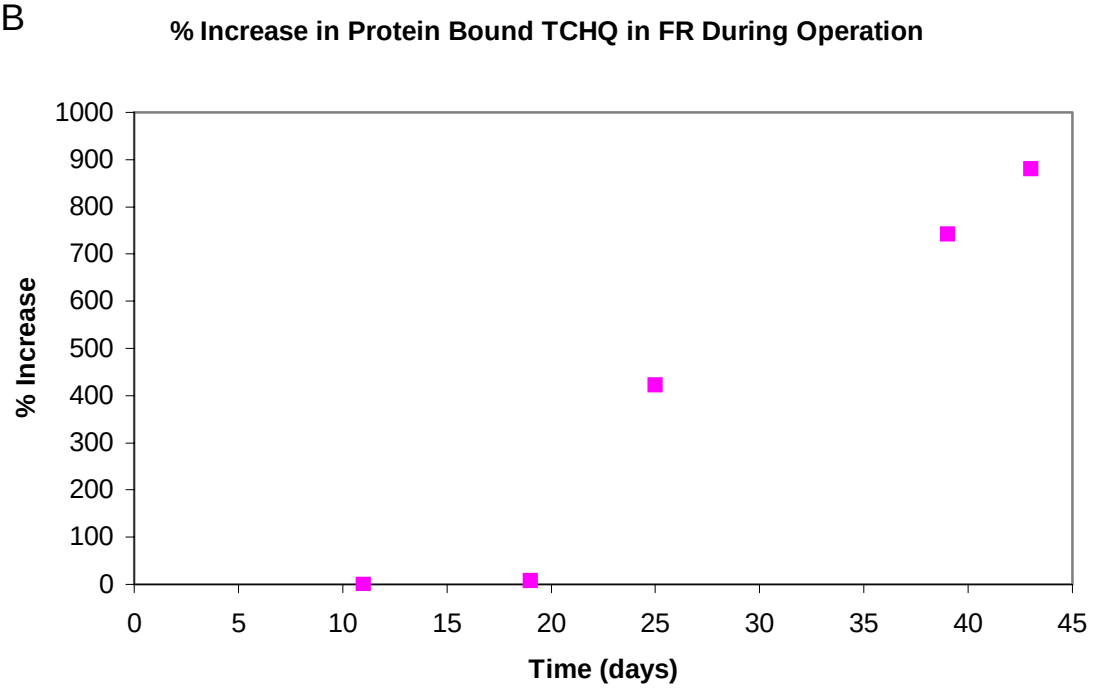
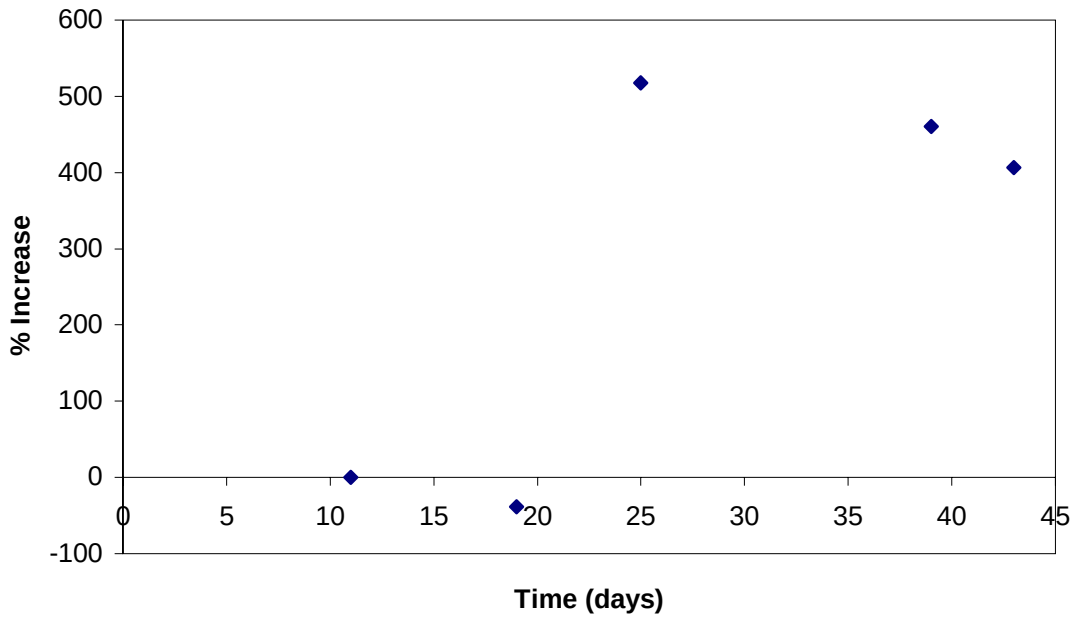


Figure 40. %Increase in protein bound PCP in FR (A) and % Increase in protein bound TCHQ in FR (B) during reactor operation ◆-% Increase in protein bound PCP in FR; ■-%Increase in protein bound TCHQ in FR.

A



B

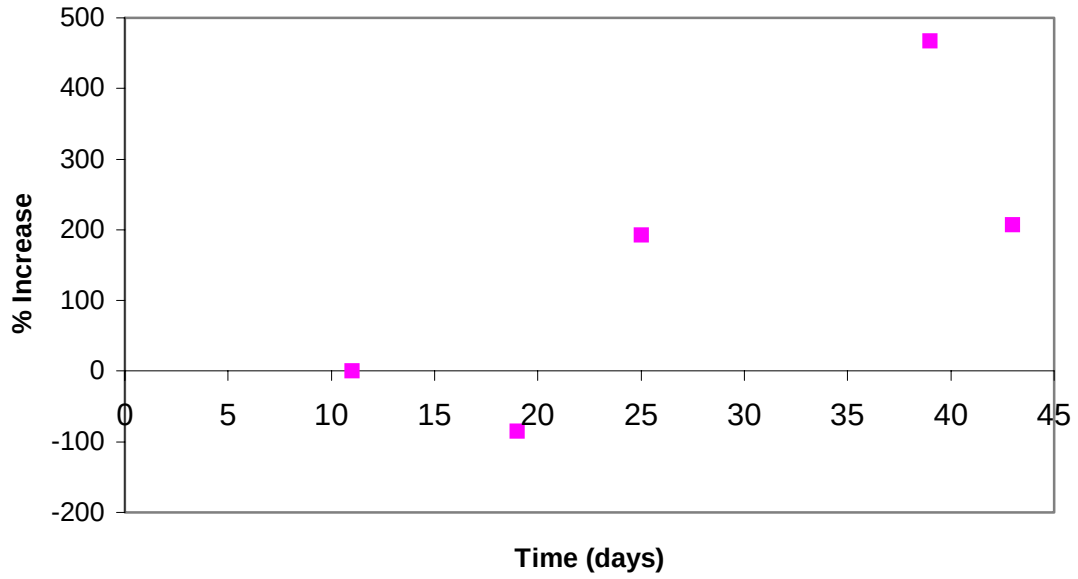


Figure 41. % Increase in protein bound PCP in CEFR (A) and % Increase in protein bound TCHQ in CEFR (B) during reactor operation ◆-% Increase of protein bound PCP in CEFR; ■- %Increase of protein bound TCHQ in CEFR.

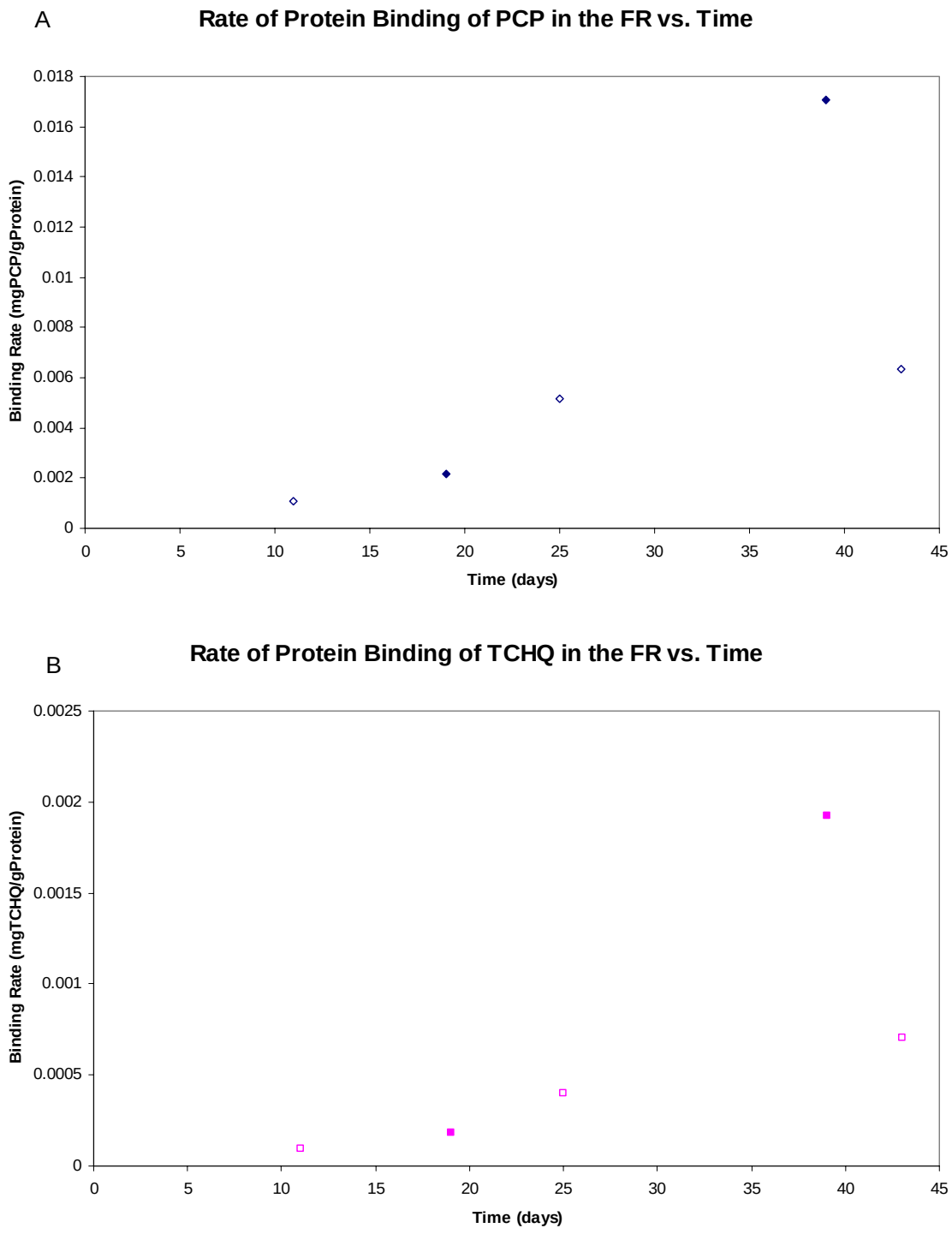


Figure 42. Protein binding of PCP in FR (A) and protein binding of TCHQ in FR (B) during reactor operation ◆-Protein binding of PCP in FR; ■- Protein binding of TCHQ in FR.

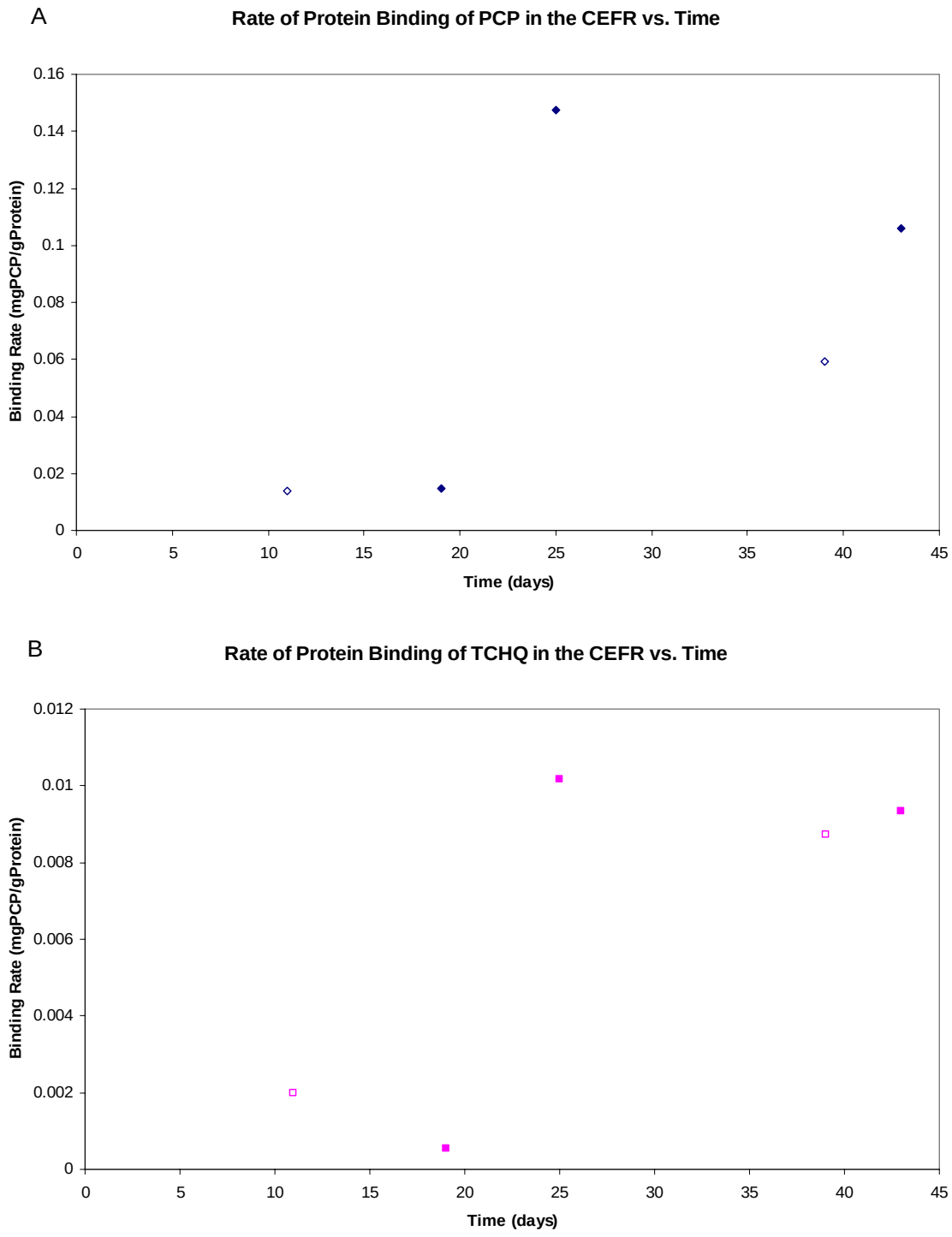


Figure 43. Protein binding of PCP in CEFR (A) and protein binding of TCHQ in CEFR (B) during reactor operation ◆-Protein binding of PCP in CEFR; ■- Protein binding of TCHQ in CEFR.

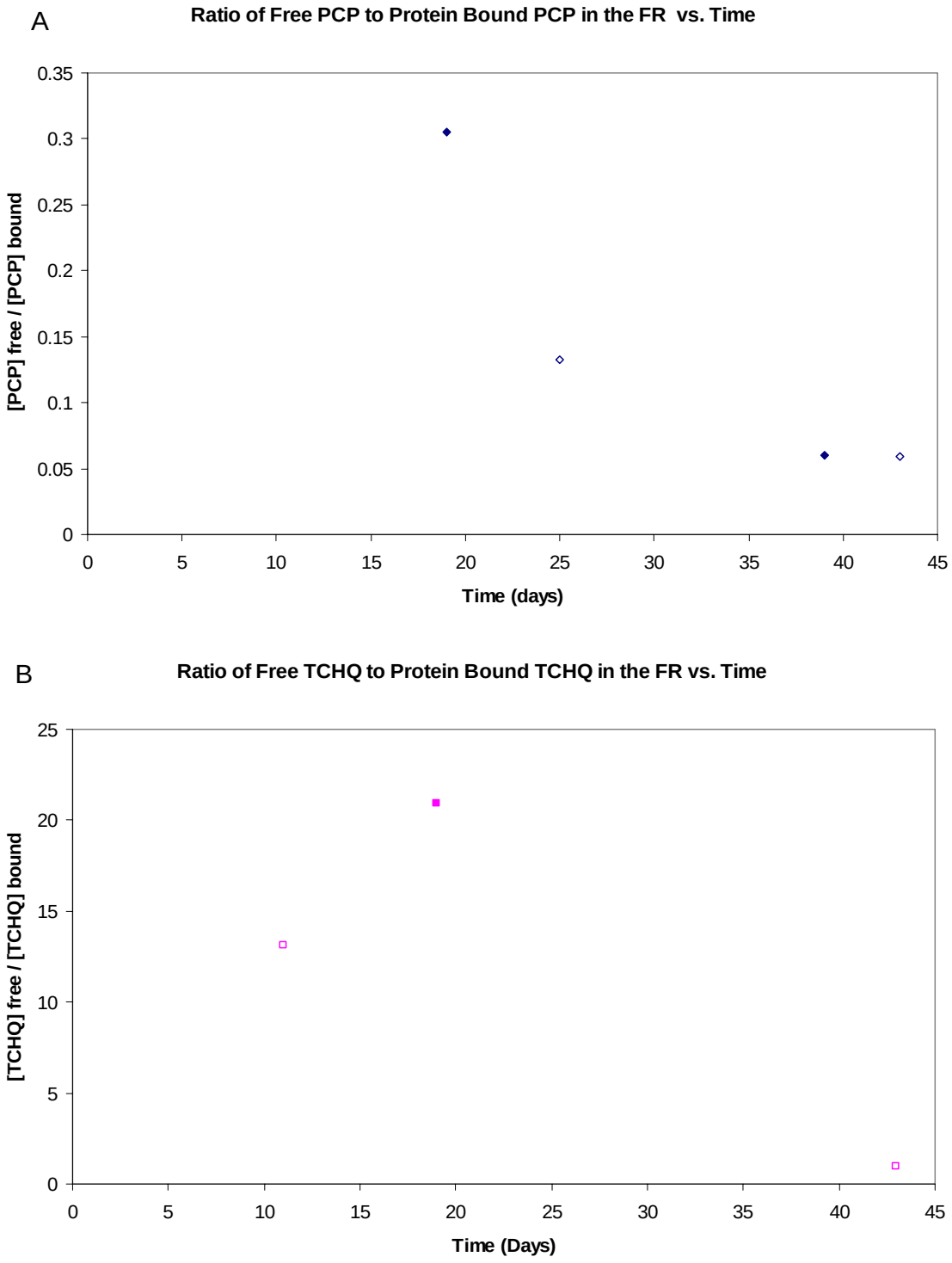


Figure 44. Ratio of free PCP to protein bound PCP in the FR (A) and ratio of free TCHQ to protein bound TCHQ in the FR (B) during reactor operation. ◆-Ratio of free PCP to protein bound PCP; ■- Ratio of free TCHQ to protein bound TCHQ.

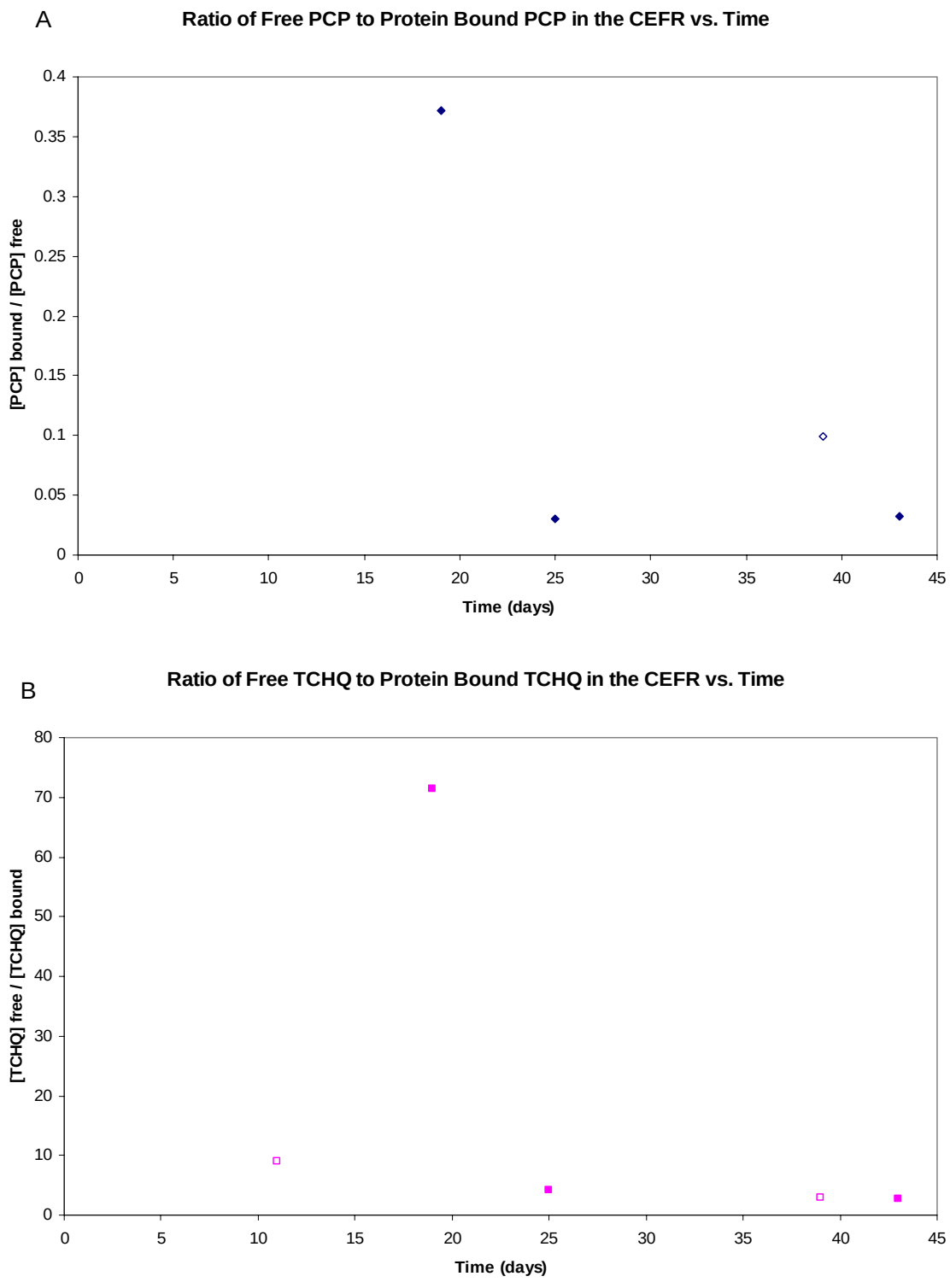


Figure 45. Ratio of free PCP to protein bound PCP in the CEFR (A) and ratio of free TCHQ to protein bound TCHQ in the CEFR (B) during reactor operation. ◆-Ratio of free PCP to protein bound PCP; ■- Ratio of free TCHQ to protein bound TCHQ.

CHAPTER IV

USE OF SULFHYDRYL CONTAINING MODEL COMPOUNDS TO REMOVE PCP FROM AQUEOUS SOLUTIONS

ABSTRACT

Earlier studies have shown that protein binding contributes to the removal of the chlorinated compounds, PCP and TCHQ, from aqueous solutions. Sulfhydryl binding of the protein seems to be the mechanism for this binding to occur. Model compounds containing sulfhydryl groups were reacted with FR and CEFR solutions to determine the effects of different types of molecules that contain sulfhydryl groups on the concentrations of free and bound PCP and TCHQ. Dithiothreitol (DTT), glutathione (GLUT) and cysteine (CYS) removed PCP from aqueous solution by forming conjugates and bovine serum albumin (BSA) appeared to remove PCP by competitive binding or affecting solubility of PCP. Inhibition of binding of PCP occurred by the adding BSA or DTT with GLUT or CYS. However inhibition of binding of TCHQ only occurred with the addition of DTT with GLUT or CYS, which also increased the concentration of free TCHQ. The addition of BSA greatly enhanced the amount of binding with TCHQ.

INTRODUCTION

Experiments have been conducted which indicate that sulfhydryl binding between protein and chlorinated compounds as an important mechanism for removing chlorinated compounds from aqueous solutions. Several studies have been conducted which show that sulfhydryl binding occurs between protein and chlorinated compounds in biological systems (Rizzardini & Smith, 1982; Lilienblum, 1985; Van Ommen et al., 1986, 1988; den Besten et al., 1989; Waidyanatha et al., 1994, 1996; Lin et al., 1993, 1997, 1999). Goldfish have been shown to form sulfate bound PCP conjugates (Akitake & Kobayashi, 1975). Other animals, such as mice, metabolize PCP into TCHQ which binds to liver and blood protein by covalent sulfhydryl binding (Van Ommen, 1986, 1988; den Bensten et al., 1989; Waidyanatha et al., 1994).

Studies have shown that adding different sulfhydryl contained compounds to reactions where covalent protein binding is occurring has different effects on binding. The addition of bovine serum albumin (BSA) has been shown to increase protein binding of laccase catalyzed reaction, depending on BSA concentration and activity of laccase (Shuttleworth & Bollag, 1986). Glutathione and cysteine inhibit covalent binding of TCHQ by forming adducts with TCHQ (Van Ommen et al., 1988, den Besten et al., 1989).

Objectives of this part of the study were to verify that sulfhydryl interactions were responsible for protein binding of PCP and TCHQ and to determine the effects of adding model protein compounds and model sulfhydryl containing compounds had on protein

binding. BSA was chosen to study the effects of adding a known protein which contains one sulfhydryl group and 17 intrachain disulfide bounds (Hirayama, K., 1990). The molecular weight of BSA has been determined to be 66430 and consists of 583 amino acid residues and no carbohydrates as a single chain polypeptide (Hirayama, K., 1990). Cysteine and glutathione were selected because cysteine is a single amino acid that contains a sulfhydryl group and glutathione is a tripeptide containing a sulfhydryl group. Dithiothreitol was used in these experiments because it contains two sulfhydryl groups, it is a reagent for maintaining SH groups in reduced state (Cleland, 1964) and has been used for reducing protein disulfide bonds.

By determining the effects of reactions with model compounds the mechanisms of protein binding with chlorinated phenolic compounds as a method to remove them can better understood, and the remediation of chlorinated phenolics can be better understood. By studying reactions with molecules that contain sulfhydryl groups, protein or another type of compound, other removal technologies can be developed.

METHODS AND MATERIALS

Analytical Methods

Determination of reactor protein, unbound PCP and TCHQ and protein bound PCP and TCHQ were described in Chapter II.

Experimental Approach

Bovine serum albumin (Sigma) was prepared as a 400μg/ml standard solution. Dithiothreitol (DTT) (Fisher Scientific), glutathione (GLUT) (Fisher Scientific) and cysteine (CYS) (Fisher Scientific) were prepared as a 0.02M stock solutions. Dithiothreitol and glutathione were prepared in distilled water and cysteine was prepared in 0.5M HCl. A 1.0mg/L PCP solution was prepared with 0.16% $(NH_4)_2SO_4$, 0.3% K_2HPO_4 and 0.2% KH_2PO_4, and pH was adjusted to 3.8. The two-stage bioreactor system was used to treat 2.5mg/L PCP feed solutions as described in Chapter II. Reactor samples were taken from the FR and CEFR and reacted with BSA, DTT, GLUT and CYS to determine the effects of adding several different sulfhydryl containing compounds. Reactor protein concentration and pH was measured for each sample.

Prior to reacting DTT with reactor solutions or buffer solution 25 μl of DTT stock solution was mixed with 1 ml of GLUT stock solution, CYS stock solution or distilled water. Reactions with bioreactor samples or the 1.0mg/L PCP buffer solution contained 5 ml of FR, CEFR or 1mg/L PCP solution and 0.5ml of the DTT/GLUT, DTT/CYS, DTT/Water, GLUT stock or CYS stock solution. Samples described as having no

treatment contained 5 ml of the FR, CEFR or 1mg/L PCP solution and 0.5ml distilled water. The sample mixtures were adjusted to the measured reactor pH. Samples were continuously stirred and allowed to react for 10min. After the reaction time was complete, the amounts of unbound compounds or protein bound compounds were determined following the procedures described in Chapter II.

A 400μg/ml standard solution of BSA was prepared to add to reactor samples. The average reactor protein concentrations in the FR and CEFR were determined and concentrations of BSA were chosen to add 50% or 25% more protein to the reaction mixture. Reactor solution from the FR (5ml) was mixed with either 625μl or 313μl of the BSA standard solution and the mixture was adjusted to the measured reactor pH. Reactor solution from the CEFR (5ml) was mixed with either 250μl or 125μl of the BSA standard solution and the mixture was adjusted to the measured reactor pH. Samples with no treatment were not diluted any further. Samples were continuously stirred and allowed to react for 10min. After the reaction time was complete, the amount of unbound compounds or protein bound was determined following the procedures described in Chapter II.

RESULTS AND DISCUSSION

Effects of adding sulfhydryl containing model compounds on free PCP in reactor and buffered PCP solutions

Solutions from the FR and CEFR were reacted with mixtures of DTT and GLUT or DTT and CYS. The effects that this treatment had on the concentration of free PCP in solution are shown in Table 47. In the CEFR, the concentration of free PCP in the reaction mixture was reduced considerably. Treatment of the CEFR solution with DTT and GLUT decreased free PCP by 49%, and treatment with DTT and CYS decreased free PCP by almost 38%. The results with the CEFR solution are expected since PCP has been shown to form conjugates with sulfhydryl compounds (Akitake & Kobayashi). The additional experiments of DTT, GLUT and CYS with the 1mg/L PCP, results presented in Table 48, provided more insight into the action of these compounds on removing PCP from aqueous solution. GLUT and CYS stock solutions only reduced free PCP by about 6%. However, DTT alone was able to reduce PCP by 28%, the DTT and GLUT solution reduced PCP by 44% and the DTT and CYS solution reduced PCP by 38%. The structures of DTT, GLUT and CYS are shown in Figure 46. DTT contains two sulfhydryl groups, which are capable of binding PCP. DTT tends to remain in reduced form and is capable of reducing sulfhydryl groups (Cleland, 1964) and reacting with oxygen or other oxidizing compounds. Conjugation of PCP with GLUT or CYS seems to require the presence of DTT to reduce these compounds to their reduced, active form and DTT may react with oxidizers that may interfere with GLUT or CYS forming conjugates when DTT is not in the mixture.

In the FR, the amount of free PCP in the reaction mixture was actually 31% higher when treated with DTT and GLUT, as well as with DTT and CYS, though at a lesser extent at 6%. An increase in PCP in the FR was also observed when reacted with BSA. The addition of 50% more protein in the form of BSA resulted in a 78% increase in free PCP and addition of 25% more protein resulted in 28% more free PCP in reactions with FR solutions. However, reactions of BSA reduced PCP by 22% in the CEFR, shown in Table 49. The opposite effects of the treatment on the two different reactor solutions are perplexing. Removal of phenols in laccase-catalyzed reactions has been enhanced by the addition of BSA, however laccase must be active (Shuttleworth & Bollag). One possible explanation is that the higher concentration of reactor protein in the FR is due to higher molecular weight protein that is not present in the CEFR. Some PCP may be entrapped amongst protein molecules, in a micellar fashion. The addition of DTT and GLUT, DTT and CYS and BSA to this protein may decrease solubility of the proteins present and cause the proteins to release entrapped PCP into solution as free PCP. These compounds may also interact with the proteins entrapping PCP, being more attractive to the proteins due to the similar structures, causing the protein to release entrapped PCP. This could explain why the higher concentration of BSA results in more PCP in solution and why the larger molecule GLUT has a greater effect on increasing PCP than CYS. The protein in the CEFR is more soluble than that in the FR therefore the protein in the CEFR is likely involved in other types of interactions with PCP. There is also fresh unbound PCP fed to the CEFR, so more PCP is available to form conjugates with sulfhydryl compounds.

Effects of adding sulfhydryl-containing model compounds on bound PCP in reactor solutions

Reactions of FR and CEFR solutions with DTT and GLUT, DTT and CYS, and BSA inhibited binding of PCP, shown in Tables 50 and 51. GLUT and CYS with DTT had the greatest effect on inhibiting binding of PCP in the CEFR solutions about 60%, while BSA greatly inhibited binding of PCP in the FR solutions by over 85%. Van Ommen et al.(1988) also found that GLUT and CYS inhibited covalent binding during binding of TCBQ to protein. GLUT and CYS were determined to form conjugates with TCHQ to inhibit binding. Since the concentration of free PCP was decreased significantly when CEFR solutions were reacted with GLUT or CYS with DTT, this is a likely explanation for the inhibition of binding of PCP. Inhibition of binding of PCP by BSA could also be due to competitive binding or solubility effects.

Effects of adding sulfhydryl containing model compounds on free and protein bound TCHQ in reactor solutions

When GLUT or CYS with DTT was reacted with solutions from the FR and CEFR most samples experienced an increase in free TCHQ, shown in Table 52, and a decrease in bound TCHQ, shown in Table 53. These effects were exhibited most strongly in the CEFR reactions, with a 63% increase in free TCHQ and 90% inhibition of binding when reacted with GLUT and DTT, and a 27% increase in free TCHQ and 92% inhibition of binding when reacted with CYS and DTT. Inhibition of binding of TCHQ, along with an increase in TCHQ, was reported by Van Ommen et al. (1986) when ascorbic acid was added to solutions, where binding of TCBQ occurred, however no increase in TCHQ was

seen when reacting GLUT or CYS. These researchers determined ascorbic acid inhibited binding by reducing TCBQ to TCHQ. In the reaction mixture using GLUT or CYS, the strong reducing agent DTT was also present. This could explain the inhibition of protein binding and the increase of TCHQ. While GLUT and CYS with DTT were able to form conjugates with PCP, the reductive nature of DTT was able to reduce TCHQ before it was able to bind, therefore the concentration of free TCHQ increased while binding was inhibited.

Reactions of BSA with the reactor solutions had a quite different effect on the concentration of bound TCHQ. The reaction mixtures did not contain detectable levels of free TCHQ therefore those results are not shown. Binding of TCHQ, shown in Table 54, was increased greatly in the presence of BSA. The addition of just 25% more protein in the form of BSA increased the amount of bound TCHQ over 125%. BSA apparently binds strongly to TCHQ forming covalent bounds. This significant increase in bound TCHQ could help explain why, in Chapter III, the small increase of protein produced in the FR due to the addition of BPE in the feed to the FR led to very high concentrations of bound TCHQ. BPE added more carbohydrates as well as a reactive component that increased protein production and conversion of PCP to TCHQ. A slight increase in the concentration of protein that is active and available for binding in the reactor can significantly increase the amount of binding that occurs with TCHQ.

CONCLUSIONS

Previous experiments with bioreactor solutions have suggested that protein binding is an important mechanism for removal of chlorinated compounds from aqueous solutions. These experiments have shown some of the effects that sulfhydryl containing compounds have on chlorinated compounds present in reactor solutions free in solution or protein bound.

DTT, GLUT and CYS were found to form conjugates with PCP, removing free PCP from solutions obtained from the CEFR or buffered, untreated PCP solutions. Reactions of GLUT and CYS were greatly enhanced by the presence of DTT in the solution mixture. The addition of BSA to CEFR solutions also decreased the concentration of free PCP, likely due to BSA binding to PCP or non-covalent entrapment of PCP. Reactions of the model compounds with FR solutions resulted in an increase in free PCP. Interactions of the model compounds with protein in the FR may cause this to occur. Binding of PCP was inhibited by reactions with GLUT and CYS with DTT present and BSA. Inhibition is likely due to competitive binding or reduction reactions.

The concentrations of free TCHQ and bound TCHQ were affected differently. Inhibition of binding of TCHQ occurred when reacted with GLUT and CYS in the presence of DTT and the concentration of free TCHQ actually increased. The strong reducing properties of DTT likely reduce TCBQ to TCHQ, which would inhibit binding and increase the concentration of TCHQ. Binding of TCHQ increased significantly by the addition of BSA, which seems to form covalent, sulfhydryl bounds with TCHQ.

Verification of sulfhydryl interactions occurring with PCP and TCHQ provides more evidence that the protein binding measured throughout this study was due to binding to the sulfhydryl on the protein binding. Solubility of the protein in the reactor solution was determined to be very important for binding to occur. The concept of sulfhydryl binding could be used to develop other types of remediation technologies.

Table 47: Effects of treatment of FR solution or CEFR solution with sulfhydryl containing model compounds on free PCP

	Sample Description	Sample pH	Reactor Protein (μg/ml)	PCP_{free} (mg/L)	% Change in PCP Due to Treatment
FR Solution					
	No Treatment	4.32	102	1.19	-
	Glutathione & Dithiothreitol	4.32		1.56	+31%
	Cysteine & Dithiothreitol	4.32		1.27	+6%
CEFR Solution					
	No Treatment	3.87	43	4.46	-
	Glutathione & Dithiothreitol	3.87		2.29	-49%
	Cysteine & Dithiothreitol	3.87		2.75	-38%

Note: 1. Each sample was performed in duplicate; 2. Final concentration model compounds in each sample: 0.045mM dithiothreitol; 1.8mM glutathione; 1.8mM cysteine

Table 48: Effects of treatment of 1mg/L buffered PCP solution or CEFR solution with sulfhydryl containing model compounds on free PCP

	Sample Description	Sample pH	Reactor Protein (μg/ml)	PCP_{free} (mg/L)	% Reduction in PCP Due to Treatment
1mg/L Buffer PCP Solution					
	No Treatment	3.8	0	1.19	-
	Glutathione	3.8		0.99	6.4%
	Cysteine	3.8		1.00	5.6%
	Dithiothreitol	3.8		0.761	28%
	Glutathione & Dithiothreitol	3.8		0.590	44%
	Cysteine & Dithiothreitol	3.8		0.661	38%
CEFR Solution					
	No Treatment	3.87	43	4.46	-
	Glutathione & Dithiothreitol	3.87		2.29	49%
	Cysteine & Dithiothreitol	3.87		2.75	38%

Note: 1. Each sample was performed in duplicate; 2. Final concentration model compounds in each sample: 0.045mM dithiothreitol; 1.8mM glutathione; 1.8mM cysteine

Table 49: Effects of treatment of FR solution or CEFR solution with bovine serum albumin on free PCP

	Sample Description	Sample pH	Reactor Protein (μg/ml)	PCP_{free} (mg/L)	% Change in PCP Due to Treatment
FR Solution					
	No Treatment	4.33	107	0.415	-
	50μg/ml BSA	4.33		0.738	+78%
	25μg/ml BSA	4.33		0.520	+25%
CEFR Solution					
	No Treatment	4.07	38	0.743	-
	20 μg/ml BSA	4.07		0.719	-22%
	10 μg/ml BSA	4.07		0.714	-22%

Note: Each sample was performed in duplicate

Table 50: Effects of treatment of FR solution or CEFR solution with sulfhydryl containing model compounds on bound PCP

	Sample Description	Sample pH	Reactor Protein (μg/ml)	PCP_{bound} (mg/L)	% Change in PCP_{bound} Due to Treatment
FR					
	No Treatment	5.01	79	0.043	-
	Glutathione & Dithiothreitol	5.02		0.030	-30%
	Cysteine & Dithiothreitol	5.03		0.080	+86%
CEFR Solution					
	No Treatment	4.13	37	0.047	-
	Glutathione & Dithiothreitol	4.02		0.020	-57%
	Cysteine & Dithiothreitol	4.02		0.016	-66%

Note: 1. Each sample was performed in duplicate; 2. Final concentration model compounds in each sample: 1.8mM dithiothreitol; 1.8mM glutathione; 1.8mM cysteine

Table 51: Effects of treatment of FR solution or CEFR solution with bovine serum albumin on bound PCP

	Sample Description	Sample pH	Reactor Protein (μg/ml)	PCP_{bound} (mg/L)	% Change in PCP_{bound} Due to Treatment
FR Solution					
	No Treatment	4.37	62	0.026	-
	50μg/ml BSA	4.37		0.004	-85%
	25μg/ml BSA	4.36		0.003	-88%
CEFR Solution					
	No Treatment	3.92	46	0.076	-
	20 μg/ml BSA	3.92		0.057	-25%
	10 μg/ml BSA	3.92		0.043	-43%

Note: Each sample was performed in duplicate

Table 52: Effects of treatment of FR solution or CEFR solution with sulfhydryl containing model compounds on free TCHQ

Sample Description	Sample pH	Reactor Protein (μg/ml)	$TCHQ_{free}$ (mg/L)	% Change in $TCHQ_{free}$ Due to Treatment
FR Solution				
No Treatment	4.322	102	0.0160	-
Glutathione & Dithiothreitol	4.322		0.0155	-3%
Cysteine & Dithiothreitol	4.322		0.0190	+19%
CEFR Solution				
No Treatment	3.87	43	0.0187	-
Glutathione & Dithiothreitol	3.87		0.0304	+63%
Cysteine & Dithiothreitol	3.87		0.0237	+27%

Note: 1. Each sample was performed in duplicate; 2. Final concentration model compounds in each sample: 1.8mM dithiothreitol; 1.8mM glutathione; 1.8mM cysteine

Table 53: Effects of treatment of FR solution or CEFR solution with sulfhydryl containing model compounds on bound TCHQ

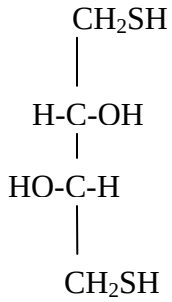
Sample Description	Sample pH	Reactor Protein (μg/ml)	$TCHQ_{bound}$ (mg/L)	% Change in $TCHQ_{bound}$ Due to Treatment
FR Solution				
No Treatment	5.01	77	0.0018	-
Glutathione & Dithiothreitol	5.02		0.0015	-17%
Cysteine & Dithiothreitol	5.03		0.0014	-22%
CEFR Solution				
No Treatment	4.13	37	0.0049	-
Glutathione & Dithiothreitol	4.02		0.0005	-90%
Cysteine & Dithiothreitol	4.02		0.0004	-92%

Note: 1. Each sample was performed in duplicate; 2. Final concentration model compounds in each sample: 1.8mM dithiothreitol; 1.8mM glutathione; 1.8mM cysteine

Table 54: Effects of treatment of FR solution or CI lution with bovine serum albumin on bound TCHQ

Sample Description	Sample pH	Reactor Protein (μg/ml)	$TCHQ_{bound}$ (mg/L)	% Change in $TCHQ_{bound}$ Due to Treatment
FR Solution				
No Treatment	4.37	62	0.0058	-
50μg/ml BSA	4.37		0.0146	+152%
25μg/ml BSA	4.36		0.0138	+138%
CEFR Solution				
No Treatment	3.92	46	0.0021	-
20 μg/ml BSA	3.92		0.0048	+128%
10 μg/ml BSA	3.92		0.0053	+152%

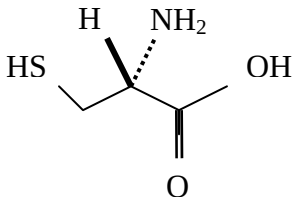
Note: Each sample was performed in duplicate



Dithiothreitol

$C_4H_{10}O_2S_2$

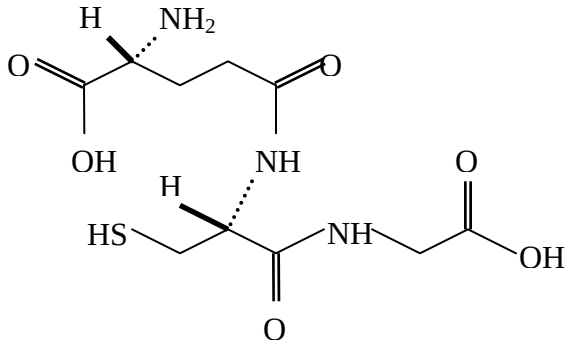
MW: 154.25



Cysteine

$C_3H_7NO_2S$

MW: 121.16



Glutathione

$C_{10}H_{17}N_3O_5$

MW: 307.32

Figure 46: Structures of the sulfhydryl containing model compounds

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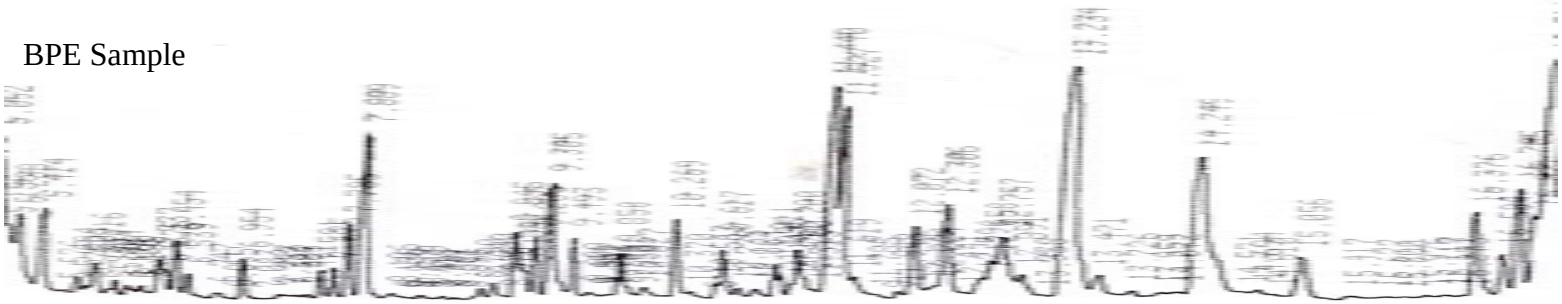
APPENDIX I

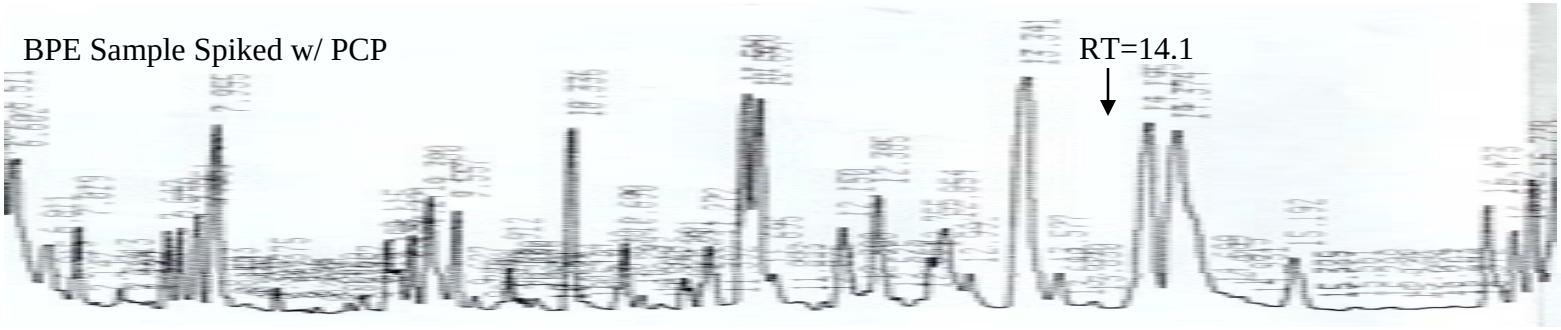
Verification of PCP and TCHQ by Spiking BPE Samples

BPE Sample



BPE Sample Spiked w/ PCP

RT=14.1

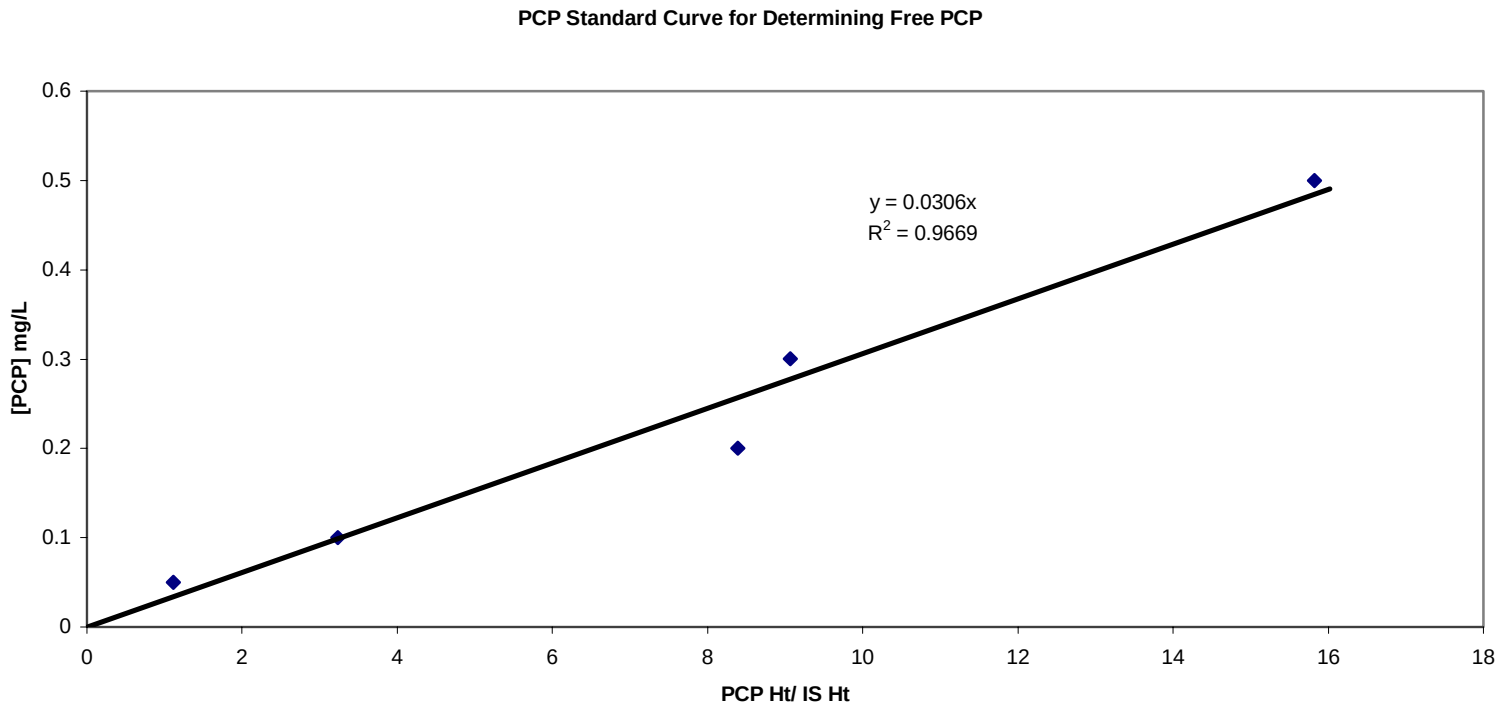


BPE Sample Spiked w/ TCHQ

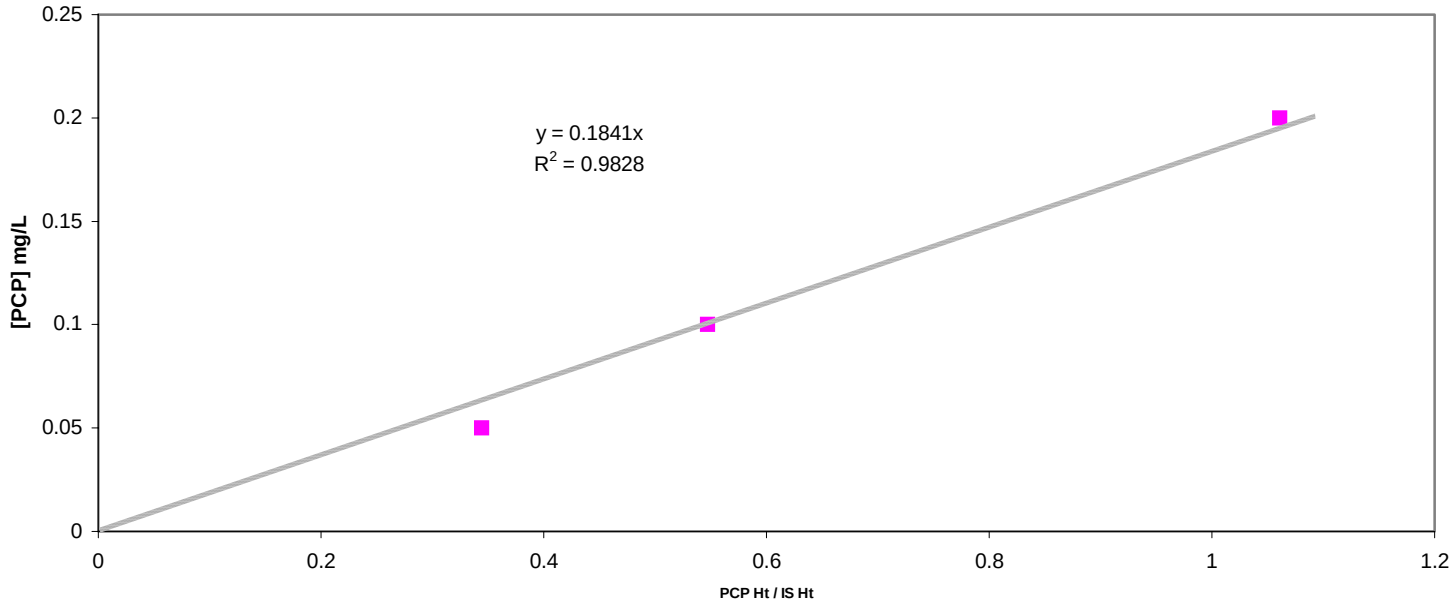
RT=12.5

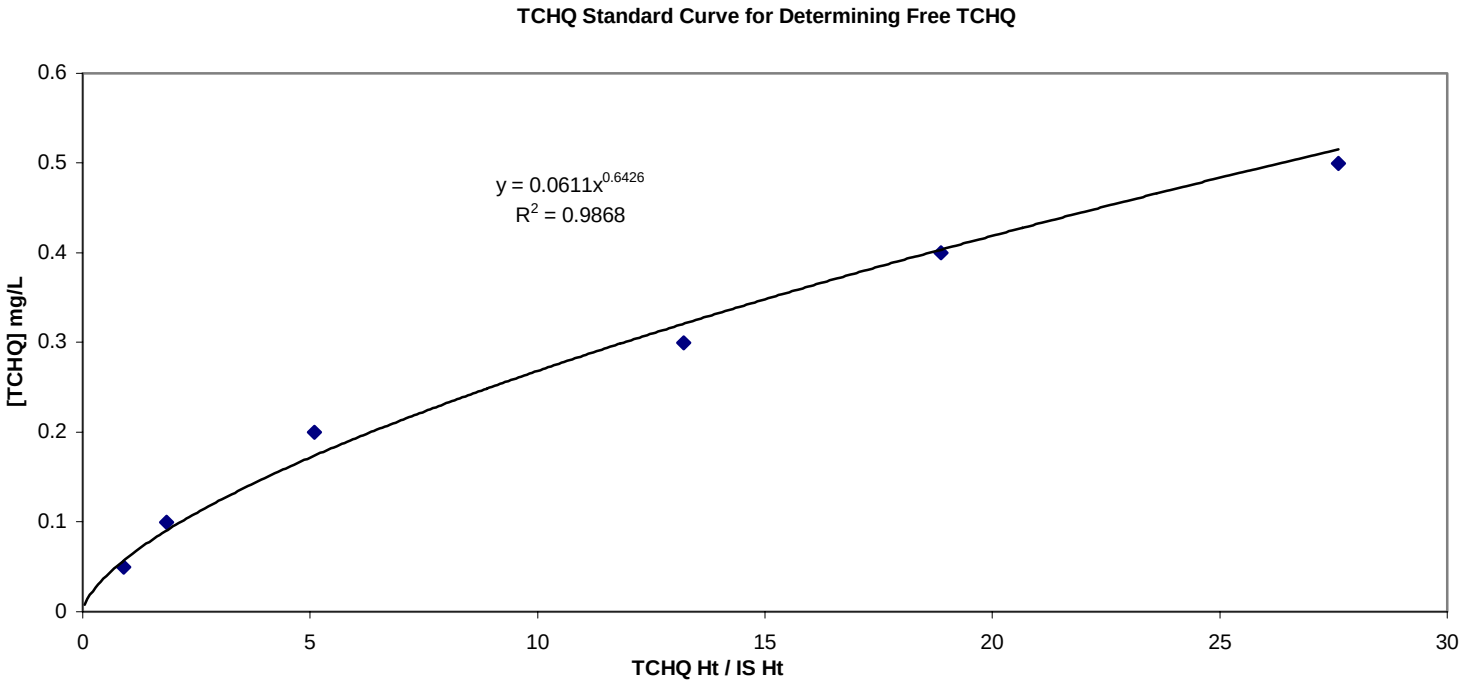
APPENDIX II

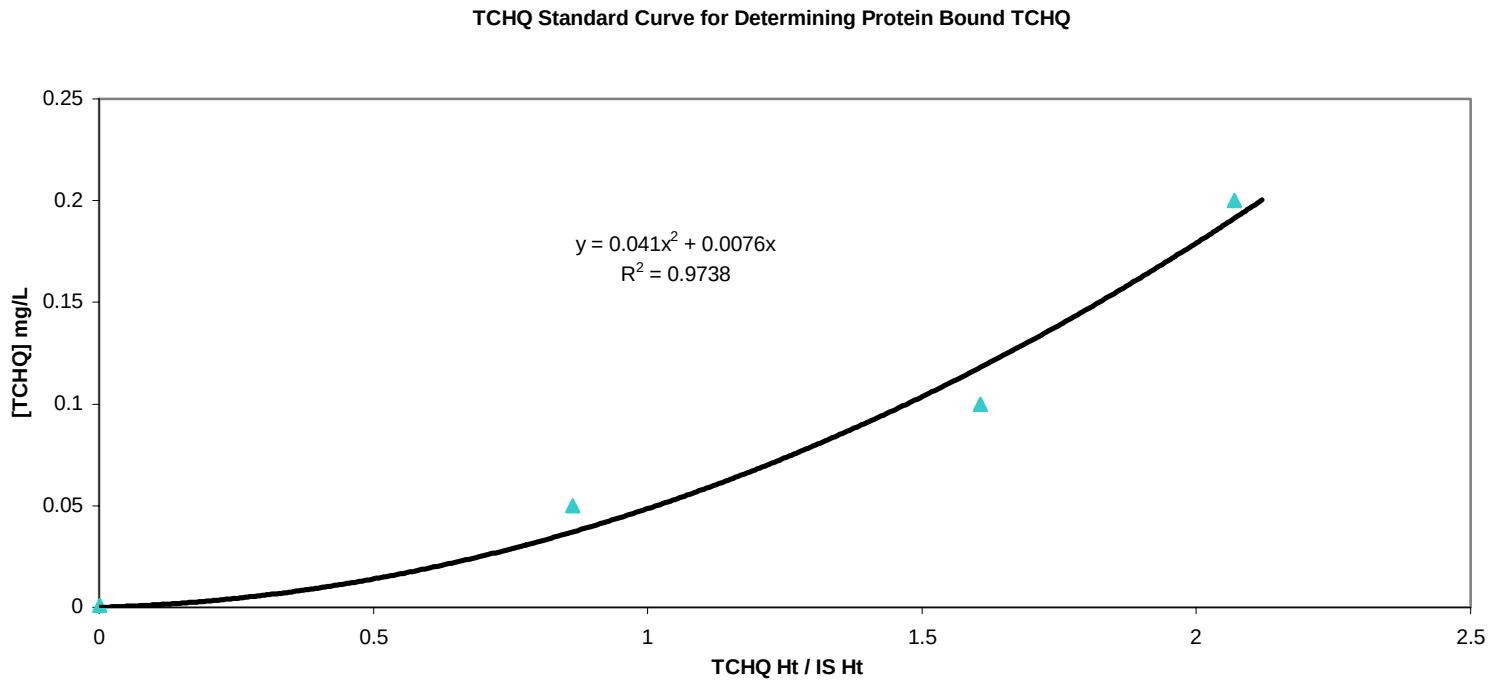
PCP and TCHQ GC/ECD Standard Curves using HFBI derivatization


PCP Standard Curve for Determining Free PCP
y = 0.0306x
R2 = 0.9669
[PCP] mg/L
PCP Ht/ IS Ht
0
0.1
0.2
0.3
0.4
0.5
0.6
0
2
4
6
8
10
12
14
16
18

PCP Standard Curve for Determining Protein Bound PCP

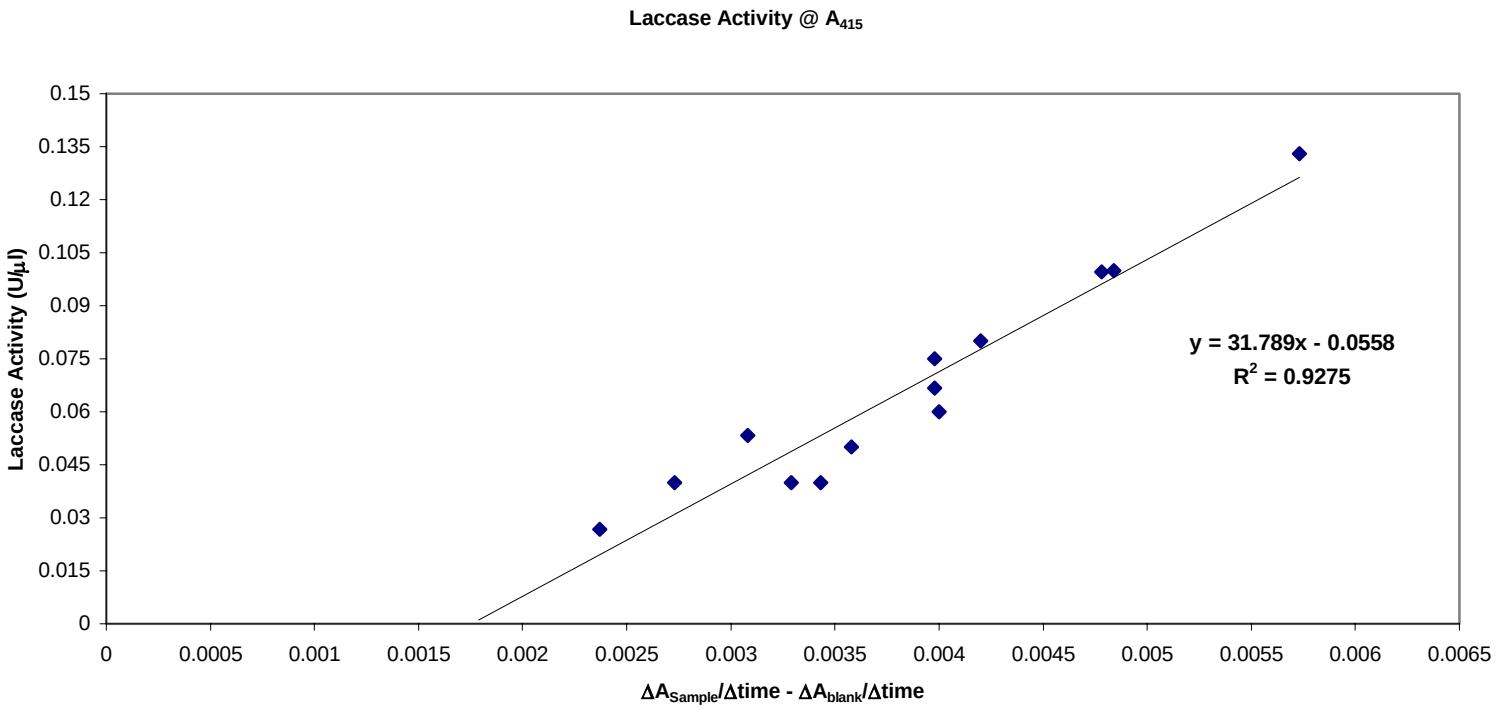



TCHQ Standard Curve for Determining Free TCHQ
y = 0.0611x^0.6426
R^2 = 0.9868
[TCHQ] mg/L
TCHQ Ht / IS Ht
0
0.1
0.2
0.3
0.4
0.5
0.6
0
5
10
15
20
25
30


TCHQ Standard Curve for Determining Protein Bound TCHQ
y = 0.041x2 + 0.0076x
R2 = 0.9738
[TCHQ] mg/L
TCHQ Ht / IS Ht
0.25
0.2
0.15
0.1
0.05
0
0
0.5
1
1.5
2
2.5

APPENDIX III

Laccase Enzyme Spectrometric Standard Curve



Note: Diluted solutions of purified laccase from *T. versicolor* were used to develop this standard curve