MODELLING AEROBIC 4-CHLOROPHENOL AND 2,4-DICHLOROPHENOL BIODEGRADATION-EFFECT OF BIOGENIC SUBSTRATE CONCENTRATION

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ABSTRACT

MODELLING AEROBIC 4-CHLOROPHENOL AND 2,4-DICHLOROPHENOL BIODEGRADATION-EFFECT OF BIOGENIC SUBSTRATE CONCENTRATION

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Aerobic biodegradation kinetics of 4-Chlorophenol (4-CP) and 2,4-Dichlorophenol (2,4-DCP) by acclimated mixed cultures were examined separately and in mixture using batch and sequencing batch reactors (SBRs). Biodegradation abilities of acclimated mixed cultures were also compared with those of isolated pure species. Complete degradation of chlorophenols and high COD removal efficiencies were observed throughout the SBRs operation. During the degradation of 4-CP, 5-chloro-2-hydroxymuconic semialdehyde, (the -meta cleavage product of 4-CP), accumulated but was subsequently removed completely. Chlorophenol degradation

rates increased with increasing chlorophenols concentration in the feed of the SBRs. Gradually decreasing feed peptone concentration did not adversely affect chlorophenol degradation profiles in SBRs. Only competent biomass was thought to be responsible for chlorophenol degradation due to required unique metabolic pathways. It was assumed that the fraction of competent biomass (specialist biomass) is equal to COD basis fraction of chlorophenols in the feed of the reactors as competent biomass grows on chlorophenols only. Models developed using this assumption agreed well with experimental data.

The performance of a two stage rotating biological contactor (RBC) was also evaluated for the treatment of synthetic wastewater containing peptone, 4-CP and 2,4-DCP at 5 rpm. High chlorophenols (>98%) and COD (>94%) removals were achieved throughout the reactor operation up to 1000 mg/L 4-CP and 500 mg/L 2,4-DCP in the feed. Results showed that RBC is more resistant than suspended growth reactors to high chlorophenols load.

The change of dominant species during the operation of SBRs and RBC was also followed using API 20NE identification kits.

Keywords: Chlorophenols, Biodegradation Kinetics, Competent Biomass, Sequencing Batch Reactor, Rotating Biological Contactor

ÖZ

AEROBİK 4-KLOROFENOL VE 2,4-DİKLOROFENOL BİYODEGRADASYONUNUN MODELLENMESİ- KOLAY AYRISABİLEN BESİ MADDESİNİN ETKİSİ

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Aerobik 4-Klorofenol (4-KF) ve 2,4-Diklorofenol biyogiderim kinetiği ayrı ayrı ve karışım halinde, kesikli ve ardışık kesikli reaktörler (AKR) kullanılarak araştırılmıştır. Aklime edilmiş karışık kültürün klorofenol arıtım yeteneği, izole edilen saf kültürlerinki ile de karşılaştırılmıştır. AKR lerin işletilmesi sırasında, klorofenollerin tamamen arıtıldığı ve yüksek KOİ arıtım verimi gözlenmiştir. 4-KF`ün parçalanması sırasında, 5-kloro-2-hidroksimukonik semialdehit (4-

klorofenolün, -meta bağının parçalanması ile oluşan ara ürün) birikimi gözlenmiş, fakat sonradan tamamen giderilmiştir. AKR lerin girişinde klorofenol konsantrasyonu artıkça, klorofenol arıtım hızı da artmıştır. AKR lerin girişinde pepton konsantrasyonunun kademeli olarak azaltılması, klorofenol giderim profilini etkilememiştir. Klorofenol arıtımı için özel bir biyolojikyol (pathway) gerektiğinden, sadece yetenekli biyokütlenin klorofenol arıtımından sorumlu olduğu düşünülmüştür. Yetenekli biyokütle sadece klorofenol üzerinden büyüyebildiği için, çamur içindeki yetenekli biyokütle oranının reaktör girişindeki KOİ bazlı klorofenol oranına eşit olduğu kabul edilmişitir. Bu varsayımlara dayanılarak kurulan modeller, deneysel verilere yüksek düzeyde uygunluk göstermiştir.

4-KF, 2,4-DKF ve peptone içeren sentetik atıksuyun arıtılabilirliliği, iki kademeli ve 5 rpm de işletilen dönen biyolojik disk reaktörde (DBD) de araştırılmıştır. Reaktör işletimi süresince, 1000 mg/L 4-KF ve 500 mg/L 2,4-DKF giriş konsantrasyonlarına kadar yüksek düzeyde klorofenol (>%98) ve KOİ (>%94) giderimi gözlenmiştir. Sonuçlar DBD'in yüksek klorofenol yüklemelerine bakterilerin askıda büyüdüğü reaktörlerden daha dayanıklı olduğunu göstermiştir.

Ayrıca, AKR ve DBD işletimi sırasında baskın kültürlerin değişimi API 20NE belirleme kiti kullanılarak takip edilmiştir.

Anahtar Kelimeler: Klorofenoller, Biyogiderim Kinetiği, Yetenekli Biyokütle, Ardışık Kesikli Reaktör, Dönen Biyolojik Disk Reaktör

To My Family and Nihan

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ABBREVIATIONS

μ	: Specific growth rate
μ_{max}	: Maximum specific growth rate
2,4-DCP	: 2,4-Dichlorophenol
4-CP	: 4-Chlorophenol
a	: Fraction of chlorophenols in feed on COD basis
AOX	: Adsorbable organic halogen
BKME	: Bleached kraft mill effluent
CHMS	: 5-chloro-2-hydroxymuconic semialdehyde
COD	: Chemical oxygen demand
CSTR	: Completely stirred tank reactor
Culture4	: Culture acclimated to 4-CP
CultureM	: Culture acclimated to mixture of 4-CP and 2,4-DCP
DCG	: Dichloroguaiacol
FBR	: Fluidized bed reactor
FED4	: Instantaneously fed SBR operated at 10 d SRT and fed with 4-CP
FED4-2	: Instantaneously fed SBR operated at 20 d SRT and fed with 4-CP
FEDM	: Instantaneously fed SBR operated at 10 d SRT and fed with mixture of 4-CP and 2,4-DCP
HRT	: Hydraulic retention time
I	: Inhibitor
k	: Zero order degradation rate constant of 2,4-DCP
K_I	: Inhibition coefficient in Andrews equation
K_{ii}	: Competitive inhibition constant

K_s : Half saturation rate constant

MLVSS : Mixed liquor volatile suspended solid

PCP : Pentachlorophenol

PNP : Paranitrophenol

q : Specific substrate utilization rate

q_m : Maximum specific substrate utilization rate

RBC : Rotating biological contactor

S : Substrate concentration

SBR : Sequencing batch reactor

S_{cr} : Critical substrate concentration

SDR : Specific degradation rate

SDRc : Specific degradation rate based on competent biomass concentration

S_{min} : Minimum substrate concentration

SOC : Synthetic organic chemical

SRT : Sludge retention time

TCG : Trichloroguaiacol

TCP : Trichlorophenol

TCS : Trichlorosyringol

TeCP : Tetrachlorophenol

TOC : Total organic compound

UASB : Up-flow anaerobic sludge blanket reactor

V : Volume

X : Biomass concentration

Xc : Competent biomass concentration

X_{sus} : Suspended biomass concentration

X_t : Total biomass concentration

Y : Yield coefficient

CHAPTER 1

INTRODUCTION

1.1.General

The development of industrial and agricultural human activities leads to the synthesis of new organic compounds known as xenobiotics (Lora et al., 2000). Chlorophenols are introduced into the environment through various human activities such as waste incineration, uncontrolled use of wood preservatives, pesticides, fungicides and herbicides, as well as via bleaching of pulp with chlorine and the chlorination of drinking water. Many forms of chlorinated aromatics or chlorinated polyaromatics are widely utilized to control microbial contamination and degradation. It is, therefore, not surprising that these compounds are very inhibitory to bioremediation (Hill et al., 1996; Valo et al., 1990). Therefore, their discharge to the environment is of great concern because of their toxicity and suspected carcinogenicity.

Despite the recalcitrance of chlorophenols, efforts are still being made to treat them biologically for economic reasons and in expectation of few byproducts. The microorganisms used are usually aerobes, including *Pseudomonas sp., Alcaligenes sp., Azotobacter sp., Rhodococcus sp.* and *Cryptococcus sp.* However, chlorinated compounds have been reported as being in general useless as carbon and energy

sources for microbial growth, and should thus be rather biodegraded by cometabolism (Wang and Loh, 1999; Tarighan et al., 2003; Saez and Rittmann, 1991 and 1993).

In most of the studies with special strains of microorganisms, necessity of phenol supplementation to the growth medium was reported to induce enzymes required for 4-Chlorophenol (4-CP) degradation (Wang and Loh, 1999 and 2000; Hill et al., 1996; Lu et al., 1996; Kim and Hao, 1999; Hao et al., 2002). The main problems with the usage of special strains are the possibility of contamination and phenol requirement, which may lead to additional pollution problem. Due to the toxicity problems of using phenol as a growth substrate and inducer, some studies have been conducted to replace toxic phenol with a non-toxic conventional growth substrate. Under these conditions, the enzymes required for chlorophenol degradation should be induced by the compound itself. In this context, Tarighian et al. (2003) pointed that the amount of waste chlorophenols generated by the industries in USA were 1,900 tones in 1999 and if the entire 1,900 tones of chlorophenols waste discharged in 1999 were subjected to cometabolic degradation using either phenol or glucose as the growth stimulant at the ratio used in their study (10 parts growth cosubstrate to 1 part chlorophenol), then the substrate cost would have been U.S. $ 17.2 million compared to U.S. $ 9.4 million using glucose, a saving of 45%. Also, as mentioned above, the addition of phenol to the waste effluent would have added toxicity potential to those streams, whereas glucose would not.

On the other hand, a wastewater treatment plant generally receives influent containing a mixture of recalcitrant synthetic organic chemicals (SOCs) and biogenic substrates. Interactions among these multiple substrates are complex, partially due to their toxicity and, competition for enzymes and cofactors (Hu et al. 2005b). This raises the question of how does biogenic substrate concentration affect the removal efficiency of SOCs present in the feed mixture. Many researchers claim that a specific competent biomass fraction is responsible for the degradation of

specific compounds, and which is equal to the fraction of COD contributed to the feed by that compound (Hu et al., 2005b; Hu et al., 2005a; Ellis et al., 1996; Tomei et al., 2003). This means that the presence of a biogenic substrate does not guarantee the enhanced biodegradation of SOCs (Hu et al. 2005b). For example, Kulkarni and Chaudhari (2006) reported that degradation rate of p-nitrophenol decreased with the addition of glucose. In another study, Hu et al. (2005b) reported that at standard oxygen conditions 4-CP specific degradation rate (SDR) decreased with the supplementation of biogenic substrates (7 g/L milk powder plus 1 g/L yeast extract), whereas, the specific degradation rate of 2,4-DCP increased in the presence of biogenic substrate. They also reported, that at elevated oxygen conditions, although biogenic substrate addition did not affect 4-CP degradation rate, it caused increase in the specific degradation rate of 2,4-DCP. These results showed that different chemicals might give different responses to the presence of biogenic substrate depending on assay conditions.

Another important point is that mixed cultures are particularly important if we aim at complete mineralization of toxic organics to CO_2. Many pure-culture studies have shown that toxic intermediates accumulate during biodegradation, because a single organism may be incapable of fully mineralizing the xenobiotics (Buitron and Gonzalez, 1996). Therefore, treating chlorophenols with activated sludge that contains an active mixed culture but lacks a special growth substrate would be more meaningful, informative, and practical. The advantage in using a microbial consortium formed by activated sludge is that the species present in the flocks would interact. Furthermore, the degrading capabilities of an activated sludge system can be enhanced by acclimation (Buitron et al., 1998 and Kim et al., 2002). For example, Buitron et al. (1998) reported that acclimated activated sludge degraded a chlorophenols mixture up to two orders of magnitude faster than pure strains obtained from an acclimated consortium. Wiggings et al. (1987) suggested that during acclimation, physiological transformations in the metabolism of microorganisms, such as changing enzyme levels, regulation and production, and mutations, lead to selection and multiplication of specialized microorganisms. In

aerobic microbial communities, acclimation periods range typically from several hours to several days. Thus by definition, acclimation is any response that ultimately leads the community to eliminate stress or find a way to maintain its function despite the stress (Rittmann and McCarty, 2001). Several studies have showed that acclimated mixed bacterial culture has ability to use chlorophenols as sole carbon and energy sources (Quan et al., 2005; Moreno and Buitron, 2004; Buitron et al., 2005).

Moreover, generally stirred tank reactors or flasks as batch reactors were utilized in the treatability studies of chlorophenolic wastes. The advantage of these reactors is that operating and adjusting the detention time is simple. Whereas, it is vulnerable to shock and washout and it takes a long time to recover from perturbation (Kim et al., 2002). On the other hand, use of attached growth reactors overcome most of the problems encountered in suspended cells bioreactors. For example, Kim et al. (2002) reported that packed bed reactor is more effective compared to CSTR in simultaneous degradation of 4-CP and phenol as although washout occurred in CSTR at a dilution rate of 0.36 per hour, efficient treatment was observed in packed bed reactor at the same dilution rate.

Another point to consider is that knowledge of microbial growth and substrate utilization kinetics is important for the accurate prediction of effluent quality from engineered treatment processes (Ellis et al., 1996a,b; Ellis and Anselm, 1999; Grady et al., 1996). Accurate kinetic parameters also help to operation engineers to optimize operational conditions to meet discharge requirements (Ellis and Anselm, 1999). The Andrews equation (Andrews, 1968) is one of the most commonly used models to describe self-inhibitory effect of a compound on its own transformation (Hao et al., 2002, Ellis et al., 1996b). In recent years great effort has been put on the modeling biodegradation of phenol (Monterio et al., 2000; Kim and Hao, 1999; Hao et al., 2002) and cometabolic degradation of 4-CP in the presence of phenol as a growth substrate (Hill et al., 1996; Sae´z and Rittmann, 1993; Kim and Hao, 1999; Hao et al., 2002; Wand and Loh, 2001). Most of these studies were carried out

using pure cultures under sterilized conditions. Therefore, the observed kinetic parameters cannot be applicable to model a full-scale application, in which mixed culture is responsible for substrate utilization.

As it can be concluded from the literature survey, although kinetics of chlorophenols degradation has been deeply investigated using pure cultures, there is no noteworthy kinetic study with acclimated mixed culture especially when chlorophenols are present in mixtures. Another important point is that the presence of biogenic substrate may improve or impede the degradation of SOC of interest. Therefore, the effect of biogenic substrate concentration on the degradation kinetics of chlorophenols mixtures is worth studying.

Also, the feed composition of an industrial wastewater treatment plant may show great fluctuation. Therefore, it will be interesting to investigate whether a culture previously acclimated to a specific type of chlorophenol has ability to degrade another structurally similar chlorophenol. If biomass has such ability, the extent of this should be deeply investigated.

1.2. Purpose and Scope

The proposed research is based on the central hypothesis that the use of acclimated and/or cross-acclimated culture especially in attached growth reactors would enhance the performance of the system, which may allow the decrease or completely avoid the necessity of biogenic substrate supplementation. The main objective of the study is to comparatively investigate chlorophenol biodegradation in long-term operated suspended and attached growth systems. Sequencing batch reactors (SBRs) and rotating biological contactor (RBC) were used as suspended and attached growth reactors, respectively. In this context, the specific objectives of the research can be listed as:

1- To investigate the biodegradation kinetics of 4-CP and 2,4-DCP separately and in mixture using batch and SBRs.
2- To investigate 2,4-DCP degradation kinetics by culture acclimated to 4-CP and the culture acclimated to 4-CP and 2,4-DCP mixture in batch reactors.
3- To investigate the effect of acclimation levels, filling time and feed chlorophenol concentrations on the chlorophenols degradation kinetics in SBRs.
4- To reduce competitive and self inhibition of chlorophenols in an attach growth reactor (RBC).
5- To investigate the effect of biogenic substrate concentration on the biodegradation kinetics of chlorophenols using SBRs and RBC.
6- To isolate and identify the pure strains from suspended and attached cultures.
7- To compare chlorophenols degradation rates of mixed and pure cultures.

1.3. Literature Review

The most commonly disrupted xenobiotics are the pesticides, which are common components of toxic wastes. Over 1000 pesticides have been marketed for chemical pest control purposes. These include primarily herbicides, insecticides, and fungicides (Madigan et al., 1997). Chlorophenols are one of the most commonly used pesticides and they constitute a series of 19 compounds consisting of mono-, di-, tri-, -tetra and pentachlorophenol (PCP). PCP and the lower chlorophenols have been used as biocides to control bacteria, fungi, algae, mollusks, insects, slime, and other biota (Hale et al., 1994).

Potential environmental sources of chlorinated phenols include:

- Direct soil applications as biocides;
- Leaching or vaporizing from treated wood items;

- Synthesis during routine chlorination process in water and wastewater treatment plants, since both water sources can contain aromatic compounds of natural origin;
- Synthesis during production of bleached pulp in which chlorine is used;
- Releases from factories into air and water;
- Incineration of waste materials and burning of fresh lignocellulosic biomass, e.g. forest fires.

A considerable amount of information is available regarding the stability of chlorinated phenols in the environment. The following generalization can be made:

- Chlorophenols are much more environmentally stable than the parent unsubstituted phenol.
- As the number of chlorine substituents increases, the rate of aerobic decomposition decreases, whereas, opposite is generally true for anaerobic decomposition.
- Compounds containing a meta-chlorine (i.e. 3-chloro- or 2,4,5-trichlorophenol) are more persistent under aerobic conditions than compounds lacking a chlorine substituent in -meta positions to the hydroxyl group.

Chlorinated phenols may be removed from water body via:

- Volatilization,
- Photodegradation,
- Adsorption onto suspended or bottom sediments, and
- Microbial degradation (Hale et al., 1994).

It is apparent from the studies on contaminated wood-preserving sites that polychlorinated phenols are persistent in soil and ground water. Self-cleaning systems are slow, and the contamination may thus persist for decades. While

degradation of chlorophenols may be slow in the environment, it is, however, possible to isolate microbes capable of utilizing PCP and other types of chlorinated phenols as a source of carbon and energy. In addition to aerobic isolates, obligate anaerobic bacteria that reductively dechlorinate chlorophenols have been isolated.

Chlorophenols are among the most widely used industrial organic compounds and listed as priority pollutants by the U.S. Environmental Protection Agency (U.S.EPA) (Jin and Bhattacharya, 1997). The reported levels of chlorophenol in contaminated ground water range from 150 μg/L (Valo et al., 1990) to 100-200 mg/L (Ettala et al., 1992). The concentration and the types of chlorophenols in industrial effluents may also show great variation depending on the type of industry and process used. Some typical values of phenolic compounds in chemical industry wastewaters were reported to be 400 mg/L for phenolic resin production; 50 mg/L for refineries; 12 mg/L for naphthalenic acid production and 200 mg/L for shale dry distillation (Chen et al., 1997). Low concentrations of chlorophenols were also reported for some industrial wastewaters. For example, the average chlorophenol concentration in a bleached kraft mill effluent may range from 39-236 μg/L (Rempel et al., 1992). In another study, Hall and Randle (1994) used a simulated bleached kraft mill effluent with an average chlorophenols concentration of 155 μg/L. Many forms of chlorinated aromatics or chlorinated polyaromatics are widely utilized to control microbial contamination and degradation. It is, therefore, not surprising that these compounds are very inhibitory to bioremediation (Hill et al., 1996; Valo et al., 1990). Therefore, their discharge to the environment is of great concern because of their toxicity and suspected carcinogenicity.

1.3.1. Microbial Degradation of Recalcitrant Synthetic Organic Chemicals (SOCs)

At first glance, it might seem strange to try and use biological treatment systems to destroy recalcitrant synthetic organic chemicals (SOCs). After all, the destruction of organic matter in such systems is caused by microorganisms that use the organic

matter as a carbon and energy source. The pathways employed for degrading biogenic organic matter have developed over long periods of time and are quite effective. Since SOCs have only been on the earth for a very short time period (most for 50 years or less), how is it possible for microorganisms to posses enzymatic pathways that can act on them? The answer to that question is two fold. First, most SOCs have structural similarity to biogenic materials, allowing them to fit into active sites on enzymes that did not evolve with them as substrates. Second, the specificity of enzymes is not exact. Although the type of reaction catalyzed by a given enzyme is very specific; the range substrate upon which it can act is not. In other words, biodegradation of SOCs are fortuitous. Because the initiation of biodegradation of a SOC requires the presence of an enzyme that is able to perform a transformation reaction, it follows that biodegradation of the SOC requires the presence of a microorganism with the genetic capability to synthesize that enzyme. Furthermore, if mineralization of SOC to occur, the transformation product from the first reaction must serve as the substrate for another transforming enzyme, etc., until ultimately a biogenic product is formed that will funnel into common metabolic pathways. The enzymes catalyzing reactions subsequent to the initial reactions may not reside in the same microorganism as the ones producing the compounds upon which they act, so that mineralization may require the concerted action of a community rather than a single type of organism (Grady et al., 1999).

The requirement to maintain appropriate microbial community can cause serious problems if the SOC is only intermittently discharged to a bioreactor. The capability to degrade any given organic chemical is not necessarily widely distributed in the microbial world. While the ability to use common sugars like glucose and fructose is widespread, other biogenic organic compounds are utilized by only a fraction all isolates tested. Thus, one would anticipate that the ability to use a SOC would be even less widely distributed. When the discharge of a SOC to a bioreactor stops, the niche by which the capable microorganisms are maintained may be eliminated, causing them to be lost from the system. The time constant for their loss is the SRT of the system, with the result that about 95% of them will be

lost in three SRTs. Thus, it can be seen that most of the microorganisms capable of degrading a SOC can be lost if SOC is absent from the bioreactor feed for only a few weeks, unless there is some other means of preserving their niche. This means that the capable population must be rebuilt upon reintroduction of the SOC in the influent, causing time lags during which release of the SOC in the effluent occurs. Even if the capable organisms are retained, chances are that the required enzymes will not be synthesized in the absence of the SOC. This follows from the fact that with a few exceptions, most organic compounds are degraded by inducible enzymes, i.e., those that are synthesized only when they are needed. When the inducer is no longer present, their synthesis is stopped and the unused enzymes are degraded, freeing their amino acids for use in the synthesis of new enzymes. Because proteins have a relatively high turnover rate in bacteria, this loss can be quite rapid, causing a culture to lose the ability to degrade a SOC much faster than would be predicted from a simple washout of the enzyme. Then, when the inducer is reintroduced into the system, new enzyme synthesis must occur, leading to another type of lag. While this lag is considerably shorter than the lag associated with the regrowth of a population, it can still be sufficiently long to allow discharge of a SOC following its reintroduction in feed to a bioreactor. So, biological systems will be most effective in destroying SOCs when the SOCs are continuously discharged to treatment systems (Grady et al., 1999). Unlike to inducible enzymes, constitutive enzymes are always produced by cells independently of the composition of the growth medium. The enzymes that operate during glycolysis and the TCA cycle are generally constitutive and they are present at more or less the same concentration in cells.

Alexander (1965) described factors causing organic compounds to resist biodegradation in the environment, which termed “molecular recalcitrance”.

1. A structural characteristic of the molecule prevents an enzyme from acting.
2. The compound is inaccessible, or unavailable.
3. Some factor essential for growth is absent.

4. Environment is toxic.
5. Requisite enzymes are inactivated.
6. The community of microorganisms present is unable to metabolize the compound because of some physiological inadequacy.

The first factors suggest that, for biodegradation to occur, the molecular structure must be inherently biodegradable. The next four factors are related to environmental conditions that are necessary for biodegradation to take place. The last indicates that appropriate organisms must be present. If biodegradation does not occur naturally, then at least one of the factors listed is likely to be the cause. In order to bring about degradation, the factors imparting recalcitrance must be overcome. The objective of engineering strategies for enhancing biodegradation is to overcome the factor effecting recalcitrance (Rittmann and McCarty, 2001).

The nature, number, and position of xenophores all influence the biodegradability of a SOC. Biodegradability of halogenated compounds is lower than the unsubstituted compounds. Furthermore, the greater the number of xenophores, the less susceptible the SOC is to biodegradation. However, it is difficult to generalize about the effect of the position of a xenophore. A given xenophore in a given position may have a strong effect, whereas another xenophore in the same position may have none. Because of these widely diverse effects, there is strong interest in the development of structure-biodegradability relationships that can be used to deduce biodegradability from a compound's molecular structure. It must be recognized, however, that the knowledge base for such relationships is still limited, thereby restricting their utility at this time. Prediction of biodegradability is a new science and much is still to be learned. With a few exceptions, microorganisms are thought to degrade only organic compounds that are dissolved in the aqueous phase. This means that solubility has a profound effect on the biodegradability of any organic compound, whether xenobiotic or biogenic. Many xenophores reduce aqueous solubility, thereby reducing bioavailability and the rate of biodegradation.

Furthermore, if a compound has very low solubility, it may be difficult to induce the enzymes required for its biodegradation (Grady et al., 1999).

The environment has a strong effect on biodegradability. In addition to the usual effects of pH, temperature, and the availability of nutrients and electron acceptor, the presence or absence of molecular oxygen can have a strong effect. For example, some enzymatic steps, such as those carried out by oxygenase enzymes require the presence of oxygen, whereas others, such as reductive dehalogenation, require its absence. The engineer must be knowledgeable about the nature of the pathways for biodegradation of a given SOC so that the appropriate environment can be provided. Failure to do so will result in an inadequate system that can meet effluent goals (Grady et al., 1999). Considering this, anaerobic reductive dehalogenation and aerobic degradation of chlorophenols were deeply discussed.

1.3.1.1. Anaerobic Reductive Dechlorination of Chlorophenols

Chlorinated organic compounds can be used as electron acceptors, a process termed *reductive dehalogenation* (Rittmann and McCarty, 2001). For example, a sulphate-reducing bacterium implicated in this process, *Desulfomonile*, reduces 3-chlorobenzoate to benzoate and CI^-.

$$C_7H_4O_2CI^- + 2H \longrightarrow C_7H_5O_2^- + HCI$$

Electrons for this reaction can come from acetate, formate, or H_2, and the reductive reaction is linked to establishment of a proton gradient across the cytoplasmic membrane, which *Desulfomonile* uses to drive adenosine triphosphate (ATP) synthesis. Thus the reductive dechlorination is a type of anaerobic respiration (Madigan et al., 1997). Nearly 30% of the electrons available in mixed organic material are converted to hydrogen, which serves as electron donor in reductive dehalogenation reactions (Rittmann and McCarty, 2001).

Aerobic microorganisms can metabolize mono- and di-chlorinated substances, but aerobic attack becomes less effective with more highly chlorinated compounds. On the other hand, the rate of dechlorinations under anaerobic conditions is actually greatest for more heavily chlorinated compounds, but progressively slower as the chlorophenol molecules become less chlorinated. In particular, although monochlorophenols and more heavily chlorinated phenols can be degraded under anaerobic conditions, anaerobic cultures capable of dechlorinating more heavily chlorinated compounds have a limited ability to degrade monochlorophenols (Armenante et al., 1999).

Efficiency of thermophilic anaerobic process (55^0C) to remove chlorinated phenolic compounds from bleached kraft mill effluent (BKME) was studied by Lepisto and Rintala (1994). In this study, four different types of anaerobic processes: an upflow anaerobic sludge blanket (UASB) reactor; an UASB reactor enriched with sulfate; an UASB reactor with recirculation and a fixed bed reactor with recirculation at total influent chlorophenols concentration varying between 75.3 and 1010 μg/L. 2,4-DCP accumulation was observed. However, it was completely removed when loading rate of reactors was increased. Contrary to AOX (adsorbable organic halogen) removal (AOX removal during the study varied between 19 and 67% depending on loading rate and reactor type), the COD reduction efficiency in the four reactors decreased as the concentration of the influent COD increased (COD removal efficiency showed variation between 32 and 72 %).

Jin and Bhattacharya (1997) investigated the toxicity and treatability of 12 chlorophenolic compounds consisting of mono-, di- and trichlorophenolic compounds in anaerobic propionate enrichment culture in batch reactors. It was observed that trichlorophenols was the most toxic among the examined mono-, di- and trichlorophenolic compounds and 2,3,5-Trichlorophenol (TCP) was the most toxic among the examined trichlorophenolic compounds. The toxicity of selected chlorophenols to the propionate-fed culture decreased in the following order: 2,4,5-TCP > 2,3,5-TCP > 2,4,6-TCP> 2,3,6-TCP > 2,3-DCP > 2,6-DCP, 3-CP, 4-CP, 2-

CP, 2,5-DCP, 3,4-DCP, 2,4-DCP. Most chlorophenols were dechlorinated reductively to less chlorinated compounds and/or phenol. Dechlorination at the ortho position was observed most frequently for TCPs. During the degradation studies in batch experiments, it was observed that 2-CP degraded faster than 3-CP, which degraded more rapidly than 4-CP. Phenol also was detected during the degradation of 2,3-DCP; 2,6-DCP; 3,5-DCP; 2-CP; 3-CP; and 4-CP. The overall removal of selected chlorophenols in anaerobic propionate enrichment systems were in the following order: 2,3,5-TCP > 2,3,6-TCP > 3,5-DCP > 2,4-DCP > 2-CP > 3,4-DCP, 2,3-DCP, 2,5-DCP > 3-CP > 2,4,5-TCP > 2,6-DCP > 2,4,6-TCP > 4-CP.

Puhakka et al. (1994) studied continuous removal of chlorophenols in the anaerobic fluidized-bed reactor at influent concentration of 7.1 mg/L PCP and 2,4,6-TCP and 1.0 mg/L TCG. During steady operation at 24 h HRT, the mean total organic carbon (TOC) removal was 92% with simultaneous mean methane production of 250 mL/gCOD added. In the study, 99% removals for TCP and PCP and over 95% removal of TCG were achieved. This study also demonstrated that sequential anaerobic-aerobic treatment of pulping effluents, which is often proposed for overall waste management economy, was an attractive combination for mineralization of chlorinated monoaromatics as well.

Fang et al. (1999) investigated the efficiency of treating wastewater containing medium-strength of phenolic pollutants and nitrate in both continuous and batch reactors. The influence of a carbonhydrate co-substrate and interaction between methanogens and denitrifiers were also investigated. Continuous experiments were conducted in UASB reactor at different COD/NO_3-N concentration containing 200 mg/L phenol and 100 mg/L m-cresol. Results showed that denitrifiers out-competed methanogens for carbon and electron supplies. At the COD/NO_3-N ratio of 3.34, all electrons were utilized by denitrification, as evidenced by the cease of methane production. The fraction of electron flow to methogenesis increased with the COD/NO_3-N ratio. It was shown that denitrifiers had superior m-cresol degrading

capability than methanogens. When COD/NO_3-N ratio was 3.34 or lower, denitrifiers were able to degrade nearly 100% of phenol and m-cresol with 1 day of hydraulic retention even in the absence of additional carbon source. Batch studies showed that degradation of m-cresol was enhanced by the presence of either sucrose or phenol as cosubstrate.

Shin et al. (1999) used tire chips as packing material for sequential anaerobic and aerobic biofilm reactors to remove persistent chlorinated hydrocarbons. In this study, 2,4-DCP was added at concentration of 10, 20, 40 and 70 mg/L, total influent COD was 1000 mg/L. Adsorption experiments for 4-CP and 2,4-DCP in batch reactors showed that maximum adsorption capacity of 2,4-DCP was only 0.3% of granular activated carbon. For 4-CP, maximum adsorption capacity was only 0.04% of granular activated carbon because 4-CP has greater polarity than DCP. Therefore, it was concluded that removal of 2,4-DCP and 4-CP through adsorption could be neglected. Biomass attachment tests showed that tire chips has not inhibitory effect on biofilm and can be used as carrier in anaerobic and aerobic reactors. 2,4-DCP was completely degraded in anaerobic reactor but 4-CP was produced as a byproduct. 4-CP could not be removed in anaerobic reactor and it was further removed in aerobic reactor with the removal efficiencies of 98 and 70% at concentrations of 0.16mM and 0.31mM, respectively. Therefore, waste tires, which are difficult to dispose of or treat, can be effectively used as a medium for biofilm reactors.

Armenante et al. (1999) studied a two-stage anaerobic-aerobic process (including both batch and continuous reactors) to completely degrade 2,4,6-TCP. Anaerobic batch experiments in serum bottles showed that lag time for TCP degradation was about 40 h. After lag period, 2,4,6-TCP was degraded to 2,4-DCP, which was degraded to 4-CP. Anaerobic batch studies showed that reduction of 4-CP much more slower than that of more chlorinated phenolic compounds. Also, parachlorophenol (4-CP) are frequently reported as the accumulated intermediates

during reductive dechlorination of more heavily chlorinated phenols (Bali and Sengül, 2003).

1.3.1.2. Aerobic Microbial Degradation of Chlorophenols

Oxygen has two important roles in enzymatic reactions. First, it can be used as an electron acceptor in energy generating reactions. Although this is by far the most important role of O_2 in cellular metabolism, O_2 plays an interesting and important role as a direct reactant in certain types of anabolic and catabolic processes. Oxygenases are enzymes that catalyze the incorporation of oxygen from O_2 into organic compounds. There are two kinds of oxygenases: *dioxygenases*, which catalyze the incorporation of both atoms of O_2 into the molecule; and *monooxygenases*, which catalyze the transfer of only one of the two O_2 atoms to an organic compound as a hydroxyl (OH) group, with the second atom of O_2 being reduced to water, H_2O. Because monooxygenases catalyze the formation of hydroxyl group (OH) in organic compounds, they are sometimes called *hydroxylases*. In most monooxygenanses, the electron donor is NADH or NADPH (Madigan et al., 1997).

Two major steps occur in the biodegradation of aromatic compounds (1) conversion of aromatic compounds into central intermediates with peripheral pathways and (2) conversion of the central intermediates to biomass and end products with central pathways. In the peripheral pathways, the aromatic compounds are converted to hyroxylated intermediates by monooxygenases or dioxygenases. In the central pathways, the aromatic ring in the intermediates is broken. Three different dioxygenases are involved in the cleavage of the central intermediates. First, the intradiol (ortho) cleaving dioxygenases (e.g. catechol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase). The second class is extradiol (meta) cleaving dioxygenases (e.g., catechol 2,3-dioxygenase, procatechuate 4,5-dioxygenase). The third groups is the dioxygenases (e.g., gentisate 1,2-dioxygenase) cleaving the

aromatic ring of para-hydroxylated benzoic acid between carbon 1 and 2 (Çinar, 2002).

Despite the recalcitrant nature of chlorophenols, efforts are still being made to treat them biologically for economical reasons and in expectation of few byproducts formation. The microorganisms used are usually aerobes, including *Pseudomonas* sp., *Alcaligens* sp., *Azotobacter* sp., *Rhodococcus* sp., and *Cryptococcus* sp. Aerobes are more efficient at degrading toxic compounds because they grow faster than anaerobes and usually achieve complete mineraralization of toxic organic compounds, rather than transformation as in the case of anaerobic treatment (Kim et al., 2002).

Bacteria use different strategies to degrade chlorophenols:

- Mono- and dichlorophenols are usually degraded aerobically by hydroxylation to chlorocatecols and by a spontaneous dechlorination after ortho- or meta- cleavage of the chlorocatecols.
- Thrichloro- and polychlorophenols are degraded aerobically via parahydroquinones, which are subsequently dechlorinated before ring cleavage.
- All chlorophenols (mono- to PCP) are degraded under anaerobic conditions by a variety of microbial communities; degradation is initiated by reductive dechlorination followed by ring cleavage (Hale et al., 1994).

Aerobic microorganisms can metabolize mono- and di-chlorinated substances, but aerobic attack becomes less effective with more highly chlorinated compounds. On the other hand, the rate of dechlorinations under anaerobic conditions is actually greatest for more heavily chlorinated compounds, but progressively slower as the chlorophenol molecules become less chlorinated (Armenante et al., 1999).

For complete degradation of chlorinated aromatic compounds, two steps are necessary, cleavage of the aromatic ring and removal of chlorine atom. The initial step in the aerobic degradation of mono-chlorophenols is their transformation to chlorocatechols, ring cleavage by deoxygenates may proceed. In the aerobic biodegradation of 4-CP, the first step is conversion of 4-CP to 4-chlorocatechol. The meta-cleavage of 4-chlorocatechol results in the production of a chlorinated aliphatic compound; 5-chloro-2-hydroxymuconic semialdehyde (CHMS), widely reported to be a dead-end metabolite especially when pure species were used (Tarighan et al., 2003; Saez and Rittmann, 1991 and 1993). However, recent reports have shown that further metabolism of CHMS may occur resulting in complete degradation of 4-CP via meta-cleavage pathway with mixed cultures (Sahinkaya and Dilek, 2002 and 2005; Moreno-Andrade and Buitron, 2004a,b; Buitron et al., 2005; Farrell and Quilty, 1999). CHMS is metabolized leading to the production of pyruvic acid and chloroacetic acid. Chloroacetic acid may then be dehalogenated to form glycolate, which may be utilized along with pyruvic acid in the TCA cycle (Figure 1.1) (Bali and Sengül, 2002; Farrel and Quilty). The degradation of 2,4-DCP via –ortho cleavage pathway is illustrated in Figure 1.2.

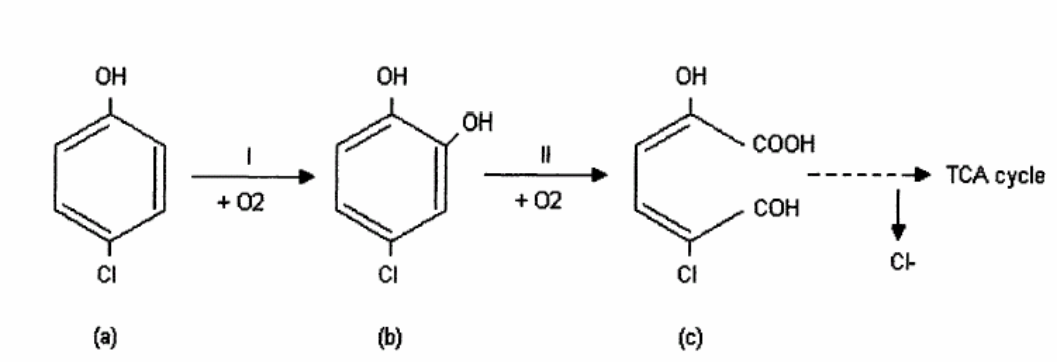

Figure 1.1. Degradation of 4-CP via meta cleavage pathway, (a) 4-CP, (b) 4-chlorocatechol, (c) 5-chloro-2-hydroxymuconic semialdehyde (CHMS).

The major aerobic kinetic studies on chlorophenol treatment conducted so far have focused on the use of 4-CP as a model nongrowth substrate using special strains (Hill et al., 1996; Sae´z and Rittmann, 1991 and 1993; Kim and Hao, 1999; Hao et

al., 2002; Wand and Loh, 2001). Many pure-culture studies have shown that toxic intermediates accumulate during biodegradation, because a single organism may not have the ability to completely mineralize the xenobiotic (Buitron and Gonzalez, 1996). Therefore, if pure species are used in the degradation of SOCs, bacteria need a primary biogenic substrate to grow on it, which is known as co-metabolism, whereas, complete degradation of chlorophenols with mixed culture is possible even in the absence of externally added biogenic substrate.

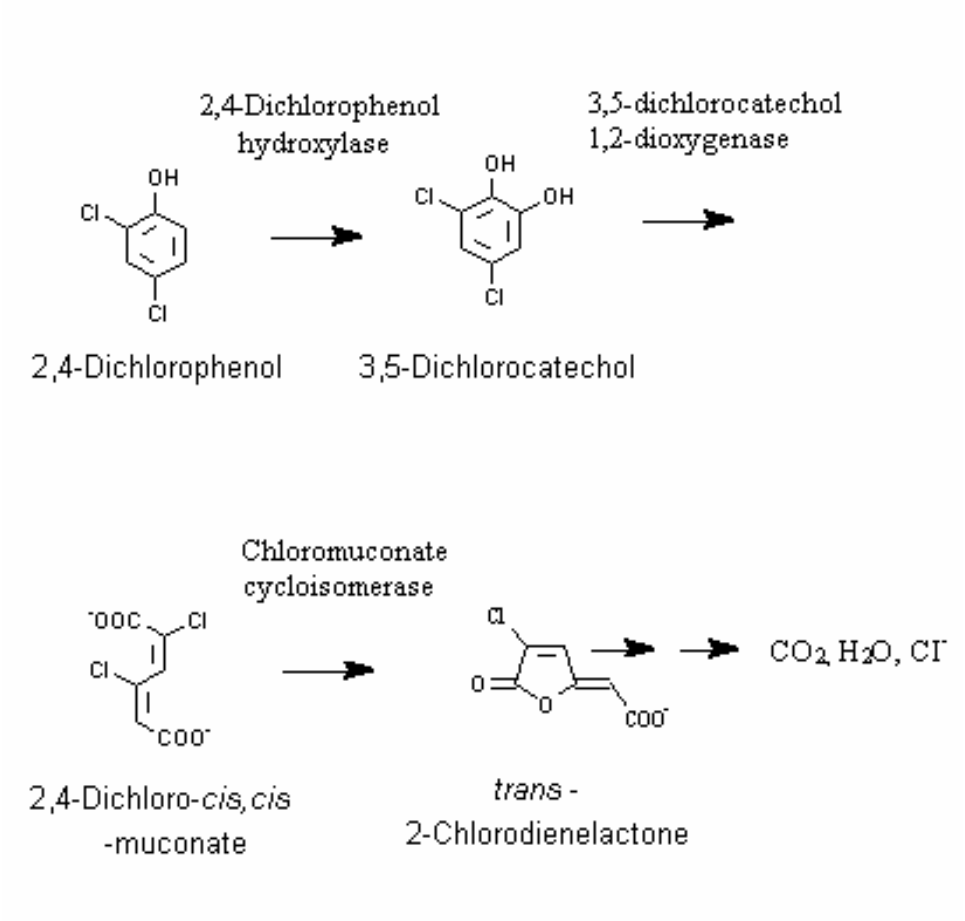

Figure 1.2. Degradation of 2,4-DCP via ortho cleavage pathway

1.3.1.2.1. Degradation of SOCs in Multisubstrate Environment and Co-metabolic Degradation of Chlorophenols

The ability of microorganisms to degrade xenobiotic organic compounds when present at low concentrations, typical of those in the environment, is open to question. Organism growth rate is a function of substrate concentration. At sufficiently low concentrations, the organism growth rate may be exceeded by the organism decay rate so that no net growth can occur. The concentration at which growth is just balanced by decay is termed the minimum substrate concentration (S_{min}). Therefore steady-state biomass can be sustained only when $S>S_{min}$. If $S<S_{min}$, the cell net specific growth rate is negative and biomass will not accumulate or will gradually disappear (Laura et al., 1984; Rittmann and McCarty, 2001). Although some microorganisms have the ability to use SOCs as a sole carbon and energy source, addition of some growth supporting compounds may be necessary if SOCs concentration is below S_{min}. It has been postulated that bacteria can, however, consume a xenobiotic compound at low concentrations if the population is supported through energy obtained from another substrate that is present in concentrations above its S_{min} level. The compound at high concentration is termed the primary substrate, and that at low concentration is termed the secondary substrate (Laura et al., 1984). In this case, the primary substrate should be selected carefully as it should not impede the degradation of SOC of interest. If primary substrate is present in the medium, its presence should also be considered in the development of the model equation.

Co-metabolism (co-oxidation or co-transformation) refers to the ability of microorganisms to metabolize a compound that cannot be used as an independent source of energy or growth. Co-metabolism is thus a fortuitous process in which microorganisms while growing at the expense of one substrate also have the capacity to transform another compound without driving any benefit from its metabolism. Thus, microorganisms would need another substrate as a carbon and energy source on which to grow. This special type of metabolism has been named

as co-metabolism. In co-metabolism, organisms use one substrate as primary energy source and metabolize another compound utilizing the enzymes, which are synthesized to degrade the primary substrate (Atlas, 1993). In other words, primary substrate may also induce enzymes required for the degradation of target compound. However, non-growth substrates have been shown to inhibit the oxidation of the growth substrate. As such, the rate and efficiency of cometabolism are always dependent on a complex interaction between the growth substrate and non-growth substrate. Because some of the most common chlorinated solvents are known to be biodegraded through cometabolic pathways, the degradation behavior of cometabolised compounds is of great importance to the biological treatment of polluted groundwater, industrial effluent, hazardous waste sites, and so on (Bali and Sengül, 2002).

Wang and Loh (2000) reported that 4-CP degradation at high concentrations using *P. putida* were only possible when phenol was supplemented in addition to a readily degradable substrate (sodium glutamate was used in the study) to induce enzymes required for the degradation of 4-CP. They stated that for cometabolism of 4-CP, phenol and sodium glutamate play quite different roles although both of them are growth substrates and are responsible for the production of new cells. As a specific growth substrate, phenol initiates the key enzymes for its own degradation and 4-CP transformation. Phenol oxidation efficiently regenerates the reducing power and continuous phenol degradation sustains the cells ability to cometabolically transform 4-CP. On the other hand, the major contribution of sodium glutamate is to increase the specific growth rate and regenerate more active cells through its metabolism (Wang and Loh, 2001). In another study, Hill et al. (1996) studied the degradation of 4-CP using a pure culture of *Alcaligenes eutrophus*. They reported that 4-CP was found to decrease biomass yield and increase lag time before logarithmic growth occurred. Both phenol and 4-CP were found to inhibit the growth rate linearly with maximum concentrations of 1080 ppm and 69 ppm respectively, beyond which no growth occurred. Although, efficient degradation of 4-CP was observed in the presence of phenol, no growth or 4-CP

consumption occurred in the absence of phenol as the primary growth substrate. Similar to these observations, Hao et al. (2000) and Kim and Hao (1999) reported synchronous phenol and 4-CP utilization by the *Acinetobacter* isolate for sufficiently high initial phenol:4-CP concentration ratios (>200:50 mg/L). Under such conditions, sufficient energy from phenol utilization was available for 4-CP transformation.

Although the removal of 4-CP in the presence of phenol as a primary substrate by special strains of microorganisms has been studied extensively, it is well known that phenol is a toxic compound and supplementation of phenol may lead to another pollution problem (Wang and Loh, 1999; Tarighan et al., 2003; Bali and Sengül 2002). In addition to potential environmental problems caused by phenol, competition between phenol and 4-CP was observed especially at high phenol concentrations. In cases where growth and nongrowth substrates share the same key enzymes, competitive inhibition is commonly observed, and cometabolic transformation of the nongrowth substrates is strongly affected by the presence of the growth substrates. To exclude such competitive inhibition, two-stage or sequencing reactor systems have been proposed to decouple cell growth and transformation of the nongrowth substrates. Nevertheless, in addition to the systems being inherently complex, the degradation activity of the cells in such systems for the nongrowth substrates cannot be maintained due to an absence of growth substrates. Alternatively, the competitive inhibition between growth and nongrowth substrates can be avoided if different enzymes catalyze their respective transformations. This is possible if the cometabolic enzymes were induced by the nongrowth substrate. Then, the growth substrate is only responsible for cell growth and regeneration of necessary co-substrates like NADH or NADPH. This being the case, growth substrates can be optimally chosen from a wide range of carbon sources, including nontoxic, readily degradable organic compounds. Especially when the selected growth substrate is a conventional carbon source, the design of cometabolic systems can be facilitated with reduced cost and risks associated with the addition of toxic growth substrates (Wang and Loh, 1999). In this context, some

studies have been conducted in order to replace phenol with a conventional non-toxic organic substrate as a growth substrate to degrade mono chlorophenols (Wang and Loh, 1999; Bali and Sengül, 2002, Tarighian et al., 2003). In the study of Wang and Loh (1999), 4-CP degradation by *Pseudomonas putida* was complete up to 200 mg/L when growth medium was supplemented with 1000 mg/L glucose. However, when the concentration of 4-CP was raised to 300 mg/L, cells could not grow even after extended period of incubation. Therefore, the ratio of glucose to 4-CP concentration was found to be 1000:200 or higher in order to achieve complete degradation of 4-CP. Compared with phenol as the added growth substrate, cells grown on glucose displayed a longer acclimation phase and, in general, a lower specific transformation rate. The volumetric transformation rate of 4-CP, however, was greatly enhanced due to the increased cell density. The results of this work suggest that 4-CP itself induced the enzymes necessary for its cometabolism. With NADH regenerated effectively through metabolism of glucose, 4-CP was transformed in the absence of added phenol. Consequently, the competitive inhibition involved in cometabolism was avoided and the risks associated with addition of toxic growth substrates such as phenol were eliminated. Similar to these results, Bali and Sengül (2002) reported that maximum 4-CP removal rates were achieved at glucose concentrations between 1000 and 4000 mg/L for 200 mg/L 4-CP, with an effluent 4-CP concentration below detection limits (0.1 mg/L). Complete removal of 300 mg/L of 4-CP was achieved at glucose concentrations of 2000 and 3000 mg/L. Increase in 4-CP effluent concentration accompanied with increase in the influent glucose concentration was observed. Also they observed an intermediate having a yellow color at the effluent of reactor in cases where complete degradation of 4-CP was not possible. This intermediate had all characteristics of 5-chloro-2-hydroxymuconic semialdehyde. The same intermediate was also reported by some other investigators (Tarighan et al., 2003; Saez and Rittmann, 1993). Although this intermediate was observed at the effluent, Tarighian et al (2003) reported negligible toxicity of the intermediate towards *Artemia salina,* unlike phenol and 4-CP.

In another study, Tarighian et al. (2003) reported that 4-CP was consumed as a cometabolite with either glucose or phenol acting as the primary growth substrate. Surprisingly, glucose was found to be the superior growth cosubstrate, suggesting that inexpensive sugars can be used to enhance the biodegradation of chlorophenol-contaminated sites. Another advantage of using conventional carbon source as a primary substrate in the cometabolic degradation of 4-CP is the lower cost of conventional growth substrate compared to phenol. Tarighian et al. (2003) pointed that the amount of waste chlorophenols generated by the industries in USA were 1,900 tones in 1999 and if the entire 1900 tones of chlorophenol waste discharged were subjected to cometabolic degradation using either phenol or glucose as the growth stimulant at the ratio used in their study (10 parts growth cosubstrate to 1 part chlorophenol), then the substrate cost would have been U.S. $ 17.2 million compared to U.S. $ 9.4million using glucose, a saving of 45%. Also, as mentioned above, the addition of phenol to the waste effluent would have added to the toxicity potential of those streams, whereas glucose would not.

It is also important to state that a wastewater treatment plant generally receives influent with a mixture of SOCs and biogenic substrates. This means that SOCs and biogenic compounds often coexist in many wastewater reactors. Interactions among these multiple substrates are complex, partially due to the toxicity, competition for enzymes and cofactors (Hu et al., 2005b). Therefore, the question should be answered is how biogenic substrate concentration affects the removal efficiency of SOCs especially when they are present in mixture. Many researchers claim that a fraction of biomass is responsible for the degradation of specific compound, which is equal to the fraction of COD contributed to the feed by that compound (Hu et al., 2005b; Hu et al., 2005a; Ellis et al., 1996; Tomei et al., 2003). This means that the presence of biogenic substrate does not guarantee the enhanced biodegradation of SOCs (Hu et al., 2005b). For example, Kulkarni and Chaudhari (2006) reported that degradation rate of p-nitrophenol decreased with the addition of glucose. In another study, Hu et al. (2005b) reported that at standard oxygen conditions 4-CP degradation rate decreased with the supplementation of biogenic substrate, whereas,

2,4-DCP degradation rate increased in the presence of biogenic substrate. In the same study, it was also reported that at elevated oxygen conditions, although biogenic substrate addition did not affect 4-CP degradation rate, it increased the degradation rate of 2,4-DCP. These results showed that different chemicals might give different response to the presence of biogenic substrate depending on assay conditions. In our previous studies, we have investigated the effect of biogenic substrate availability on 2,4-DCP (Sahinkaya and Dilek, 2002) and 4-CP (Sahinkaya and Dilek, 2005) degradation for short term experiments. We have observed a positive effect of biogenic substrate on the chlorophenol removal rate, although complete degradation was observed even in the absence of biogenic substrate. However, the impact of biogenic presence should be investigated in long-term operated reactors.

Zaidi and Mehta (1995) have reported that different bacteria may give different response to the presence of alternative carbon source. They reported that addition of glucose did not enhance the p-nitrophenol (PNP) degradation of *Corynebacterium* strain Z4 and the bacterium utilized the two substrates simultaneously. Glucose used at the same concentration, inhibited PNP degradation by *Pseudomonas* strain MS. However, glucose increased the extent of PNP degradation by *Pseudomonas* strain GR. Phenol also enhanced the degradation of PNP in wastewater by *Pseudomonas* strain GR, but had no effect on PNP degradation by *Corynebacterium* strain Z4. They also reported that the concentration of toxic compound may also play important role as the addition of glucose increased the PNP degradation by *Corynebacterium* strain Z4 when PNP concentration was too low to support growth.

Yang et al. (2004) investigated the ability of PCP degrading bacterium *Sphingomonas chlorophenolica* to degrade and dechlorinate other chlorophenols. Their results indicated that *Sphingomonas chlorophenolica* was able to completely degrade 2,3,6-TCP, 2,4,6-TCP, 2,3,4,6-TeCP and PCP within 38.1, 15.1, 11.8 and 11.8 hours, and to release 50.1, 60.9, 63.7 and 58.5 mg/L chloride at the same

period of time. Furthermore, the results showed that four kinds of chlorophenols were completely dechlorinated by the cell suspensions. In the presence of supplementary carbon sources (300 mg/L glucose, pyruvate, sodium acetate and 150 mg/L 2,4,6-TCP, PCP), PCP removal efficiency increased with the presence of glucose or pyruvate, but the removal efficiency of 75 mg/L 2,4-dichlorophenol did not raise with the supplement of carbon sources as 75 mg/L of 2,4-DCP was inhibitive to the growth and activity of cells. Although glucose and pyruvate increased the removal of PCP, sodium acetate did not have any effect on PCP removal. The first possible reason of increasing PCP degradation rate in the presence of glucose and pyruvate suggested by the authors is that the metabolic pathway of PCP and glucose or pyruvate was partially the same. The second suggested reason is that the metabolism of glucose and pyruvate could provide more NADH to facilitate the degradation of PCP.

Although effects of phenol addition on 4-CP degradation kinetics have been extensively evaluated, less study is available on other types of chlorophenols. In the study of Quan et al. (2004b), the effect of phenol addition on 2,4-DCP degradation was evaluated using inner-loop bioreactor immobilized with *Achromobacter sp.* They reported that the presence of phenol had inhibitory effect on the enzyme responsible for 2,4-DCP degradation and the degradation rate decreased in the presence of phenol. The reason of this observation was that the major carbon source shifted from toxic compound, 2,4-DCP, to relatively less toxic compound, phenol. It was also interesting to observe that after complete 2,4-DCP degradation, the biodegradation rate of phenol increased rapidly.

The same authors (Quan et al., 2004c) also studied the removal of multiple chlorophenols using activated sludge system bioaugmented with a 2,4-DCP degrading mixed culture. Results showed that bioaugmented system not only enhanced the removal of 2,4-DCP, but also improved the removal of other chlorophenols such as 4-CP and 2,4,6-TCP. They reported that the three types of chlorophenols could be degraded in the bioaugmented bioreactor in the sequence of

2,4-DCP>4-CP>2,4,5-TCP. They also reported that both 4-CP and 2,4,5-TCP retarded the degradation of 2,4-DCP, but the effect of glucose on 2,4-DCP removal was not obvious.

In another study, Mangat and Elefsiniotis (1999) investigated the effect of HRT, the presence and absence of supplemental substrate (phenol or dextrose) and variation in feed concentration on the biodegradation potential of 2,4-dichlorophenoxyacetate (2,4-D) in SBR. They reported a need of long acclimation period (around 4 months) to observe efficient 2,4-D removal. They observed a sequential utilization pattern of supplemental substrate and 2,4-D. The 2,4-D specific removal rates were affected by the type of supplemental substrate used (phenol or dextrose), being significantly lower (30-50%) in the case of dextrose, whereas, they reported the highest specific degradation rates when 2,4-D was supplemented as a sole carbon and energy source.

Although there are some studies on the effect of a biogenic substrate concentration on the degradation rate of toxic organic compounds, their conclusions are often conflicting as also reported by Wang et al. (1996). In the study of Wang et al (1996) they pointed different results found in the literature and discussed the possible reasons of these observations. They stated that confusion seems to stem from the fact that in some studies well-specified cultures have been used while in others widely heterogeneous cultures have been employed in the experiments and from the use of overall rather than specific (i.e., per-unit amount of biomass) biodegradation rates. If we give some examples from the literature, Lackmann et al. (1981) have performed experiments with 2,4-D and glucose using a mixed culture which consisted of three to four dominant Pseudomonas sp. They found that when simultaneous use of glucose and 2,4-D by the culture occurred, the rates of 2,4-D biodegradation were the same with those observed when the medium did not contain glucose, whereas the rate of glucose utilization was lower than that observed in the absence of 2,4-D. The author's conclusion was that 2,4-D inhibits glucose removal while glucose does not affect 2,4-D biodegradation. However,

Wang et al. (1996) pointed that this conclusion is not valid when specific growth rates are used. If 2,4-D biodegradation rate is the same in the presence (implying higher biomass levels) and absence of glucose, the specific biodegradation rate of 2,4-D is inhibited by the presence of glucose.

In another study, Orhon et al. (1989) studied the effect of the presence of a nutrient broth on the biodegradation of 2,4-D using a heterogeneous culture. They observed that the rate of nutrient broth utilization was severely reduced in the presence of 2,4-D while 2,4-D removal (in acclimated cultures) was unaffected by the presence of the nutrient broth. They explained the reason of this observation is that a specific fraction of biomass was only responsible for 2,4-D degradation and addition of biogenic substrate did not affect 2,4-D degradation rate. It seems that presence of nutrient broth inhibited 2,4-D degradation if the rates had been measured on a per-unit amount of biomass basis (Wang et al., 1996). In their study, Wang et al. (1996) concluded that the use of easily degradable substrates did not enhance the specific degradation rate of phenol.

Contrary to these findings, Schmidt et al. (1987) indicated that glucose enhances the specific rate of p-nitrophenol degradation by *Pseudomonas sp.*

Therefore, the effect of other organics on the degradation kinetics of SOCs should be considered in the development of models used to predict the system performance.

1.3.1.2.2. Use of Mixed Microbial Communities in Aerobic Biodegradation of Chlorophenols

Most biodegradation occurs by growth-linked metabolism. This means that the microorganisms performing the biodegradation receive their carbon and energy from degradation of the SOC and the SOC can serve as the sole carbon and energy source for microbial growth. As a consequence, the transformation products from

the initial biodegradative reactions with the SOC ultimately enter into the normal metabolic pathways wherein some of the carbon is incorporated into new cell material and the remainder is released as carbon dioxide. The result is mineralization of SOC because it has been converted into innocuous products. Engineered systems almost always employ natural microbial communities, rather than pure microbial cultures, and so it is not unusual for biodegradation of SOCs to occur by the coordinated action of several species within that community. Such assemblages are called consortia (Grady et al., 1999).

Although pure cultures may exhibit a range of chlorophenol degradation capabilities, the use of pure culture in a real out door system would be impractical due to the dominance of other strains on the special strains that are better equipped to degrade the target compound. Despite the mentioned disadvantage of using pure culture in out door degradation systems, in most of the studies discussed above, special strains of microorganisms were utilized for cometabolic degradation of chlorophenols in the presence of phenol as a growth substrate (Wang and Loh, 1999 and 2000; Wang et al., 2000; Hill et al., 1996; Lu et al., 1996; Kim and Hao, 1999; Hao et al., 2002). Another disadvantage of using pure culture is the incomplete degradation of target compound, as the selected specie may not have the ability to degrade all intermediate coming out from the conversion of target compound. Mixed cultures are particularly important when the emphasis is placed on complete mineralization of toxic organics to CO_2. Therefore, treating chlorophenols with activated sludge that contains an active mixed culture but lacks a special growth substrate would be more meaningful, informative, and practical. Also, it is well known that the capacities of an activated sludge system can be enhanced by acclimation (Buitron et al., 1998 and Kim et al., 2002). Buitron et al. (1998) reported that acclimated activated sludge degraded chlorophenol mixture by one to two orders of magnitude faster than pure strains obtained from the acclimated consortium. Sahinkaya and Dilek (2002) reported that although no removal was observed with an unacclimated culture, almost complete removal of 2,4-DCP, up to 148.7 mg/L, was achieved with the acclimated culture. Similar result was also

observed for 4-CP degradation as the degradation rate sufficiently increased with the acclimation (Sahinkaya and Dilek, 2005). Kim et al. (2002) observed that 4-CP and phenol degradation by a single pure culture was seriously inhibited in the presence of 2,4,6-TCP and when another microorganism having the ability of 2,4,6-TCP degradation added, the concentration of 2,4,6-TCP decreased and the degradation of 4-CP and phenol began. Therefore, by using a mixed culture, the severe effects of one compound could also be relieved.

The first step to biodegrade toxic substances in a wastewater treatment plant is the acclimation of the microorganisms. When microorganisms are put in contact with toxic compounds, in a favorable environment, acclimation to these compounds may occur. Different phenomena have been described to explain the acclimation phase. Wiggings et al. (1987) suggested that there is a selection and a multiplication of specialized microorganisms during this phase and physiological transformations in the metabolic system of the microorganisms, i.e., alterations in the enzymatic level, regulation and production, mutations, etc. In aerobic microbial communities, the acclimation periods typically range from several hours to several days. Therefore, acclimation (adaptation) is any response that ultimately leads the community to eliminate stress or find a way to maintain its function despite the stress (Rittmann and McCarty, 2001). However, not much information is available concerning the activity evolution of a microbial consortium during the acclimation to toxic wastewater. On the other hand, it has been shown that the acclimation is not permanent. The exposition of the acclimated population to prolonged periods of starvation produces a decrease of the bacterial activity and, even the death of some of them (Moreno-Andrade and Buitron, 2004a).

The variation of the microbial activity during the acclimation and deacclimation process in the degradation of 4-CP was studied in a sequencing batch reactor (SBR) by Moreno-Andrade and Buitron (2004a). The influence of initial toxic concentration on acclimation of activated sludge from a municipal wastewater treatment plant was studied. The experimental design considered three different sets

of initial concentration of 4CP: 50, 100 and 200 mg/L. After acclimation, the biomass was exposed to different periods of starvation to study the deacclimation. The results showed a reduction in the degradation time as the acclimation process occurred. Degradation time was reduced from 40 h to 50 min, in the case of 50 mg 4-CP/L and from 52 to 1.16 h, for the case of 100 mg/L. The metabolite production during the acclimation has been observed. As the acclimation took place, it was found that the affinity of the consortia to biodegrade the toxic increased, whereas the ability to biodegrade acetate decreased. This may be due to increase in fraction of specialist biomass grown on 4-CP (Magbanua et al., 1998). Moreno-Andrade and Buitron (2004a) also observed that starvation generates a decrease on the microbial activity. The extent of the deacclimation of starved microorganisms seems to be affected by the history of the culture. Degradation rates during the acclimation and deacclimation process followed an exponential model.

Sudden introduction of some unexpected recalcitrant compounds or increase of their loading rate may cause failure of a conventional activated sludge system easily and result in toxicity breakthrough. This system failure is mainly due to the low population of bacteria capable of degrading these recalcitrant compounds and inhibition of these pollutants to the biomass in the system. One of the most straightforward strategies to remediate such system failure is bioaugmentation using a group of specific bacteria (Quan et al., 2004a). In this context, Quan et al. (2004a) extensively evaluated enhancement of 2,4-DCP degradation in conventional activated sludge systems bioaugmented with mixed special culture. Results showed that the two bioaugmented conventional activated sludge systems, which were inoculated with 5% and 15% 2,4-DCP degrading mixed culture, respectively, demonstrated and maintained stronger ability to degrade 2,4-DCP than the non-supplemented control one during the first three shock loading periods. For the fourth 2,4-DCP shock loading which occurred 100 days after inoculation, the advantages demonstrated by the bioaugmented systems were greatly reduced compared to those of the previous three runs, which may be due to acclimation of culture to 2,4-DCP in non-augmented reactor.

In another study, Quan et al. (2003) studied biodegradation of 2,4-DCP in SBR augmented with immobilized mixed culture. They reported that inoculum size affected the start-up time of the SBR systems. For the non-augmented system more time was needed to start up. In addition, bioaugmented SBR systems demonstrated stronger capacity to cope with 2,4-DCP shock loading. The control SBR failed to treat 2,4-DCP at 166 mg/L in influent, while the SBR including 1.9% and higher fraction of special sludge achieved 166 and 250 mg/L 2,4-DCP degradation, respectively. It was also found that augmented culture played the primary role in degrading 2,4-DCP at the beginning of system start-up, but after one-month operation, both the indigenous and the introduced culture posed strong ability to degrade 2,4-DCP.

Bioaugmention to improve chlorophenol removal was also studied by Farrell and Quilty (2002). Degradation of 2-chlorophenol by the commercial mixture via a meta-cleavage pathway led to incomplete degradation, while *P. putida* CP1 was shown to be capable of completely degrade 2-chlorophenol via an ortho-cleavage pathway. Augmentation of the commercial mixed culture with *P. putida* CP1 caused complete degradation of 2-chlorophenol via an ortho-cleavage pathway. The ability of *P. putida* CP1 to degrade 2-chlorophenol increased with the addition of mixed culture. Increasing the mixed culture inoculum size added to *P. putida* CP1 decreased lag periods and increased rates of degradation, resulting in decreased times of degradation.

Biodegradation of 2,4-DCP using *Pseudomonas putida* and *Pseudomonas putida* CP1 was studied by Kargi and Eker (2004 and 2005). They observed low 2,4-DCP removal efficiencies with the pure cultures. For example, complete 2,4-DCP removal was only observed when initial concentration is 30 mg/L at the end of 60 h of incubation and DCP removal decreased to 32% and 22% for initial DCP concentrations of 125 and 240 mg/L, respectively. As for *Pseudomonas putida* CP1, the maximum removal efficiency was only 35% when initial DCP concentration was 51 mg/L. Although no significant degradation of 2,4-DCP by

pseudomonas putida was observed in the study of Kargi and Eker (2004), the same authors reported high removal efficiency of 2,4-DCP with acclimated mixed culture (Kargi et al, 2005 and Kargi and Eker, 2006). Kargi et al. (2005) investigated biological treatment of synthetic wastewater containing 2,4-DCP of varying concentrations using an activated sludge unit operated at 10 d of SRT. They observed almost complete removal of 2,4-DCP when its concentration was below 150 mg/L, whereas, the effluent 2,4-DCP, COD and toxicity levels increased when 2,4-DCP inlet concentration exceeded 150 mg/L. Also, sludge volume index (SVI) increased when feed 2,4-DCP concentration was above 150 mg/L. Similar result was also observed in the study of Uysal and Turkman (2005) as the effluent 2,4-DCP increased from 2.6 to 113.7 when the feed 2,4-DCP was increased from 100 to 150 mg/L in an activated sludge unit operated at 20 days of SRT. They also reported that addition of biosurfactant slightly increased the reactor performance as the COD and 2,4-DCP removal efficiencies increased from 50 and 24.2% to 55.3 and 32.9%, respectively, with the addition of biosurfactant.

In another study, Kargi and Eker (2006) investigated the effect of SRT on the performance of an activated sludge unit treating 2,4-DCP containing synthetic wastewater. They kept influent wastewater characteristics constant, while changed SRT between 5 and 30 days. Increase in sludge age caused significant increases in biomass concentration in the aeration tank, which resulted in increase in percent COD, DCP and toxicity removals. COD removal increased from 58 to 90%, while DCP and toxicity removals increased from 15 to 100% and from 38 to 100%, respectively, when the sludge age was raised from 5 to 30 days. They also reported improved sludge settling characteristics at high SRT values.

Activated sludge treatment of ground water contaminated with chlorophenols at low concentrations (4-120 μg/L) was investigated by Ettala et al. (1992). They reported that high removal was achieved with steady dosing of chlorophenols at high SRTs (8-10 d). According to the mass balance, 71% of chlorophenols was

removed and 68% was biodegraded. A chlorophenols uptake rate of 29 mg/kg MLVSS.d was achieved.

Wang et al (2000) studied the biodegradation of 2,4,6-TCP in the presence of primary substrate by immobilized pure cultures (*Pseudomonas* spp. strain 01, *Pseudomonas* spp. strain 02 and *Agrobacterium* spp.) isolated from acclimated mixed culture. Whether in suspended or immobilized forms, all strains have poor removal efficiencies of 2,4,6-trichlorophenol. Addition of a substrate such as 2-CP, 3- CP, 4-chlorophenol, or 2,4-DCP did not have any benefit to the bacteria to decompose 2,4,6-TCP. However, in the presence of phenol (200 mg/L), the immobilized *Pseudomonas* spp. strain 01 can decompose 63% of 2,4,6-TCP (5 mg/L) at the end of 15-day monitoring period indicating that addition of phenol will assist the immobilized strain in removing 2,4,6-TCP. Therefore, results showed that high phenol concentrations (200-400 mg/L) are required to remove 2,4,6-TCP even at a very low initial concentration, 5 mg/L. On the other hand, Vallecillo et al. (2000) reported high 2,4,6-TCP removal efficiency even at inlet concentration of 100 mg/L when acclimated mixed culture was used.

Vallecillo et al. (2000) studied the aerobic biodegradability and toxicity of some chlorophenolic compounds (PCP, 2,4,6-TCP, 2,4-DCP and 4-CP) in batch reactors and the behavior of a continuous activated sludge reactor feeding with 2,4,6-TCP and acetic acid in order to determine the feasibility of this treatment for degradation of such recalcitrant compounds. Activated sludge treatment of 2,4,6-TCP was studied at different CP and COD concentrations. At 1500 mg/L COD and 25 mg/L TCP, COD removal was higher than 95% and TCP removal was 90% with an HRT of 1.25 d. Operating with the same HRT, removal higher than 85% was reached for TCP concentration of 100mg/L. They reported that TCP removal was dependent on feed COD concentration, and TCP removal increased strongly when feed COD was increased from 600 to 1000 mg/L.

Farrell and Quilty (1999) used a mixed microbial community, specially designed to degrade a wide range of substituted aromatic compounds, in the degradation of mono-chlorophenols as sole carbon source in aerobic batch cultures. The mixed culture degraded 2-, 3-, and 4-CP (1.56 mM) via meta cleavage pathway. During the degradation of 2- and 3-chlorophenol, 3-chlorocatechol accumulated in the medium. Further metabolism was toxic to cells as it caused inactivation of the catechol 2,3-dioxygenase enzyme upon –meta cleavage of 3-chlorocatechol resulting in incomplete degradation. Inactivation of the –meta cleavage enzyme led to an accumulation of brown colored polymers. Degradation of 4-CP by the mixed culture led to an accumulation of CHMS, which was further metabolized, indicating complete degradation of 4-CP by the mixed culture via a -meta cleavage pathway.

1.3.1.3. Chlorophenol Degradation in Aerobic Attached Growth Reactors

Generally stirred tank reactors or flasks as batch reactors were utilized in the treatability studies of chlorophenolic wastes. The advantage of these reactors is that operating and adjusting the detention time is simple. Whereas, it is vulnerable to shock and washout and it takes a long time to recover from perturbation (Kim et al., 2002). On the other hand, use of attached growth reactors overcome most of the problems encountered in suspended cells bioreactors. For example, Kim et al. (2002) reported that packed bed reactor is more effective compared to CSTR in simultaneous degradation of 4-CP and phenol as although washout occurred in CSTR at a dilution rate of 0.36 per hour, efficient treatment was observed in packed bed reactor at the same dilution rate. The advantages of immobilized cell cultures over suspended cultures are given by Shuler and Kargi (1992) as:

- Immobilization provides high cell concentrations.
- Immobilization provides cell reuse and eliminates the costly process of cell recovery and cells recycle.
- Immobilization eliminated cell washout problems at high dilution rates

- The immobilization of high cell concentrations and high flow rates allows high volumetric productivities.
- Immobilization may provide favorable environmental conditions (that is, cell-cell contact, nutrient-product gradients, pH gradients) for cells, resulting in better performance of the biocatalysts (for example, higher product yields and rates).
- In some cases, immobilization improves genetic stability.
- For some cells, protection against shear damage is important.

In contrary to reported partial treatment efficiencies and deterioration in treatment system at low temperatures when activated sludge process was used (Ettala et al., 1992), Jarvinen et al. (1994) reported that chlorophenols could be degraded even at low temperatures in aerobic FBR. In this study, degradation of chlorinated phenolic compounds was investigated at different temperatures and HRTs. Concentrations of chlorophenols in contaminated ground water were as follows: 7-11 for 2,4,6-TCP; 32-36 mg/L for 2,3,4,6-TeCP and 1.8-2.3 mg/L for PCP. Experiments were carried out in four aerobic FBRs with different carrier materials. Temperature of reactors was gradually decreased even to ambient temperature of ground water and results demonstrated that fluidized bed treatment of CP-contaminated ground water produced close to drinking water quality effluent at ambient ground water temperature and CP loading rate as high as 740 mg CP/ L/d (HRT = 1.4 h). Fluidized bed treatment resulted in 80% CP removal at loading rate of 2130 mg CP/L/d and 0.44 h HRT. At the temperature below $7^{o}C$, suspended solid concentration slightly increased but did not affect the CP degradation. This study showed that aerobic FBR can be used to treat ground water contaminated with chlorophenolic compounds at the ambient temperatures of ground water, thus, avoids the heating expenses.

In another study, Puhakka and Jarvinen (1992) studied the degradation of polychlorinated phenols in continuous-flow fluidized-bed reactors using pure oxygen for aeration and celite carrier for cell immobilization. Chlorophenols were

fed as the only source of carbon and energy for the mixed biofilm culture. No supplemental carbon or energy source was required for continuous operation and maintenance of the mixed chlorophenol degrading culture and the possibility of o-methylation would be expected to be minimal. Initial concentration of 2,4,6-TCP was set to 20 mg TOC/L. Inorganic chloride releases indicated 100% dechlorination of 2,4,6-TCP in all pseudo-steady-state experiments. TOC removals were 82-83% for 2,4,6-TCP. The effluent 2,4,6-TCP concentration was 0.1 mg/L or less. These results demonstrated that 2,4,6-TCP was readily biodegradable by the immobilized aerobic microbes. After then the reactor was switched to 2,3,4,6-TeCP solution containing 11% PCP as impurity. The feed concentration was set at 20 mg TOC/L and fed at 5 h HRT. Then, concentration of TeCP was increased to 30 mg TOC/L while HRT remained at 5 h. These feed patterns corresponded to loading rates between 230 and 400 g chlorophenol/m^3/d and mean TOC removals were observed to be 99.3% for 2,3,4,6-TeCP and 74.5% for PCP. In this study, treatment of simulated ground water contaminated with chlorophenolic compounds was also investigated and results showed that the mean chlorophenols removals were higher than 90% when the reactor was fed with a mixture of 15±0.3 mg/L 2,4,6-TCP, 26.3±1.9 mg/L 2,3,4,6-TeCP and 3.8±0.6 mg/L PCP at chlorophenol loading rate of 217g/m^3/d and HRT of 5 h for 5 weeks.

The same research group also investigated the aerobic and anaerobic biotransformation of several chlorinated, methylated monoaromatic compounds in batch and continuous-flow FBRs (Puhakka et al., 1994). They reported that under aerobic condition PCP, 2,3,4,6-Tetrachlorophenol (TeCP), 2,4,6-TCP, 4,5-DCC (dichlorocatechol) were completely degraded in batch reactors within 8 d. However, methoxylated chlorophenols, dichloroguaiacol (DCG), trichloroguaiacol (TCG), trichlorosyringol (TCS) were recalcitrant towards aerobic degradation, whereas 5,6-dichlorovanilin was partially degraded in aerobic mixture. The addition of tryptone yeast extract did not affect the removal of chlorophenols. Under anaerobic conditions, these compounds were dechlorinated and transformed into aerobically degradable forms. It was postulated that O-methylation products

resulting in aerobic conditions may resist to aerobic microbial attack and have a high potential for bioaccumulation. The high rate operation (HRT 5 h) of aerobic FBR resulted in the mean removal of 99.6% for 2,3,4,6-TeCP, 98.4% for 2,4,6-TCP and 83.5% for PCP over a period of 235 d. Initial chlorophenols concentrations in aerobic FBR were 40 mg/L for 2,3,4,6-TeCP, and 2,4,6-TCP, 43 mg/L for PCP.

The ability of immobilized cells grown under oxic and fluidized-bed conditions to degrade 4-CP and 2,4-DCP was investigated by Shieh et al. (1990). The pseudo-steady-state performance of chlorinated phenol degradation was evaluated under different dilution rate conditions, with chlorophenols as the sole organic carbon sources. Good TOC removal was observed: 85-88% for 4-CP and 80-83% for 2,4-DCP (initial TOC concentration was 20 mg/L). This TOC removal performance remained unchanged despite a five-fold increase in dilution rate. Results indicated that, with feed 4-CP and 2,4-DCP concentrations at 35.7 and 45.3 mg/L, respectively, good and stable removal performance are achievable at empty bed HRT as low as 1 h.

In another study, Valo et al. (1990) reported that biodegradation of chlorophenols from simulated contaminated groundwater by immobilized bacteria could be achieved. The chlorophenols (3-130 mg/L) used in this study were 2,3,4,6-TeCP, 2,4,6-TCP and PCP, in a ratio of 8:1:1 by weight. Chlorophenols-mineralizing *Rhodococci* was immobilized on a polyurethane carrier. The filter was fed at 4 ^{0}C to see if the chlorophenols were adsorbed and degraded also at ground water temperature. Four periods of 1 d cold operation at 4^{o}C (74-426 mg chlorophenols /L/d) was followed by 5-16 d at 25^{o}C. Filter did not discharged organic chloride at 4^{o}C, but it started to release chloride immediately when the temperature was raised to 25^{o}C. Although Jarvinen et al. (1994) reported the degradation of chlorophenols even at the ambient temperatures of ground water; Valo et al. (1990) reported that chlorophenols were adsorbed at cold water onto a polyurethane bed material and then biodegraded at 25^{o}C while recycling one bed volume of water in the biofilter. Cooling (4^{o}C) and heating (25^{o}C) cycle did not lower the chlorophenols degrading

activity of the polyurethane immobilized *Rhodococci*. Chlorophenols degradation started as soon as the temperature was raised from 4^{o}C to 25^{o}C. The biofilter with *Rhodococci* remained active for over 4 months. The recirculating water became acid as a result of dechlorination at 25^{o}C after each cold adsorption period. The chlorophenols concentration of the column effluent was 1/1000-1/10000 of the influent products.

For continuous-flow biofilm systems, the treatment efficiency is limited by a minimum substrate concentration (S_{min}), which is required to maintain a steady-state biofilm. Below S_{min} concentration, the microbial decay rate exceeds the growth rate and the biofilm gradually disappears. Rittmann et al. (1980) and McCarty et al. (1981) proposed feeding a primary substrate at a concentration above S_{min} to support simultaneous xenobiotic compound (secondary substrate) utilization at sub-S_{min} concentrations. Problems with this approach include finding a primary substrate that supports simultaneous degradation of secondary substrates. This approach was unsuccessful for stimulating biodegradation of trace concentrations of PCP when acetate was the primary substrate (Speitel et al. 1989); low concentrations of 2,4-DCP also did not stimulate PCP degradation. Another approach involves the use of oligotrophic enrichment cultures able to utilize the contaminant as the sole source of carbon and energy. Oligotrophic bacteria grow at low substrate concentrations and are characterized by high substrate affinity (low K_S coefficient) and low S_{min} (Kobayashi and Rittmann 1982; Rittmann et al. 1986). With oligotrophic xenobiotic-degrading cultures it may, therefore, be possible to achieve low effluent concentrations without the use of additional substrates (Melin et al., 1997). In this context, Melin et al. (1997) used a fluidized-bed reactor to enrich an aerobic chlorophenol-degrading microbial culture. Long-term continuous flow operation with low effluent concentrations selected oligotrophic microorganisms producing good-quality effluent for PCP contaminated water. The results of the study showed that the culture has a high affinity for PCP but is also inhibited by relatively low PCP concentrations (above 1.1 mg/L PCP). This enrichment culture was maintained over 1 year of continuous-flow operation with

PCP as the sole source of carbon and energy. During continuous-flow operation, effluent concentrations below 2 µg/L were achieved at 268 min HRT when feed PCP concentration was 2.5 mg/L.

Zilouei et al.(2004) evaluated bioremediation of chlorophenols at low temperature in a packed bed bioreactor. Packed-bed reactor was tested at various HRTs at 14ºC and 23ºC for the treatment of a mixture of 2-CP, 4-CP, 2,4-DCP and 2,4,6-TCP as sole sources of carbon and energy source. A total chlorophenol removal efficiency of more than 95% was recorded at both temperatures at 3.9 hours HRT, corresponding to a total chlorophenol removal rate of more than 535 mg/L.d. The inlet pollutant concentration was then increased to reach an organic load of 60.4 mg chlorophenol/L.h at 2.5 hours HRT. Under this condition, total chlorophenol removal rates of 1230 and 1160 mg/L.d were achieved in the control and cold reactors, respectively. Under conditions when chlorophenols were not completely removed, the pollutants were biodegraded in the following order of increasing rates: TCP = DCP > 4CP > 2CP, which is the inverse of most results reported in literature.

1.3.1.3.1. Rotating Biological Contactors (RBCs)

Rotating biological contactors (RBCs) were first installed in West Germany in 1960 and were later introduced in United States. In the United States and Canada, 70% of the RBC systems installed are used for carbonaceous BOD removal only, 25% for combined carbonaceous BOD removal and nitrification, and 5% for nitrification of secondary effluent. A rotating biological contactor consists of a series of closely spaced circular discs. The disks are submerged in wastewater and rotated slowly through it (Figure 1.3).

In operation, biological growths become attached to the surfaces of the disks and eventually form a slime layer over the entire wetted surface area of the disks. The

rotation of the disks alternately contacts the biomass with the organic material in the wastewater and then with the atmosphere for adsorption of oxygen. The disk rotation affects oxygen transfer and maintains the biomass in an aerobic condition. The rotation is also the mechanism for removing excess solids from the disks by shearing. The specific surface area of the medium itself ranges from about 100 m^2/m^3 to about 170 m^2/m^3 (high density). The high-density media, which have smaller channels for liquid and gas penetration, are most susceptible to clogging and should be used only with the low loading. For full scale RBCs, the diameter of the medium is approximately 3.6 m (12 ft). The medium is attached to a steel shaft (or axle) that is supported on bearings and rotated by a direct mechanical drive in most cases. In a few cases, the trough is bubble aerated, and the bubbles are trapped in "cups" to create air-driven rotation. Modules of medium are attached to the shaft to give a total medium length of approximately 9300 m^2 (100 000 ft^2) of surface area, while a high-density medium has approximately 14000 m^2 (150 000 ft^2). Generally, the high-density media are used only in the later stages of an RBC system, when the BOD concentration has been reduced enough that the smaller pore openings are not clogged.

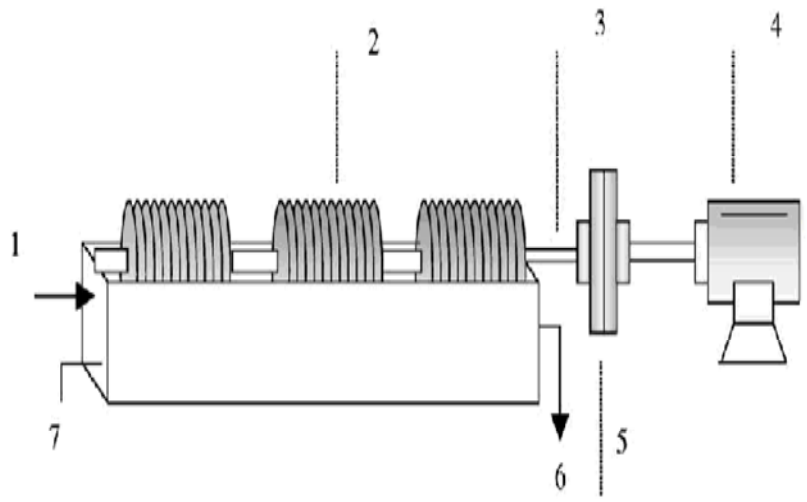

Figure 1. 3. Schematic diagram of RBC reactor; (1) inlet, (2) rotating cylinder, (3) shaft, (4) drive motor, (5) joint, (6) outlet, (7) drain (Alemzadeh et al., 2002)

The medium is partly submerged in the wastewater. A key operating parameter is the percent submergence, which is the percentage fraction of the medium diameter (measured at the centerline) that is submerged. Typical submergence is 25-40%. The rotating speed for a full-scale unit is around 2 rpm (Rittmann and McCarty, 2001).

In the design of a RBC system consideration must be given to

1. Staging of the RBC units
2. Loading criteria
3. Effluent characteristics
4. Settling tank requirement (Metcalf and Eddy, 1991; Rittmann and McCarty, 2001).

Advantages of RBC: Since the middle of the 20th century, there has been renewed interest in biofilm systems for the carbonaceous oxidation of wastewaters because of their simplicity and reliability and because of the availability of new types of media with very large surface areas per unit volume. This interest has led to upgrading of trickling filters and the development of new biofilm systems, which include the RBC, aerated filters and fluidized bed technology (Rodgers, 1999). Rotating biological contactors can be used for secondary treatment and they can also be operated in the seasonal and continuous-nitrification and denitrification modes. Rotating biological contactors generally are quite reliable because of the large amounts of biological mass present (low-operating F/M). This large biomass also permits them to withstand hydraulic and organic surges more effectively. The effect of staging in this plug-flow system eliminates short-circuiting and dampens shock loadings. Properly designed RBC system may be superior in performance to other fixed-film systems due to lower organic loading per mass of biological solids, longer detention time in the biological stage, and better control of short circuiting. A typical flow diagram of a rotating biological contactor system is given in Figure 1.4. RBC systems are almost always operated in series mode with three to five

stages. A settler follows to reduce effluent suspended solids. Control of the environmental conditions and the biofilm thickness is easier in RBCs since the liquid phase is more homogeneous and biofilm is visible and reachable during the operation as compared to biological filters. Moreover, aeration is much more effective in RBC as a result of direct contact of biofilm and air during rotation (Kargı and Eker, 2002).

RBC system can be designed to provide secondary or advanced levels of treatment. Effluent BOD_5 characteristics for secondary treatment are comparable to well-operated activated-sludge process. Where a nitrified effluent is required, RBCs can be used to provide combined treatment for BOD and ammonia nitrogen or to provide separate nitrification of secondary effluent (Metcalf and Eddy, 1991; Rittmann and McCarty, 2001).

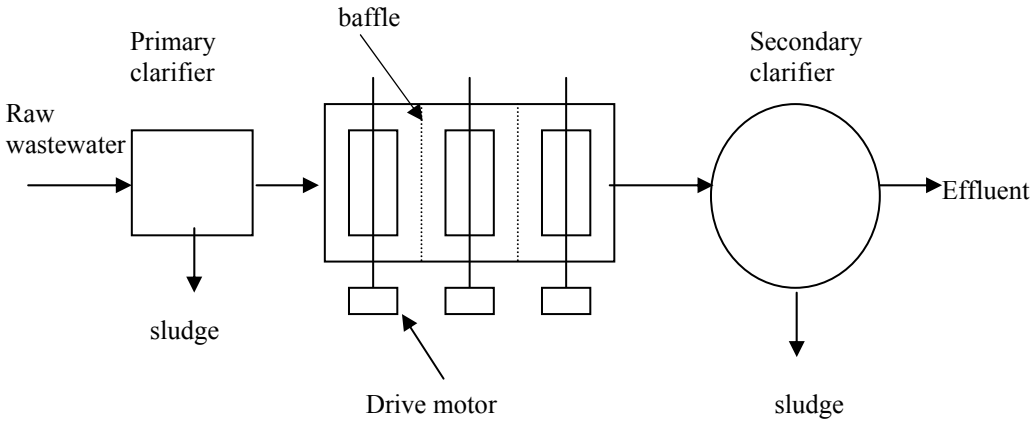

Figure 1.4. Typical RBC schematic for secondary treatment

***Disadvantages of RBC*:** Many of the early RBC units had operating problems consisting of:

1. *Shaft and Bearing Failure:* Early designs are insufficient in the structural strength, because the designers did not realize that the accumulation of

biofilm greatly increased the weight of media. Therefore, the shafts cracked, the bearings wore out and media ripped off the shaft. The components are now designed to accommodate the added weight of the biofilm.

2. *Eccentric Growth:* If the RBC stopped its rotation, the submerged portion would continue to have growth, while the exposed portion would stop growth. Then, the eccentric weight distribution required too much torque to restart the rotation. Higher capacity motors and derives are used to overcome this problem.
3. *Nuisance organisms:* Sulfate reducing bacteria fostered by too high loads and insufficient aeration, caused odors, heavy precipitates (S^0, FeS), filamentous growth, and other serious nuisances. Appropriate first-stage and overall surface loads, as well as the tip-velocity criterion, generally prevent nuisance organisms (Rittmann and McCarty, 2001).

In the literature, there are very few studies on chlorophenol treatment using RBC despite the advantages listed above. Tokuz (1991) used RBC for studying the biodegradation of phenolic compounds and reported that the phenolic compounds are biodegradable and RBC is a viable process in treating phenolic wastewaters. Radwan et al. (1997) achieved the treatment of 2,4-DCP up to a concentration of 200 mg/L in the presence of glucose as primary substrate using a modified RBC. Although, in most of the studies related to the biological treatment of chlorophenols, presence of phenol (Wang and Loh, 1999 and 2000; Wang et al., 2000; Hill et al., 1996; Lu et al., 1996; Kim and Hao, 1999; Hao et al., 2002) or another non-toxic carbon source (Wang and Loh, 1999; Bali and Sengül, 2002, Tarighian et al., 2003) is reported to be necessary in suspended culture reactors, Swaminathan and Ramanujam (1999) reported that acclimated mixed culture has the ability to use 2,4-DCP as a sole carbon source in RBC. They adapted mixed culture to 2,4-DCP, of which concentration was increased gradually, in the presence of glucose, then glucose concentration was decreased gradually and 2,4-DCP was fed to the reactor as a sole carbon source. Under these conditions, they reported a remarkably high efficiency (99.2-99.5%) of RBC in the treatment of 2,4-DCP up to

253.4 mg/L. Although complete removal of 2,4-DCP as a sole carbon and energy source was reported in this reactor, there is no enough information whether absence of primary substrate have an adverse effect on the degradation rate of 2,4-DCP. Moreover, in the study, there is no information on the fate of some intermediates coming out from the biological degradation of 2,4-DCP. Also, the ability of the system for other types of chlorophenols and for chlorophenol mixtures should be investigated further because substrate interactions are crucial for understanding and predicting the behavior of chlorophenols in biological treatment systems due to the great variation of industrial effluents in terms of both composition and concentration.

1.3.2. Modeling SOCs Degradation

Knowledge of microbial growth and substrate utilization kinetics is important for the accurate prediction of effluent quality from engineered treatment processes (Ellis et al., 1996a, 1996b; Ellis and Anselm 1999; Grady et al., 1996). Accurate kinetic parameters also help to operation engineers to optimize operational conditions to meet discharge requirements (Ellis and Anselm, 1999).

There is considerable variability in the literature concerning the values of the kinetic parameters for growth and substrate removal associated with any given compound, even with pure cultures. While there are doubtless many reasons for that variability, three appear to be particularly important: culture history, parameter identifiability, and the nature of the assay procedure employed to measure the parameters. The history of the culture is important for a variety of reasons. First, with mixed cultures, the manner in which the culture has been developed determines which species are present. Second, even with a pure culture, the way in which a culture has been grown often determines the nature of the enzyme systems that are expressed. Third, culture history also determines physiological state, which is the sum total of a cell's macromolecular composition and determines how rapidly

it can synthesize enzymes, as well as how rapidly those enzymes react (Grady et al., 1996).

Culture history (the type of environmental conditions imposed and the duration of their imposition) has been suggested as a major factor contributing to measured differences in kinetic parameters describing biodegradation. Continued cultivation of a mixed culture will result in selection for some members of the community over others, as determined by their growth kinetics. As the community make-up changes, the kinetic parameters describing it will be affected. Classical chemostat theory, for example, dictates that in a continuous flow, completely mixed environment, organisms with higher affinity enzymes are favored and will displace organisms with lower affinity enzymes. In batch assays, the chosen value of *SolXo* is also important, however, because of its impact on the influence of culture history. If *So/Xo* is very large, significant changes can occur in the culture during the assay, resulting in parameters that are not reflective of the culture as it existed in the environment from which it was removed. Conversely, if *So/Xo* is very small, it is possible for the parameters to be representative of the kinetics manifested in the source environment. One way in which the value of *So/Xo* can alter the culture history is through its effect on the composition of the microbial community, and this is the mechanism focused on most commonly in the environmental engineering literature. The meaning of the kinetics observed with a large *So/Xo* value is clear. They represent the maximum capability of the members of the microbial community with the fastest growth kinetics. Such kinetics may shed light on the ultimate reactor performance under different conditions, e.g. at a lower solids retention time. Similarly, the meaning of the kinetics observed with a small *So/Xo* value is also clear. They represent the capability that the culture had when it was in the continuous culture reactor from which it was obtained (Grady et al., 1996).

Grady et al. (1996) suggested that the term "intrinsic" be used to denote kinetic parameters measured with high *So/Xo* values (>20 on COD basis). The rationale behind the use of the term intrinsic is its definition of "belonging to the essential

nature of a thing". If the kinetics truly reflects unrestricted growth they are representative only of the nature of the organisms and the substrate. They suggest the term "extant" be used to denote kinetic parameters measured with very low *So/Xo* values (<0.025 on COD basis). The rationale for use of this term is its definition of "currently existing".

Another significant point is that not all the microorganisms are responsible for the degradation of all individual substrates in a multi component feed. The fraction of biomass utilizing biogenic compounds is expected to higher compared to the fraction of those utilizing SOCs. In the kinetic studies, the concentration of biomass actually degrading the SOC of interest should be considered rather than total biomass as degradation of SOCs require unique pathways and not all bacteria in the community is responsible for the degradation of SOC of interest. Therefore, the question of how component biomass fraction can be calculated will arise at this point. Some researchers used the fraction of COD contributed to the feed by that compound in the calculation of competent biomass fraction based on the assumption that if specialists are responsible for degradation of a particular compound, the specialists grows only on SOC of interest (Magbanua et al., 1998; Hu et al. (2005b), Hu et al., 2005a; Ellis et al., 1996; Tomei et al., 2003).

The bacterial specific growth rate is often described using Monod kinetics. However, higher substrate concentrations may become inhibitory to microbial growth, which is called *self-inhibition*. For example, phenol at sufficiently high concentrations has been observed to exhibit an inhibitory effect on pure microbial cultures and activated sludge systems. Most of the equations have been adopted from models of the substrate inhibition of enzymatic reactions, and involve a common substrate inhibition term (K_I). The Andrews equation (Andrews, 1968) is one of the most commonly used models for describing the growth inhibition kinetics:

$$\mu = \frac{\mu_{\max} S}{K_s + S + (S^2 / K_I)} \qquad (1.1)$$

Where, S and K_s are the substrate concentration and the half-saturation coefficient, respectively.

Note that the ratio of the self inhibition and half saturation constants (K_I/K_s) determines the changes in the degradation rate with increasing substrate concentration. When the ratio approaches infinity, the Andrews equation becomes a Monod equation, and the substrate removal rate approaches its maximum. On the other hand, at a low K_I/K_s ratio, the substrate concentration, while increasing, reaches a critical ($S_{cr} = \sqrt{K_s K_i}$), beyond which the substrate removal rate drops (Tomei et al., 2004 and Melin, 1997). The experimental results obtained below S_{cr} can give the appearance of growth on a non inhibitory substrate and can be modeled using Monod equation. Wayman and Tseng (1976) proposed that specific growth rate can be modeled with Monod kinetics up to S_{cr} and above that with equation;

$$\mu = \frac{\mu_{\max} S}{K_s + S + K(S - S_{cr})} \qquad (1.2)$$

Where, K is a constant. Edwards (1970) compared Andrews and other four different inhibition models, given below, to model specific growth rate on different inhibitory compounds.

$$\mu = \frac{\mu_{\max} S(1 + \frac{S}{K})}{K_s + S + \frac{S^2}{K_I}} \qquad (1.3)$$

$$\mu = \frac{\mu_{\max} S}{K_s + S + \frac{S^2}{K_I}(1 + \frac{S}{K})} \qquad (1.4)$$

$$\mu = \frac{\mu_{\max} S e^{(-S/K_I)}}{K_s + S} \qquad (1.5)$$

$$\mu = \mu_{\max}\left[e^{(-S/K_I)} - e^{(-S/K_s)}\right] \qquad (1.6)$$

The superiority of the models varied for different experimental results and only equation 1.4 did not give the best fit in any case. Edward (1970) concluded that the Andrews equation is the easiest to manipulate among the equations tested and the Andrews is the best choice among the five equations tested. However, significant case-to-case variations can be observed (Melin, 1997). For example; Sokol and Howel (1981) reported that phenol degradation kinetics by *Pseudomonas putida* was better described by a two parameter model (Equation 1.7) than Andrews model.

$$\mu = \frac{\mu_{\max} K_I S}{K K_I + S^2} = \frac{K_I S}{K_2 + S^2} \qquad (1.7)$$

The presence of other organic or inorganic compounds may have inhibitory effect on the degradation of compound of interest and both substrates may compete for the same enzyme. So, the second type of inhibition is *competitive,* and a separate inhibitor is present at concentration *I*. The competitive inhibitor binds the catalytic site of the degradative enzyme, thereby excluding substrate binding. The only parameter affected by *I* in competitive inhibition is half saturation constant (K_s) in Monod equation:

$$K_{eff} = K\left(1 + \frac{I}{K_I}\right) \qquad (1.8)$$

Where, K_I = an inhibition concentration of the competitive inhibitor. A small value of K_I indicates a strong inhibitor. The rate reduction caused by competitive inhibitor can be completely offset if S is large enough. Competitive inhibitors usually are substrate analogues.

A third type inhibition is *noncompetitive inhibition* by a separate inhibitor. A noncompetitive inhibitor binds with degradative enzyme at a site different from the reaction site, altering the enzyme conformation in such a manner that substrate utilization is slowed. The only parameter affected is q_{eff}:

$$q_{eff} = \left(\frac{q}{1 + I / K_I} \right) \qquad (1.9)$$

In the presence of a noncompetitive inhibitor, high S cannot overcome the inhibitory effects, since the maximum utilization rate is lowered for all S. This phenomenon is sometimes called allosteric inhibition, and allosteric inhibition need not have any structural similarity to the substrate.

In some cases, competitive and noncompetitive impacts occur together. This situation is termed *uncompetitive inhibition.* Effective parameters (q_{eff} and K_{eff}) vary as they do for the individual cases.

Mixed inhibition is a more general form of uncompetitive inhibition in which the K_I can have different values (Rittman and McCarty, 2001).

As it can be understood from inhibition models, in the presence of different toxic compounds, the system may be affected in different ways and a modification of Monod kinetic in the presence of toxic compound is necessary to model the system accurately and get biokinetic parameters, which are necessary to design a remediation process. Regarding this, although there are several kinetic studies on the cometabolic treatment of chlorinated phenols (Hao et al., 2002; Hill et al., 1996; Kim and Hao, 1999; Wang et al., 2003; Saez and Rittmann, 1993), little kinetic studies are available on chlorophenol degradation, especially when they are present in mixture, by mixed cultures.

CHAPTER 2

MATERIALS AND METHODS

In this chapter, chemicals, inocula, experimental set-ups and analytical methods are described.

2.1. Chemicals

Chlorophenols (4-CP and 2,4-DCP), the chemicals used for growth medium and methanol used for HPLC analysis were obtained from Merck Chemical Co., Germany. COD kits were obtained from Hach Co.,USA. API 20 NE identification kits were obtained from Biomerieux Co., France.

2.2. Cultures and Medium

The composition of the synthetic wastewater is given in Table 2.1. In order to adjust desired chlorophenol concentrations in the synthetic wastewater, two different stock solutions were used. The first one was 16,000 mg/L 4-CP dissolved in 0.02 N NaOH, the second one was the mixture of 16,000 mg/L 4-CP and 8,000 mg/L 2,4-DCP dissolved in 0.02 N NaOH. Peptone was utilized as biogenic carbon and nitrogen sources (N content is around 12%). For the experiments in which peptone concentration was low to supply enough N, NH_4CI was supplied as N source not to create sub-optimal environmental conditions and the COD/N content

was between 100/12 and 100/6.

Table 2.1. Composition of the Synthetic Wastewater
(adapted from Dilek et al., 1998)

Constituents	**Concentration (mg/L)**
Proteose-peptone	0 - 400 (COD =0 - 400 mg/L)
NH_4CI	0-182
4-CP	0-200
2,4-DCP	0-100
NaCl	30
$MgSO_4$	44.6
K_2HPO_4	400
KH_2PO_4	200
$MgCl_2.6H_2O$	3.7
$FeCl_2.2H_2O$	3.7
$CaCl_2.2H_2O$	3.7
$MnSO_4$	0.057
$ZnSO_4$	0.046
$CoSO_4$	0.049
$CuSO_4$	0.076

Tap water was used to prepare synthetic wastewater. Phosphate salts were utilized as a source of P and buffer. The pH of the synthetic medium was between 7.2-7.5. For the experiments in which peptone was excluded, the pH of the reactors decreased to 6-6.5 due to nitrification of NH_4CI added as a source of nitrogen. For

these set of experiments, $NaHCO_3$ (300 mg/L) was added to the synthetic wastewater and the pH within the reactors was always kept between 6.5 and 7.5 (7.0±0.5).

2.3. Experiments

2.3.1. Sequencing Batch Reactor (SBR) Experiments

These experiments were conducted using SBRs operated in two different operational modes, one as "instantaneously fed" and the other as "semi continuously fed". The former one was used where the goal is to investigate the effect of chlorophenol and biogenic substrate concentrations on the chlorophenol degradation kinetics whereas the latter mode was selected where the goal is to investigate the effect of filling time on the degradation of chlorophenols.

2.3.1.1. Instantaneously Fed Sequencing Batch Reactor (SBR) Experiments

A 2.5 L glass vessel with 2 L of working volume was used as instantaneously fed SBR. Primary settling tank effluent of Greater Municipality of Ankara Domestic Wastewater Treatment Plant was used as initial inocula. The reactors were fed with synthetic medium once a day and at the end of each cycle excess sludge was wasted from mixed liquor to adjust SRT as 10days. After settling, 1.75 L of the treated effluent was drawn and around 0.25 L sludge was maintained for the next cycle, which gives 1.14 d of HRT in the reactors. The reactors were housed in a water-bath in which temperature was kept at 26±0.5°C and were aerated with air pumps to obtain dissolved oxygen concentration of at least 3 mg/L. A schematic diagram of instantaneously fed SBRs is illustrated in Figure 2.1. The photos of the reactors are also shown in Figure 2.2.

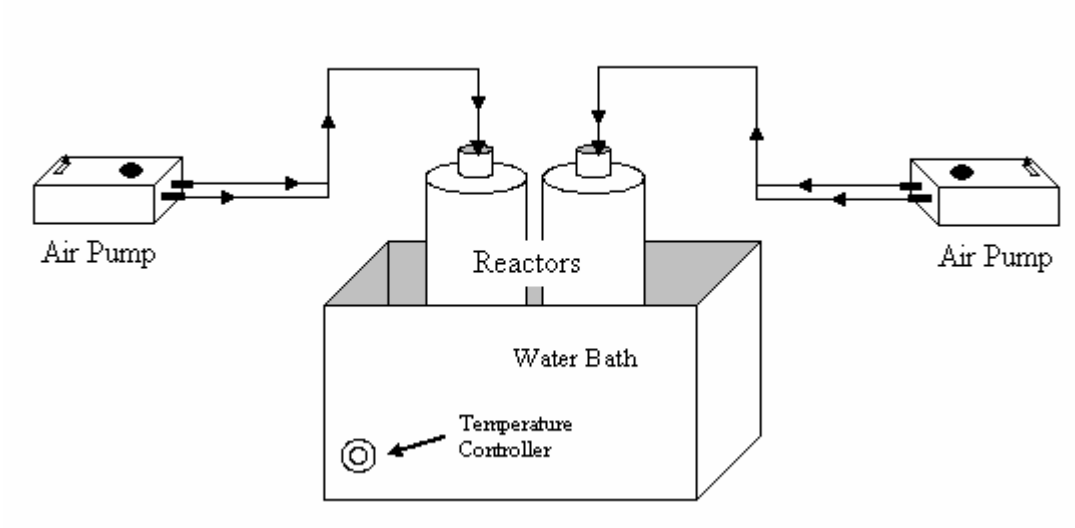

Figure 2. 1. A schematic diagram of instantaneously fed SBRs

Two reactors operated in parallel were fed with 4-CP only (FED4), whereas, the other two reactors were fed with mixture of 4-CP and 2,4-DCP (FEDM). Also, one reactor (base-line) was operated in the absence of chlorophenols in order to investigate the effect of chlorophenols addition on the reactor performance.

The feed 2,4-DCP concentrations in FEDM were always kept at one half of 4-CP concentrations as our previous study showed that 2,4-DCP was two times more toxic on biomass growth compared to 4-CP (Sahinkaya and Dilek, 2002).

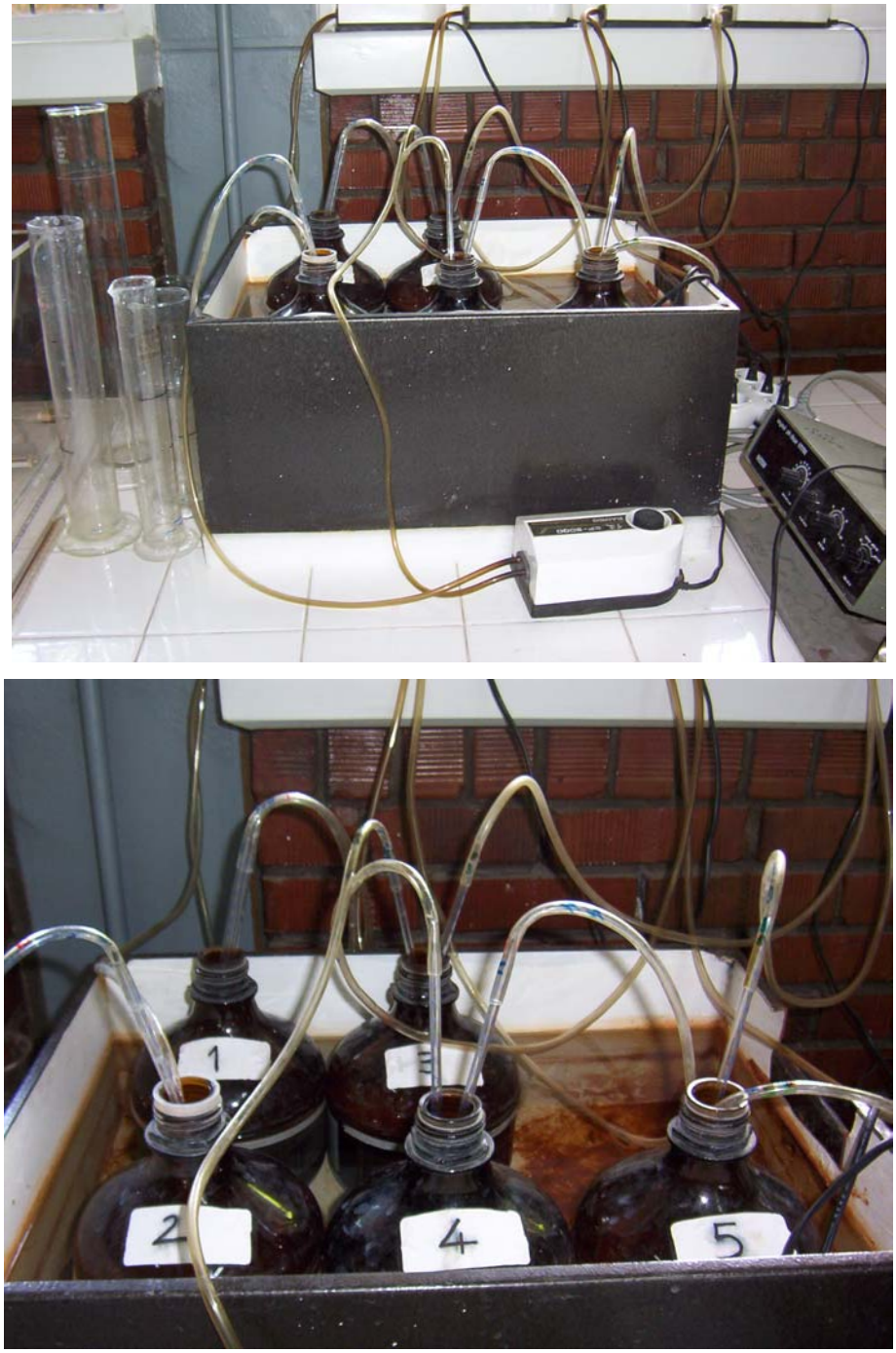

Figure 2. 2. Instantaneously fed SBRs

In the first set of experiments, peptone concentration was kept constant at 400 mg/L

and chlorophenol concentrations were gradually increased up to 200 mg/L 4-CP and 100 mg/L 2,4-DCP, which lasted around 5 months (Figure 3.1). The pH of the synthetic wastewater was 7.2-7.5 and it was 6.5-6.8 (depending on chlorophenol concentration) at the end of one-day cycle. The decrease was due to production of HCI as a result of chlorophenols degradation. When the pre-determined concentrations of chlorophenols were reached, the reactors were allowed three SRTs to reach steady state conditions, which was also checked measuring the COD and mixed liquor volatile suspended solids (MLVSS) concentrations at the end of three successive cycles. After observation of steady state-conditions, the reactors were sampled for the determination of chlorophenols, COD, 5-chloro-2-hydroxymuconic semialdehyde (CHMS) and MLVSS at different time intervals.

Also, in order to evaluate the degree of removal via adsorption, 4-CP and 2,4-DCP on biomass were extracted using 0.1 N NaOH following the centrifugation of sludge drawn from the reactors at the end of the cycle. Adsorption of 4-CP and 2,4-DCP on biomass were not detected throughout the reactors operation.

In the second set of experiments, the effect of biogenic substrate concentration on chlorophenol degradation kinetics was investigated. For that purpose, feed 4-CP and 2,4-DCP concentrations were kept constant at 200 and 100 mg/L, respectively, whereas, the feed peptone concentration was gradually decreased from 400 to 0 mg/L. When the pre-determined concentration of peptone was reached, reactors were allowed three SRTs to reach steady state condition, which was also checked measuring the COD and MLVSS concentrations at the end of three successive cycles. After observation of steady state-conditions, samples were drawn from the reactors for the determination of chlorophenols, COD, CHMS and MLVSS at different time intervals. As peptone concentration was decreased in the feed solution, the excluded amount of N was supplemented with increased concentration of NH_4CI not allow the culture to be N limited. When peptone was 100 mg/L, nitrification of NH_4CI at an elevated concentration led to the formation of H^+ ion in high enough concentration to decrease the pH value to around 6 at the end of the

cycle. To avoid creating a suboptimal environment for biomass activity, $NaHCO_3$ (300 mg/L) was added to the feed as an additional buffer to maintain a pH of 6.5-7 when the peptone concentration was 100 mg/L or less.

2.3.1.2. Semi-continuously fed Sequencing Batch Reactors (SBRs) experiments

A 2.5 L glass vessel with the 2.25 L of working volume was used as SBR. The reactor was operated in a water bath at 26±1 ºC and aerated using air pumps to keep dissolved oxygen concentration at least 3 mg/L. The SRT was kept constant at 10 d removing excess sludge from well mixed reactor content at the end of the each daily cycle. After settling, 2 L of the treated effluent was drawn and around 250 mL sludge was maintained in the reactor for the next cycle, which gives 1.125 d of HRT. Synthetic wastewater of 2 L was fed to the reactor using a peristaltic pump. A schematic diagram of SBRs used in the experiments is given in Figure 2.3.

Around 200 mL (1200 mg/L MLVSS) acclimated culture was used as initial inocula for SBRs. Acclimated culture used for the startup of SBRs was obtained from a instantaneously fed SBR receiving 220 mg/L 4-CP, 110 mg/L of 2,4-DCP and 300 mg/L peptone. Therefore, SBR operation was directly started with feeding high concentrations of chlorophenols (mixture of 220 mg/L 4-CP and 110 mg/L 2,4-DCP) without requiring further acclimation.

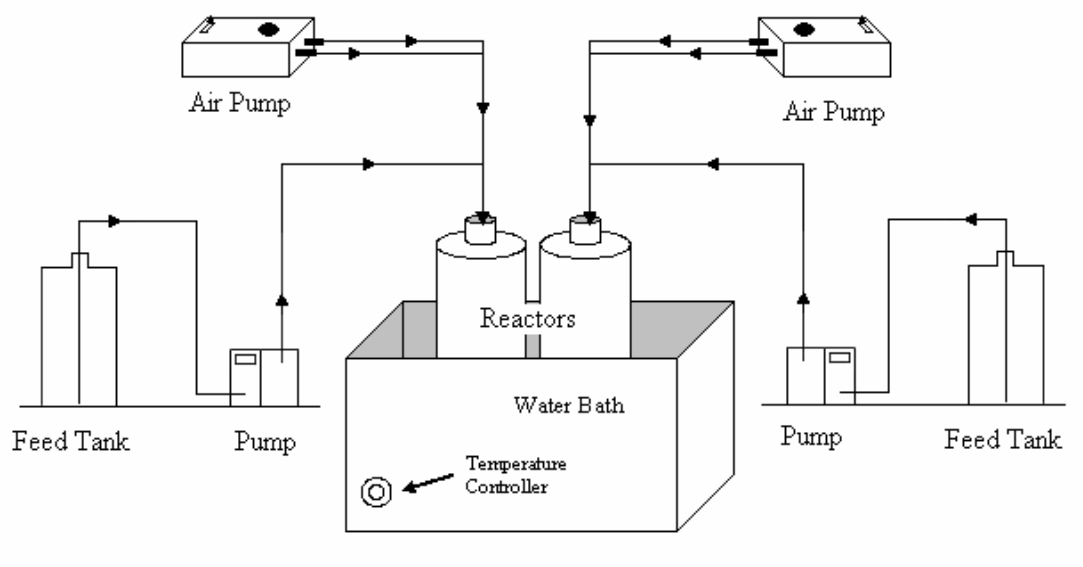

Figure 2. 3. A schematic Diagram of SBRs

Two sets of experiments were conducted with SBRs. In the first set of experiments, feed peptone (300 mg/L), 4-CP (220 mg/L) and 2,4-DCP (110 mg/L) concentrations were kept constant, while filling time was changed in order to determine the effect of it on the reactor performance. Each SBR cycle lasted one day consisted of fill (or feeding) (0-8 h), reaction (22-14 h) and settle-draw periods (2 h). The reactor was aerated in both fill and reaction periods.

The feed pH decreased from 7.0±0.2 to around 6.5±0.2 due to HCI formation during chlorophenol degradation and nitrification of NH_4CI. Though the pH dropped considerably, it was not low enough to adversely affect biomass activity and hence we did not adjust it.

During the experiments, samples were drawn from the reactors at predetermined time intervals and analyzed immediately for COD, chlorophenols and CHMS. MLVSS concentrations at the end of the cycles were also determined. Experiments were conducted when reactor reached steady-state conditions (three SRTs were allowed), which were also checked measuring the MLVSS and COD concentrations at the end of at least three successive cycles. Experiments were also run to

determine parametric variations during one cycle over two or three successive days. No significant variation occurred.

Following the centrifugation of the sludge from the reactors, 4-CP and 2,4-DCP on biomass were extracted using 0.1 N NaOH to evaluate the degree of removal via adsorption, which was not detected (detection limit of chlorophenols was 0.05 mg/L).

In the second set of experiments, the filling time was kept constant at 8 h throughout the experiments. In order to understand the effect of biogenic substrate concentration on the performance of the reactor, peptone concentration was gradually decreased from its initial value of 300 mg/L to 0 mg/L, while the chlorophenol concentrations were kept constant at 220 and 110 mg/l for 4-CP and 2,4-DCP, respectively. After any change in reactor operation, three SRTs were allowed to reach steady state conditions, which were also checked measuring MLVSS and COD concentrations at the end of at least three successive cycles. After ensuring the steady-state conditions, samples were drawn from the reactors at predetermined time intervals and analyzed immediately for COD, chlorophenols and CHMS. MLVSS concentrations at the end of the cycles were also determined.

In order to avoid creation of suboptimal environment for biomass activity, $NaHCO_3$ (300 mg/L) was added to the feed as an extra source of buffer to keep pH between 6.5 and 7 when peptone concentration was 100 mg/L or less.

2.3.2. Batch Experiments

Batch experiments were conducted in 500 mL erlenmeyer flasks stoppered with cotton plugs. The working liquid volume was 250 mL. All experiments were run in an orbital shaking incubator set at 200 rpm and 26±1°C. Biomass samples taken from FEDM and FED4 were washed two times with distilled water in order to remove organics adsorbed on biomass. Then, cultures were resuspended in distilled

water and parallel batch reactors were seeded to have biomass concentration around 200 mg/L. Batch experiments were also conducted with pure species isolated from acclimated mixed cultures in order to compare the chlorophenols degradation rate of pure cultures with those of mixed cultures.

In addition to inorganic components, reactors were supplemented with varying concentrations of 4-CP and/or 2,4-DCP as sole carbon and energy sources. The same amount of nitrogen in the excluded proteose-peptone (Oxoid) was added to batch reactors as NH_4CI.

Reactors not receiving biomass were also operated as control at the same conditions to monitor of chlorophenols removal via volatilization. Loss of 4-CP and 2,4-DCP due to evaporation was negligible (<3%).

During the batch experiments, each flask was sampled at predetermined time intervals. A 5 mL sample was filtered through a 0.45-µm filter and subjected to 4-CP and 2,4-DCP analyses. MLVSS concentrations were measured at the start and end of the reactor operation. Also, variations of pH during incubation were monitored.

At the end of the experiments, batch flasks were allowed to settle for 30 minutes and optical density (OD_{550}) of supernatants were measured at 550 nm. The observed OD_{550} values were converted to MLVSS concentrations using the linear relationship, and the floc formation ability of cultures was evaluated by the index of flocculation (IF), which is defined as (Soda et al., 1999):

$$IF(\%) = 100\frac{X_{total} - X_{sus}}{X_{total}} \qquad (2.1)$$

X_{total} indicates the total biomass concentration and X_{sus} is the biomass concentration in supernatant after 30 minutes settling.

Through these experiments, kinetics of 4-CP and 2,4-DCP degradation were deeply investigated. Following the determination of the model giving the best fit to the experimental data, kinetic constants (such as, $q_{m,}$ K_s, K_I) were determined. These kinetic constants were then used to model the chlorophenol degradation in SBRs.

Kinetic constants were determined using the nonlinear least squares technique with the help of MATLAB 6.5. Observed differential equations were numerically solved using POLYMATH 4.02. The program used the Runge-Kutta-Fehlberg (RFK) numerical integration routine.

2.3.3. Rotating Biological Contactors (RBC) Experiments

A two-stage bench-scale RBC reactor (Figures 2.4-2.6) was used to study biodegradation of 4-CP and 2,4-DCP. The diameter of each disk was 18 cm and the total surface area for biomass attachment was 0.44 m^2 per stage. Around 40% of disks were submerged in water. Total liquid volume in the reactor was 7.0 L and disk surface area per unit liquid volume was 126 m^2/m^3. The disks were made of perforated aluminum sheet because of its ease of fabrication, low cost and availability. Disks were mounted on a 25 mm diameter rigid stainless steel shaft and an inducing motor was used to rotate disks. Tank in which disks were housed was also made of stainless steel. The reactor was fed with a synthetic medium using a peristaltic pump and total HRT was kept constant at 0.70 d. The reactor was operated at room temperature (25±3ºC) and a constant disk rotation speed of 5 rpm. A control panel (Photo 2.3) was used to adjust the disk rotating speed. On day 60 and onward, the liquid phase of the reactor was aerated to have at least 3 mg/L of dissolved oxygen.

For biofilm development, reactor was filled with around 7.0 L settled sewage and it was operated in a batch manner for around 4 days. Then, the reactor was fed with synthetic wastewater devoid of chlorophenol in a fed batch manner until a slime

layer on the discs has been observed. After development of biofilm, the reactor was operated in a continuous manner.

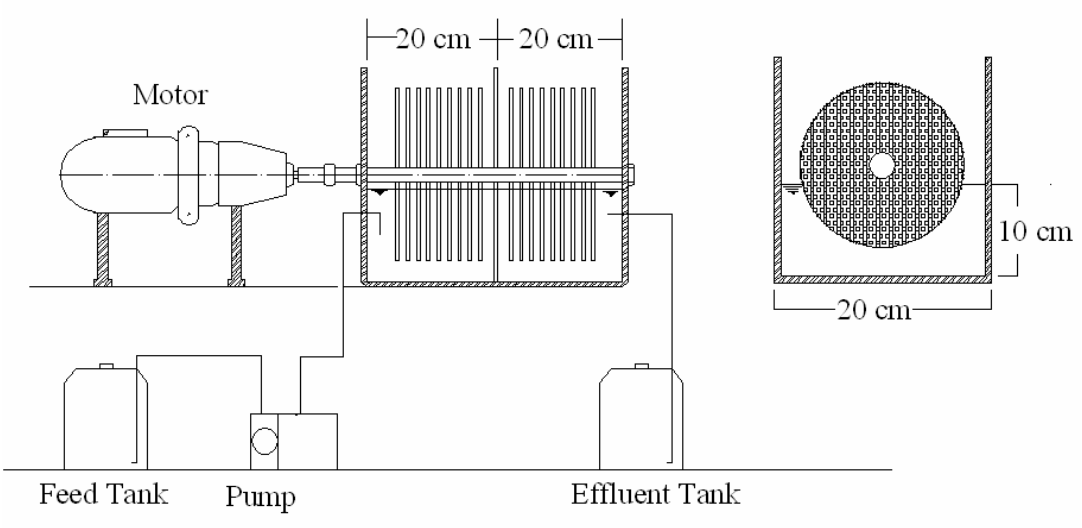

Figure 2.4. A schematic diagram of RBC

The study consists of five successive steps. First of all, the reactor performance was investigated in the absence of chlorophenols at different rotational speed of disks to optimize the operational parameters. Second part of the study was devoted to the acclimation of microorganisms to chlorophenols. To that purpose, feed chlorophenols concentrations were gradually increased, while, peptone concentration was kept constant at 400 mg/L. The reactor was fed with the mixture of 4-CP and 2,4-DCP and the concentration of 2,4-DCP was always kept as one half of 4-CP concentration. In the third step, after having an acclimated culture, peptone concentration was gradually decreased keeping the 4-CP and 2,4-DCP concentrations constant at 220 and 110 mg/L, respectively. In the fourth step, deacclimation and reacclimation of attached biomass was studied. In the deacclimation study, reactor was fed with 400 mg/L peptone in the absence of chlorophenols for around 3.5 months and then the biomass was reacclimated to chlorophenols mixture. Lastly, the effect of shock chlorophenols loadings, in the absence of peptone was extensively evaluated. The HRT of the reactor was kept at 0.7 d (hydraulic loading rate = 0.01136 $m^3/m^2.d$) throughout the study.

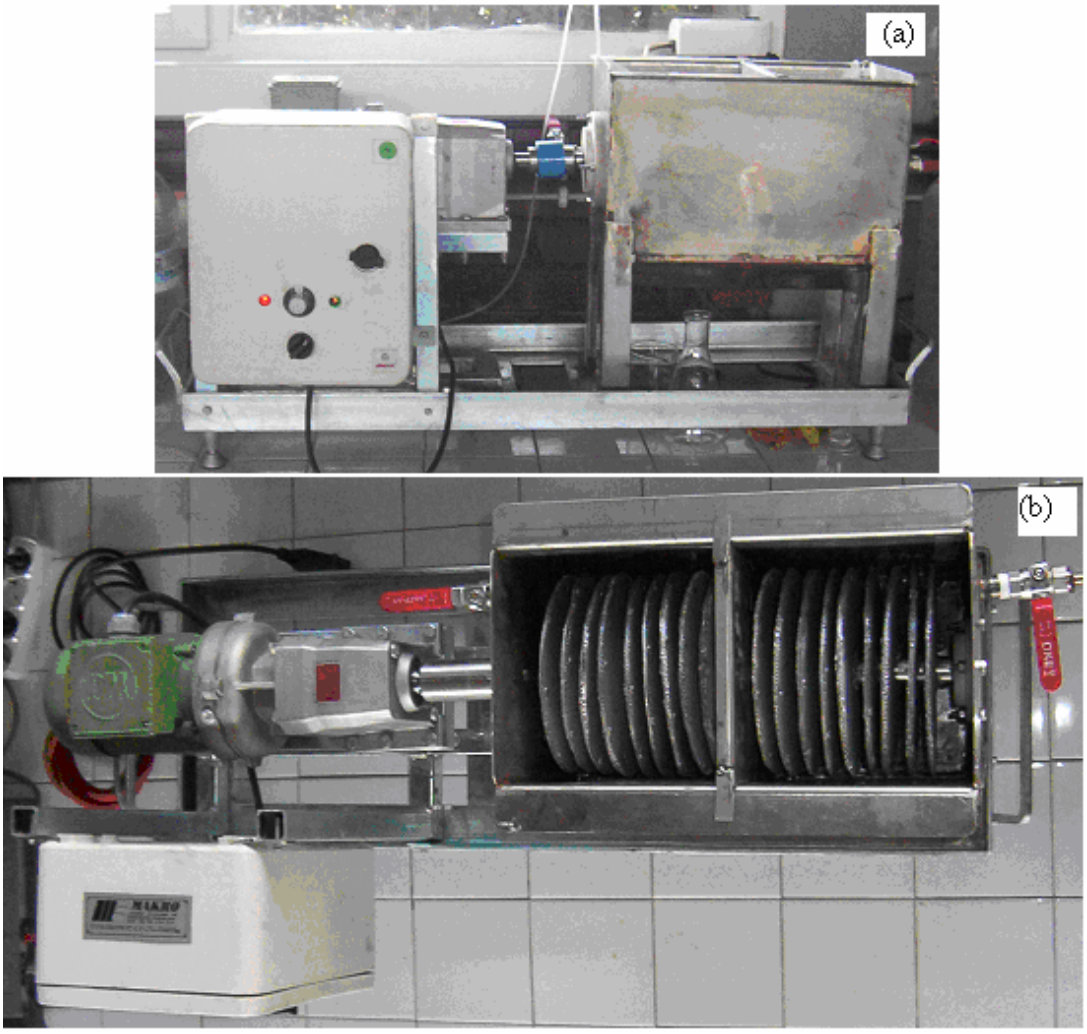

Figure 2.5. RBC used in the experiments. (a) cross view, (b) over view

When any parameter was changed to investigate the effect of corresponding parameter on the performance of the reactor, the others were kept constant. After any disturbance on the reactor operation, at least 10 days were allowed to the reactor to obtain steady-state data, which was also checked measuring the chlorophenols and COD. Samples were regularly drawn from feed tank and the effluents from stage 1 and 2 to measure chlorophenols and COD.

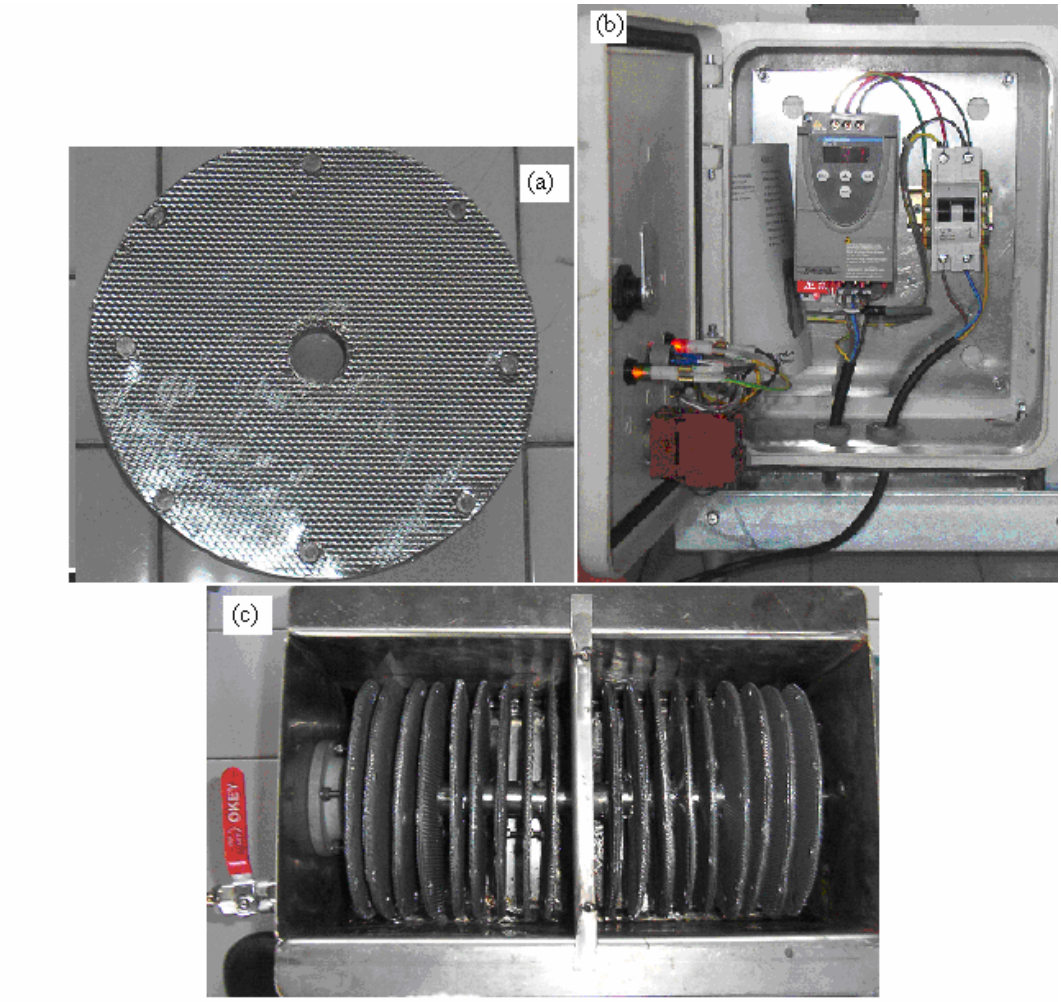

Figure 2.6. Support medium (a), control panel (b) and RBC stages (c)

2.3.4. Isolation of pure cultures

In the isolation of bacteria, the procedure given by Wang et al. (2000) was used. Mixed culture samples, obtained from reactors, were diluted to have maximum 30 colonies on solidified agar medium. The growth medium used in instantaneously fed SBRs was solidified and used during the isolation of pure strains. Agar medium was supplemented with mixture of 50 mg/L 4-CP and 25 mg/L 2,4-DCP. Streaked agar plates were incubated at 30 °C. After development of colonies, single colonies were selected and streaked to new agar plates. This procedure was repeated at least five times in order to ensure the purity of culture. API 20 NE identification kits were used to define the isolated cultures.

2.4. Analytical Techniques

During the 4-CP degradation, a yellowish color was accumulated (Figure 2.7) due to production of CHMS, indicating –meta cleavage of 4-CP (Sae´z and Rittmann, 1991; Wang and Loh, 1999; Farrell and Quilty, 1999). CHMS concentration, the –meta cleavage product of 4-chlorocatechol, was monitored by measuring OD at 380 nm (Farrell and Quilty, 1999) at which CHMS gives maximum absorbance (Figure 2.8). MLVSS and chloride concentrations (titrimetric method) were determined according to Standard Methods (APHA, 1995). COD was measured using Hach COD vials according to the EPA approved reactor digestion method (HACH Water Analysis Handbook, 1992). Accordingly, after 2 h digestion, sample CODs were directly read using Hach 45 600-02 spectrophotometer (Hach Co. Loveland, Co., USA).

A high performance liquid chromatograph (HPLC; Shimadzu, LC-10AT, Tokyo, Japan) was used to determine the 4-CP and 2,4-DCP concentrations. The HPLC was equipped with a Nucleosil C18 column (4.6 mm × 250 mm), LC-10Atvp solvent delivery module, an SC/L0Avp system controller and a SPD-10Avp UV-VIS detector set at 280 nm. Retention times for 4-CP and 2,4-DCP were 7.5 and 12 minutes, respectively. A mixture of methanol (60%), pure water (38%) and acetic acid (2%) was used as solvent at a flow rate of 0.75 mL/min. The sample injection volume was 20 μL.

The API 20 NE strip consists of 20 micro tubes containing dehydrated media and substrates. The conventional tests are inoculated with a saline bacterial suspension which reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The assimilation tests are inoculated with a minimal medium and the bacteria grow if they are capable of utilizing the corresponding substrate. The reactions are read according to reading table supplied by BioMeriux Co. and the identification is obtained by referring the API identification software (Biomeieux, Identification

And Susceptibility Testing manual methods, 1996). In Figure 2.9, two strips, which are ready for reading, are illustrated.

All the experiments and measurements were done at least in duplicate and arithmetic averages were taken throughout the data analysis and calculations. Coefficient of variations (CV) for COD and MLVSS measurements was less than 10%, whereas it was less than 5% for chlorophenols measurements.

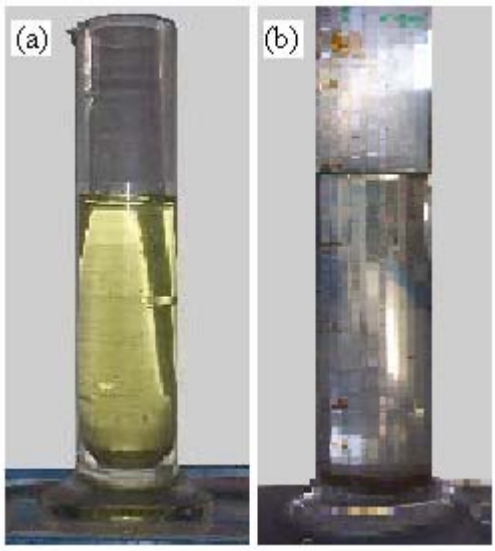

Figure 2.7. Coloration of medium due to production of CHMS (a), medium after complete removal of CHMS (b)

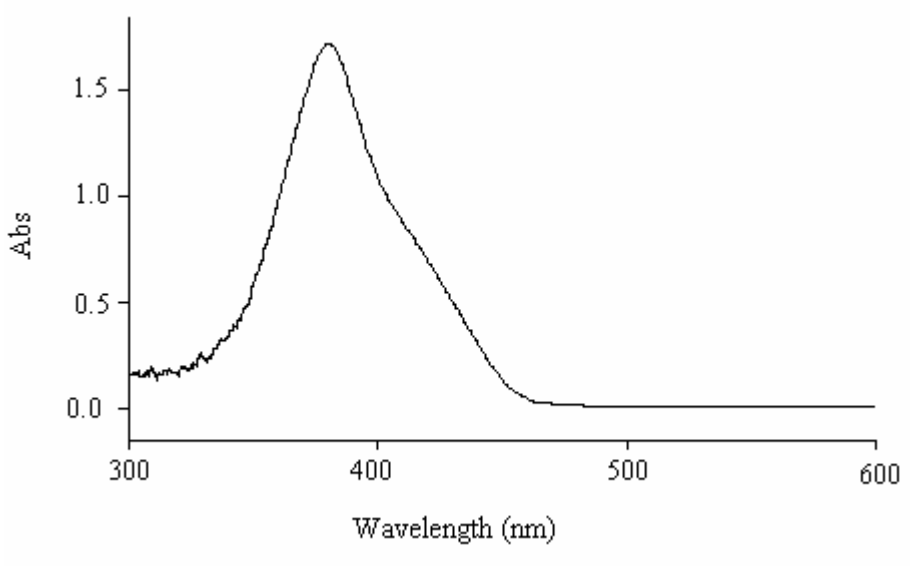

Figure 2.8. UV scan of CHMS

Figure 2.9. Two API 20NE strips after 24 h of inoculation

CHAPTER 3

RESULTS AND DISCUSSION

Experiments were conducted in suspended and attached growth reactors. Batch and SBRs were used as suspended growth reactors. Biodegradation kinetics of chlorophenols were studied in batch reactors and the observed biokinetic coefficients were tested in instantaneously fed SBRs operated at different chlorophenols and biogenic substrate concentrations. Semi continuously fed SBRs were used to investigate the effect of filling time on reactor performance and to optimize operational conditions. Chlorophenols degradation at different biogenic substrate concentrations was also comparatively studied in RBC as an attached growth reactor.

3.1. Chlorophenols Degradation In Instantaneously Fed SBRs

Despite the recalcitrant nature of chlorophenols, there are still some efforts toward their biological way of treatment with specialized culture conditions because of economical reasons and in expectation of few byproducts. The microorganisms used are usually aerobes, including *Pseudomonas* sp., *Alcaligenes* sp., *Azotobacter* sp., *Rhodococcus* sp., and *Cryptococcus* sp. (Kim et al., 2002).

Knowledge of microbial growth and substrate utilization kinetics is important for the prediction of the fate of organic compounds in natural and engineered

environments (Grady et al., 1996). Mixed microbial communities are used by environmental engineers (Templon and Grady, 1988); therefore, the kinetics of microbial growth and substrate utilization should be studied using mixed culture in order to produce data that can be used in the design of real wastewater treatment plant. The problem is that the conditions imposed prior to the kinetic test have great impact on the observed kinetics. Continued cultivation of a mixed culture will result in selection of some members of the community, which affect the observed kinetic parameters. Also, physiological adaptation (change in macromolecular composition) of bacteria to their changing environment affects the observed kinetics parameters as physiological state determines the rate of enzyme synthesis (Grady et al., 1996). Another significant point is that many researchers claim that only a specific population of microbial community is responsible for the degradation of particular compounds, especially if degradation require unique pathways (e.g. synthetic organic chemicals (SOCs)). In the literature, generally total biomass concentrations have been used for growth and substrate degradation kinetics as the measurement of competent biomass fraction is tedious and time consuming. However, models based on total biomass concentrations will fail to predict the performance of the bioreactor if it receives SOCs at varying concentrations (Magbanua et al., 1998). Also, the use of total biomass concentration in kinetic studies may be one of the reasons in the observation of considerable variability in the values of the kinetic parameters on growth and substrate removal. Some researchers used the fraction of COD contributed to the feed by that compound in the calculation of competent biomass fraction based on the assumption that if specialists are responsible for degradation of a particular compound, the specialists grows only on SOC of interest (Magbanua et al., 1998; Hu et al. (2005b), Hu et al., 2005; Ellis et al., 1996; Tomei et al., 2003). Magbanua et al. (1998) reported that generally the influent COD fraction underestimated the competent biomass fraction by a factor of about two, which may be due to the convergence of degradation pathways for different SOCs. Contrary to this finding, Hu et al. (2005b) reported that the competent fraction for 4-CP (0.01-0.03%) in sequencing batch reactor receiving multicomponent feed was two order magnitudes

lower than the contribution of 4-CP to influent COD (2.0%). Although it seems that the use of substrate specific MPN method is better approach compared to the use of COD fraction, this method may also produce results with high coefficient of variations (69–130%) due to long term variations in the microbial community of bioreactor (Magbanua et al., 1998). Therefore, use of COD fraction to roughly approximate competent biomass fraction is more realistic than assuming all biomass is responsible for SOCs degradation (Magbanua et al., 1998).

Another significant point is that as wastewater treatment systems receive multicomponent feed, the degradation of any individual organic constituent is impacted by the concurrent utilization of other substrates. The biodegradation of a recalcitrant compound may be synergistically or antagonistically affected by the presence of other compounds. The enhanced biodegradation may be due to increased biomass concentration with the utilization of other compounds or the induction of degradative enzymes, whereas, if the biomass may shift their major carbon source from recalcitrant to the relatively biodegraded compound, the utilization rate of recalcitrant compound may decrease (Lu and Tsai, 1993; Lu and Spetiel, 1988). Also, a recalcitrant compound may adversely affect the biodegradation of others inhibiting biomass growth or adversely affecting the enzymes production or reaction. In this context, Lu and Tsai (1993) reported that 2,4-DCP enhanced the biodegradation of a less chlorinated but recalcitrant compound, 2-Chlorophenol (2-CP). The same authors also reported that monochlorophenols, 3-Chlorophenol (3-CP), 2-CP and 4-CP retarded the biodegradation of 2,4-DCP. The knowledge on how substrate interacts is important to develop mathematical models in the design of a wastewater plant and estimate its performance. Although there are some studies on the effect of one chlorophenol presence on the degradation of other type of chlorophenols (Lu and Tsai, 1993; Quan et al., 2005), there is little or no kinetic study available in the literature.

Therefore, the goals of this set of experiments were to investigate chlorophenols degradation kinetics at different feed 4-CP concentrations in the presence and

absence of 2,4-DCP through long term operated instantaneously fed SBRs in order to give culture enough time for the selection of different dominant species and the changes in the physiological state of the cells involved. By this way, the use of competent biomass fraction in the degradation kinetics of chlorophenols at varying concentrations was deeply investigated. Also, dominant species in culture were determined after acclimation to chlorophenols.

One of the reactors (FED4) received 4-CP only, whereas, the other reactor (FEDM) received mixture of 4-CP and 2,4-DCP. The 2,4-DCP concentrations in the feed of FEDM was kept always one half of 4-CP concentrations. Chlorophenols were added to the feed at low concentrations and the concentrations were gradually increased up to 200 mg/L 4-CP and 100 mg/L 2,4-DCP, which lasted around 5 months (Figure 3.1). The pH of the synthetic wastewater was 7.2-7.5 and it was 6.5-6.8 (depending on chlorophenol concentration) at the end of one-day cycle. The decrease was due to production of HCI as a result of chlorophenols degradation.

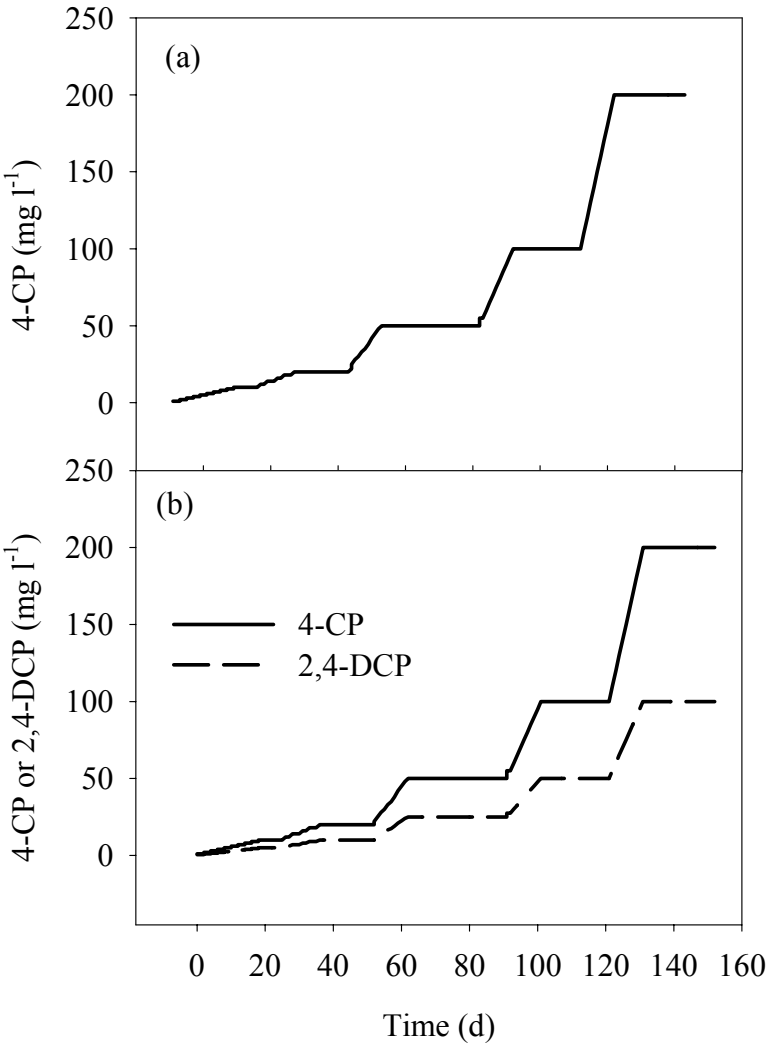

Figure 3.1. Feed 4-CP and 2,4-DCP in instantaneously fed SBRs

3.1.1. Chlorophenols Degradation at Varying Feed Concentrations

First of all, degradation of peptone in the absence of chlorophenols was studied in order to reveal the effect of chlorophenols addition on the yield coefficient (Y) and COD removal efficiency (Figure 3.2). It was observed that in the absence of chlorophenols the effluent COD concentration was around 28 mg/L corresponding to 94% removal efficiency. Also, Y value was observed to be 0.425±0.008 mg MLVSS/ mg COD.

An adequate acclimation of activated sludge has been reported to be necessary for the efficient degradation of chlorophenols (Moreno and Buitron, 2004) as unacclimated one has little or no degradation ability (Sahinkaya and Dilek, 2002 and 2005; Buitron et al., 1998). In this context, in order to have a good acclimated culture, concentrations of 4-CP and 2,4-DCP were increased in stepwise small increments (Figure 3.1).

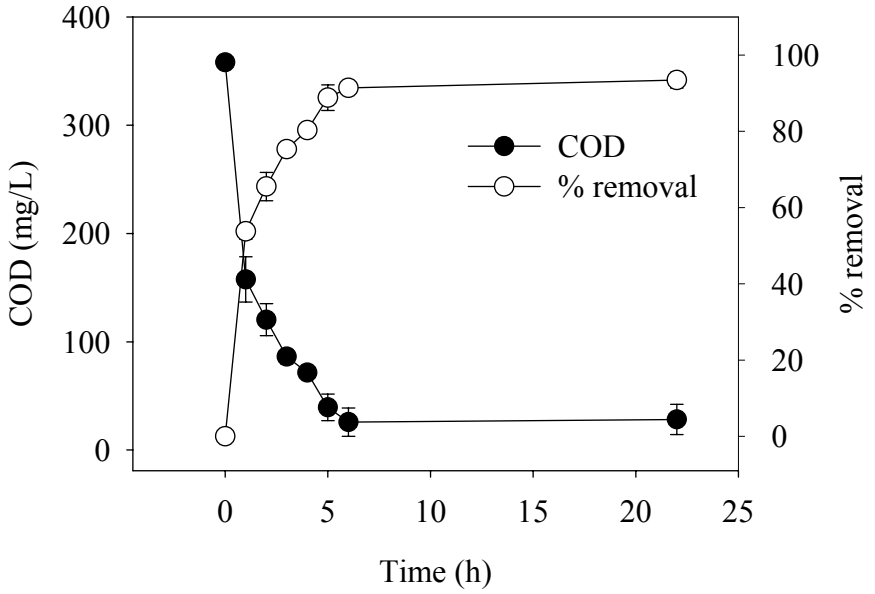

Figure 3.2. Time course variation of COD and corresponding removal efficiencies in the absence of chlorophenols

Metabolization of 4-CP results in production of 4-chlorocatechol and -meta cleavage of which causes the formation of CHMS, then the metabolization of CHMS leads to the production of pyruvic acid and chloroacetic acid. Chloroacetic acid may then be dehalogenated to form glycolate, which may be utilized along with pyruvic acid in TCA cycle (Farrel and Quilty, 1999).

During the operation of the reactors with an inlet concentration of 10 mg/L 4-CP in the presence and absence of 2,4-DCP, despite complete disappearance of chlorophenols, a yellowish color was observed at the effluent of reactors on day 21. The intermediate gave the maximum absorbance at 380 nm and have the all characteristics of CHMS, which is the –meta cleavage product of 4-chlorocatechol. The observation of the intermediate is the best evidence of meta-cleavage of 4-chlorocatechol, which is the first product in 4-CP degradation pathway. The observation of higher quantities of metabolite at the beginning of the acclimation was also reported in the study of Moreno-Andrade and Buitron (2004). Although disappearance of 4-CP was observed, the production of the intermediate proves that 4-CP was not degraded completely. However, it was observed that culture also acclimated to the intermediate and its concentration gradually decreased at the end of each daily cycle after the first observation of the intermediate on day 21 (Figure 3.3). Although, higher absorbance value, which means higher concentration of CHMS was observed in the effluent of the reactor fed with only 4-CP, complete disappearance of the intermediate took shorter time compared to the reactor fed with mixture of 4-CP and 2,4-DCP. So, the presence of 2,4-DCP slowed down the acclimation of the culture to the intermediate. Although, degradation of CHMS was achieved in our study, it had been widely reported as being a dead-end metabolite, especially for pure culture studies (Tarighian et al., 2003; Saez and Rittmann, 1993; Hill et al., 1996). Therefore, mixed cultures are particularly important when emphasis is placed on complete mineralization of toxic organics to CO2 and also, it is well known that acclimation of mixed culture can cause increase in the ability of cells to degrade toxic organic compounds.

After day 25, complete degradations of chlorophenols and the CHMS have been observed throughout the reactors operation. The change in observed yield coefficients, MLVSS concentrations and COD removal efficiencies at different initial 4-CP concentrations are shown in Figure 3.4. The Y value in the absence of chlorophenol was observed to be around 0.43 mg MLVSS/mg COD, which decreased with the addition of chlorophenols. The value of Y decreased with

increasing feed chlorophenol concentrations of FEDM. The trend was different in the case of FED4 as the addition of 4-CP caused a sharp decrease in Y value from its baseline value of 0.43 to 0.26 mg MLVSS/mg COD and after this point, Y value remained almost constant with an average value of 0.235±0.007 mg MLVSS/mg COD. Therefore, a certain effect of 2,4-DCP on Y was obvious. Similar results were also observed in batch studies (Sahinkaya and Dilek, 2002 and 2005). The average COD removal efficiency in the absence of chlorophenol was 94% and no adverse affect of chlorophenol addition on COD removal efficiency have been detected. The observation of low COD concentrations at the effluent of the reactors is an indication of complete chlorophenol removal, which was also verified with chlorophenols measurements using HPLC and CHMS measurements. Additionally, chloride ion measurements indicated an average 101±7% chlorophenols removal at the end of the cycle.

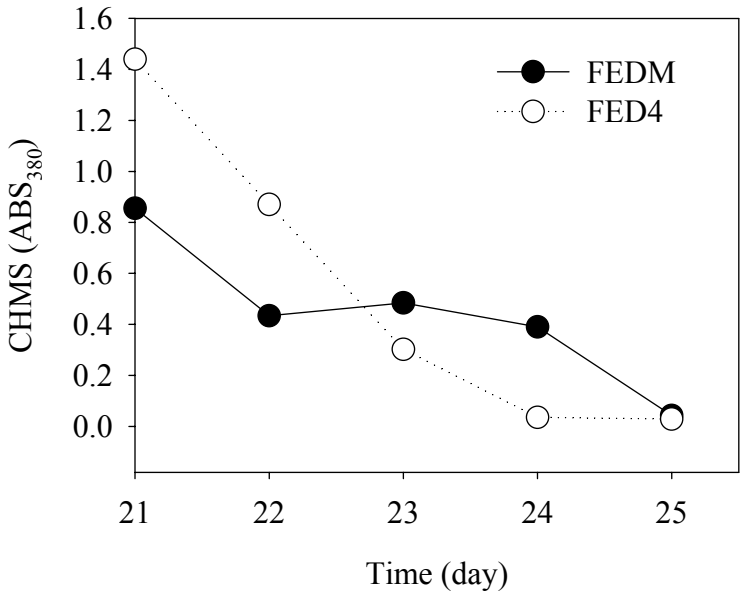

Figure 3.3. CHMS concentrations in the effluent of reactors fed with 4-CP (10 mg/L) and mixture of 4-CP (10 mg/L) and 2,4-DCP (5 mg/L)

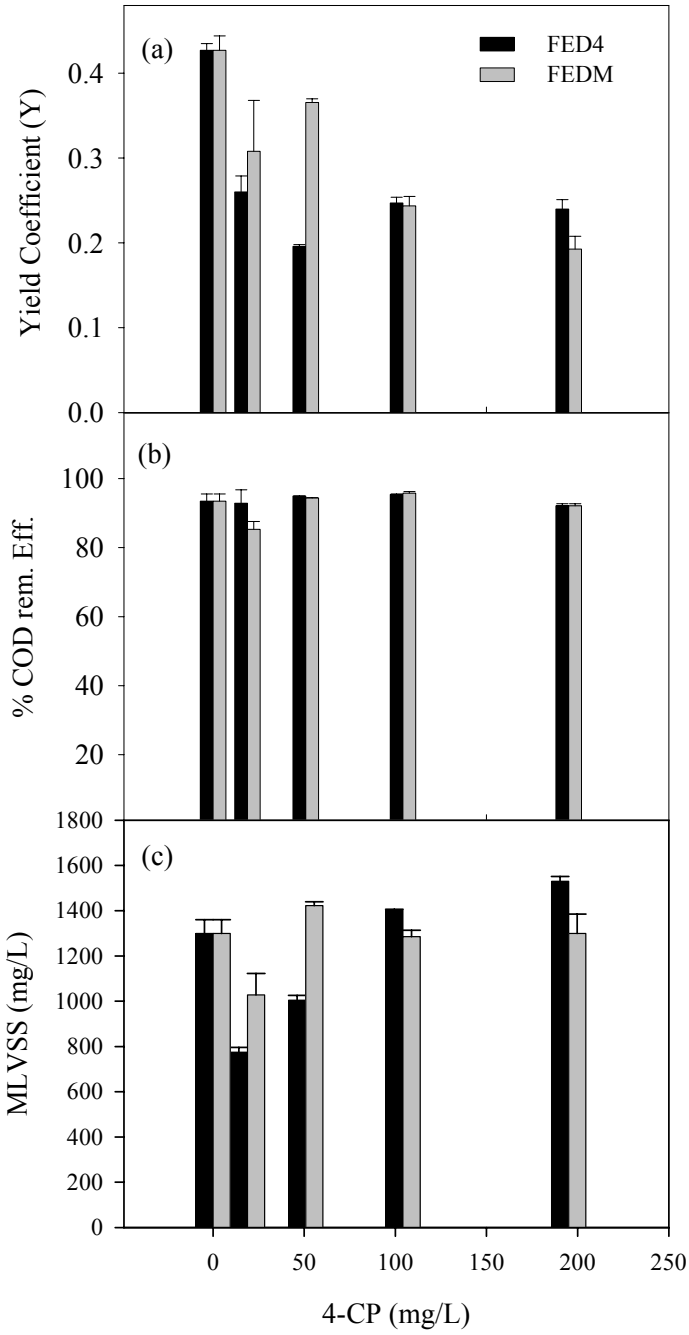

Figure 3.4. Change of Y, COD removal efficiencies and MLVSS concentrations with respect to feed 4-CP concentrations in FED4 and FEDM

3.1.1.1. Kinetics of Chlorophenols Degradation

The feed concentrations of chlorophenols were increased in stepwise small increment to acclimate biomass to high chlorophenols concentrations. When the highest chlorophenols concentrations were reached in the FED4 (200 mg /L 4-CP) and FEDM (200 mg/L 4-CP and 100 mg/L 2,4-DCP), biomass samples were drawn in order to conduct batch assays. Batch experiment results revealed that Andrews substrate inhibition model could be used to predict the degradation rates of 4-CP (batch assays will be discussed later). Also, it was assumed that only specialist biomass are responsible for chlorophenols degradation and the fraction of specialist biomass was estimated as the contribution of chlorophenols to the feed on COD basis. Therefore, competent biomass concentration in the community will increase as the feed chlorophenol concentration is increased (Magbanua et al., 1998; Hu et al., 2005a and b; Ellis et al., 1996). Based on these assumptions, the following equation was used to describe 4-CP degradation kinetics in the absence of 2,4-DCP;

$$\frac{d(S)}{d(t)} = -\frac{q_m SXa}{K_s + S + S^2/K_i} \qquad (3.1)$$

Where, q_m is maximum specific 4-CP degradation rate (mg 4-CP/(gMLVSSs.h)), K_s and K_i are half saturation and self-inhibition constants, respectively, X is total biomass concentration (g MLVSS/L) and lastly a is the COD fraction of chlorophenols in the feed of SBRs. Hence, the observed equation for 4-CP degradation in FED4 was;

$$\frac{d(S)}{d(t)} = -\frac{92SXa}{1.104 + S + S^2/194.4} \qquad (3.2)$$

The results of FEDM experiments, which will be discussed later, showed that 2,4-DCP competitively inhibited the degradation of 4-CP as the presence of 2,4-DCP

retarded the initiation of 4-CP degradation and the removal of 4-CP in the presence of 2,4-DCP was observed to be almost negligible. Similar result was also reported by Quan et al. (2005). Therefore, the proposed model is;

$$\frac{d(S)}{d(t)} = -\frac{q_m SXa}{(1+\frac{I}{K_{ii}})K_s + S + S^2/K_i} \qquad (3.3)$$

Where, I is 2,4-DCP concentration, K_{ii} is inhibition coefficient of 2,4-DCP on 4-CP degradation and *a* is the fraction of 4-CP and 2,4-DCP in the feed on COD basis. It should be noted that in the determination of competent biomass fraction having ability to degrade 4-CP, total fraction of 4-CP and 2,4-DCP in the feed was taken into consideration. The reason is that culture with the ability to degrade one type of chlorophenol may also have ability to degrade another structurally similar chlorophenol. Although such a generalization may not be true for pure cultures or sometimes for mixed cultures, in our case the validity of the assumption has also been proved in our previous studies (Sahinkaya and Dilek, 2002 and 2005). The observed competitive inhibition also claims that the same enzymes are responsible for the degradation of both chlorophenols, therefore, it is reasonable to assume that the same community is responsible for the degradation of 4-CP and 2,4-DCP.

In order to determine the $q_{m,}$ K_s and K_i values in Equation 3.3, batch experiments were carried out at different 4-CP concentrations in the absence of 2,4-DCP (Section 3.2) using biomass drawn from FEDM. After determination of $q_{m,}$ K_s and K_i, K_{ii} value was determined using the results of FEDM when it received 200 mg/L 4-CP and 100 mg/L 2,4-DCP. The best fitting value of K_{ii}, which is competitive inhibition coefficient of 2,4-DCP on 4-CP degradation, was observed to be 0.17 mg/L. The relatively small value of K_{ii} indicates a high toxic effect of 2,4-DCP on 4-CP degradation. Therefore, the resulting equation is as follows;

$$\frac{d(S)}{d(t)} = -\frac{248.49SXa}{(1+I/0.17)34.98 + S + S^2/79.74} \qquad (3.4)$$

Simply zero order degradation model was observed to be good enough to predict 2,4-DCP degradation kinetics in the presence of 4-CP and the degradation rate of 2,4-DCP can be expressed as;

$$\frac{d(I)}{d(t)} = -k \qquad (3.5)$$

Combination of the equations 3.4 and 3.5 yielded;

$$\frac{d(S)}{d(t)} = -\frac{248.49SXa}{(1+(I_0 - kt)/0.17)34.98 + S + S^2/79.74} \qquad (3.6)$$

The observed equations were solved with the help of POLYMATH 4.02 assuming that the biomass concentration was constant during the reaction time as the initial biomass concentration is very high and the average difference between initial and the final value was observed to be around 10%.

In order to evaluate the efficacy of competent biomass in the degradation models, we have estimated 4-CP concentration profile in FED4 receiving 50 mg/L 4-CP assuming specialists (Model 1) or generalists (all biomass) (Model 2) was responsible for 4-CP degradation (Figure 3.5). As clearly seen from the figure, Model 1 gave much better fit compared to Model 2, which showed that considering specialist biomass in the kinetic study is better approach, supporting the observation of Ellis et al. (1996). Hence, in the rest of our kinetic studies, specialist biomass concentration was considered.

It is also necessary to note that Ellis and Eliosov (2004) reported that the feed COD fraction gave better approximation of competent biomass fraction when the test compound's fraction in feed was high (>1%). In the literature, the efficacy of using

feed COD fraction as an indicator of competent biomass fraction was generally studied in a multisubstrate feed in which the fraction of test compound in the range of (0.1-5%) (Ellis and Eliosov, 2004; Magbanua et al., 1998; Hu et al. (2005b)). In our study, however, the fractions of chlorophenols in FED4 and FEDM varied as 10-45% and 12-53% in respective order. Therefore, based on the observation of Ellis and Eliosov (2004), the accurate estimation of competent biomass fraction using the feed COD contribution is more likely in our study due to the high fraction of chlorophenols in the feed.

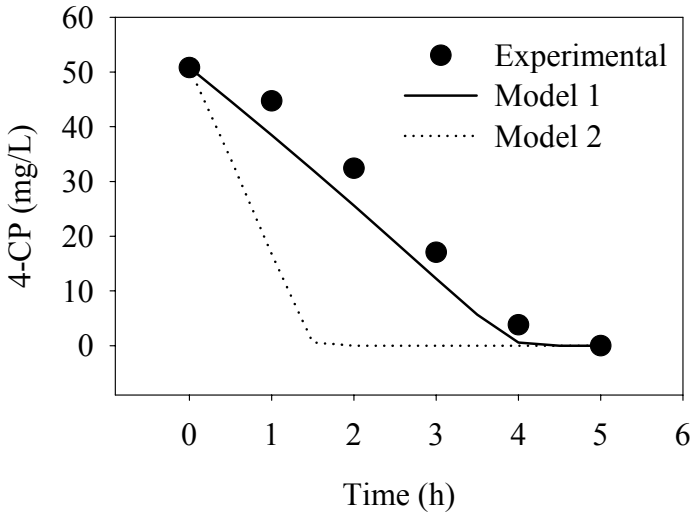

Figure 3.5. The prediction of 4-CP concentration profile considering competent (Model 1) and total biomass concentrations (Model 2)

The experimental results of FED4 receiving 100 and 200 mg/L of 4-CP were depicted in Figure 3.6. Complete 4-CP and high COD removals were observed in the reactor for both conditions. During the removal of 4-CP, CHMS concentration increased simultaneously and the highest concentration reached when 4-CP was just completely removed (Figure 3.6a and c). The COD concentration when CHMS concentration was at its maximum is close to that observed at the end of cycle (around 30 mg/L and 50 mg/L for 100 and 200 mg/L 4-CP, respectively), which indicated that the accumulated concentration of CHMS is much lower than its

theoretical COD value if all 4-CP was directly converted to CHMS (around 125 and 250 mg/L for 100 and 200 mg/L 4-CP, respectively). Using the extinction coefficient given by Farrel and Quilty (1999), the concentration of CHMS, when it reached its highest value, was calculated as 9.7 mg/L (8.77 mg/L COD) and 9 mg/L (8.16 mg/L) for 100 and 200 mg/L 4-CP, respectively. Therefore, CHMS accumulation was only 7.76 and 3.6% of calculated theoretical values for 100 and 200 mg/L 4-CP. Therefore, it can be concluded that simultaneous removal of 4-CP and CHMS occurred in the reactor although CHMS removal rate was slightly lower, which caused a slight accumulation in the medium. Another significant point is that the accumulated amount of the CHMS is nearly the same for 100 and 200 mg/L 4-CP concentrations. This may be due to enhanced rate of CHMS removal with increasing competent biomass fraction.

The developed model fitted well to the experimental data (Figure 3.6a) for both feed 4-CP concentrations. Although the model parameters were determined when reactor received 200 mg/L 4-CP, the model is applicable for different feed 4-CP concentrations (Figure 3.5 and 6a). Hence, the observed good fit between the experimental data and the model solution validated the correctness of the assumption in the calculation of competent biomass fraction.

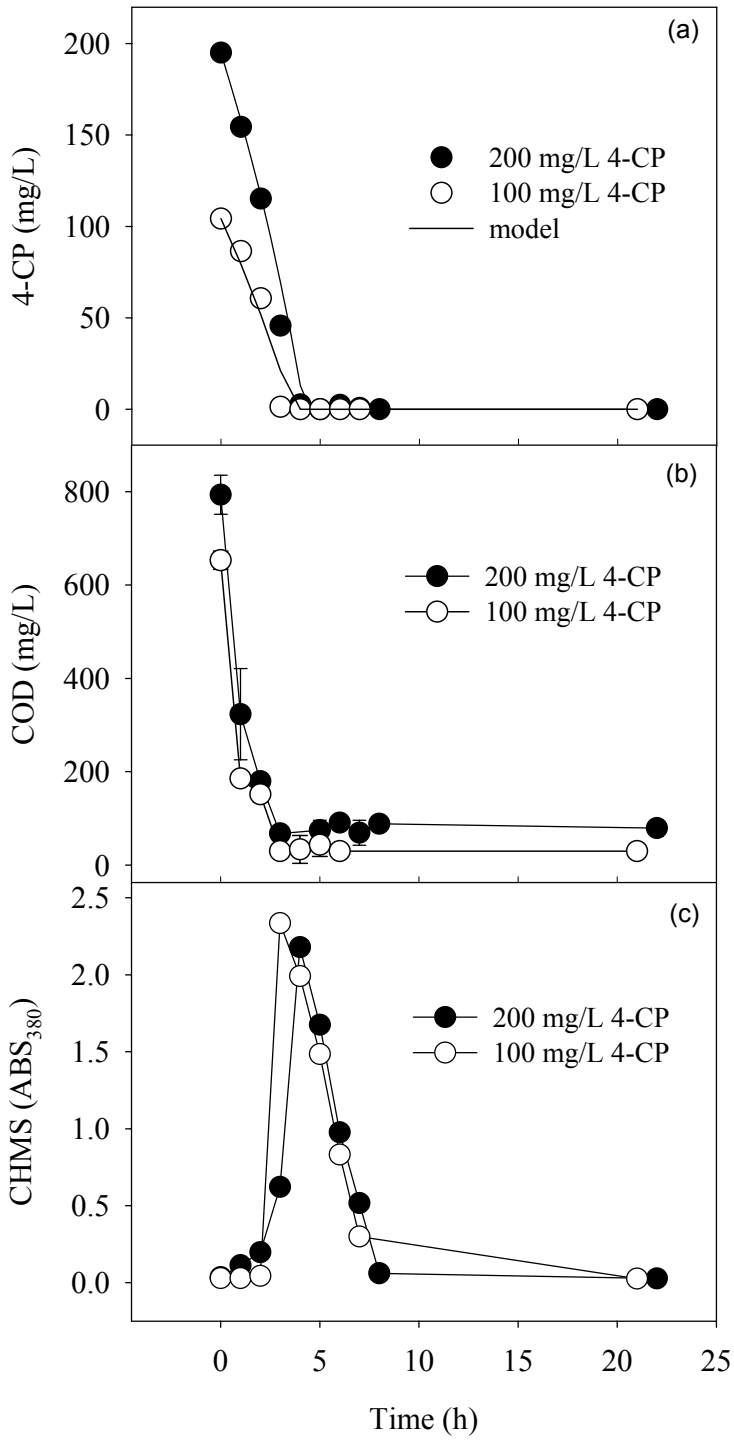

Figure 3.6. Experimental data and model results for FED4 receiving 100 and 200 mg/L 4-CP

In the case of 4-CP and 2,4-DCP mixture, competitive inhibition of 2,4-DCP on 4-CP degradation can be clearly seen from the analyses of Figure 3.7. Almost complete removal of 2,4-DCP was achieved within 4 and 6 h (Figure 3.7a) and 4-CP degradation was almost negligible up to this point. After complete degradation of 2,4-DCP, there was no any inhibitory effect on 4-CP degradation and its degradation started immediately and completed following next 5 h (Figure 3.7a). The competitive inhibitory effect of 2,4-DCP on 4-CP degradation can also be clearly seen from Figure 3.7c, which depicts normalized concentrations for FEDM receiving 200mg/L 4-CP and 100 mg/L 2,4-DCP. Zero order degradation model was good enough to predict time course variation of 2,4-DCP (Figure 3.7a). The model fitted reasonably well to the experimental data, which means the developed model can be used to predict the performance of the reactor even at different steady state feed chlorophenols concentrations.

During the degradation of 4-CP, CHMS concentration in the medium increased and it reached its maximum when 4-CP was just completely removed from the medium (Figure 3.7c). After reaching peak value, the CHMS concentration decreased sharply and complete removal was observed at the end of the cycle. The peak value of CHMS for 200 mg/L 4-CP and 100 mg/L 2,4-DCP was 3.38% of theoretical CHMS concentration. The value was observed to be 3.28% for 100 mg/L4-CP and 50 mg/L 2,4-DCP. The measured COD values are also in good agreement with those observations as the COD values when CHMS reached its maximum is almost equal to the effluent COD values. Therefore, there were almost no contributions of CHMS to COD due to its low concentration. These results indicated simultaneous removal of 4-CP and CHMS, although the removal rate of CHMS is slightly lower. Similar results were also observed for FED4.

The observed effect of 2,4-DCP on 4-CP degradation was similar to the inhibition observed in cometabolic degradation of 4-CP in the presence of phenol (Wang and Loh, 1999 and 2000; Hill et al., 1996; Lu et al., 1996; Kim and Hao, 1999; Hao et al., 2002). In 4-CP degradation, the first step is the attack of phenol hydroxylase to

4-CP, which results in production of 4-Chlorocatechol. The conversion of 4-Chlorocatechol via -*meta* cleavage pathway yields CHMS, and complete removal of the intermediate may further be observed. CHMS has been widely reported to be a dead-end metabolite when pure culture is used in the degradation process (Hill et al., 1996; Sae´z and Rittmann, 1991 and 1993; Farrell and Quilty, 1999). However, we have observed complete degradation of 4-CP and CHMS (Figures 3.6 and 3.7) with acclimated mixed culture. In the case of 2,4-DCP degradation, the first step is the production of 3,5-Dichlorocatechol with the use of 2,4-Dichlorophenol hydroxylase. Then 3,5-Dichlorocatechol is converted to 2,4-Dichloro-cis,cis-muconate, which is catalyzed by 3,5-dichlorocatechol 1,2-dioxygenase (Radjendirane et al., 1991). It was reported that phenol hydroxylase has a broad substrate specificity and it can also catalyze the turnover of 2,4-DCP (Hollender et al., 1997). It is also known that 2,4-Dichlorophenol hydroxylase has ability to convert 4-CP to 4-Chlorocatechol (Beadle and Smith, 1982). Therefore, the use of the same enzymes for the initiation of 4-CP and 2,4-DCP degradation led to observation of competitive inhibition.

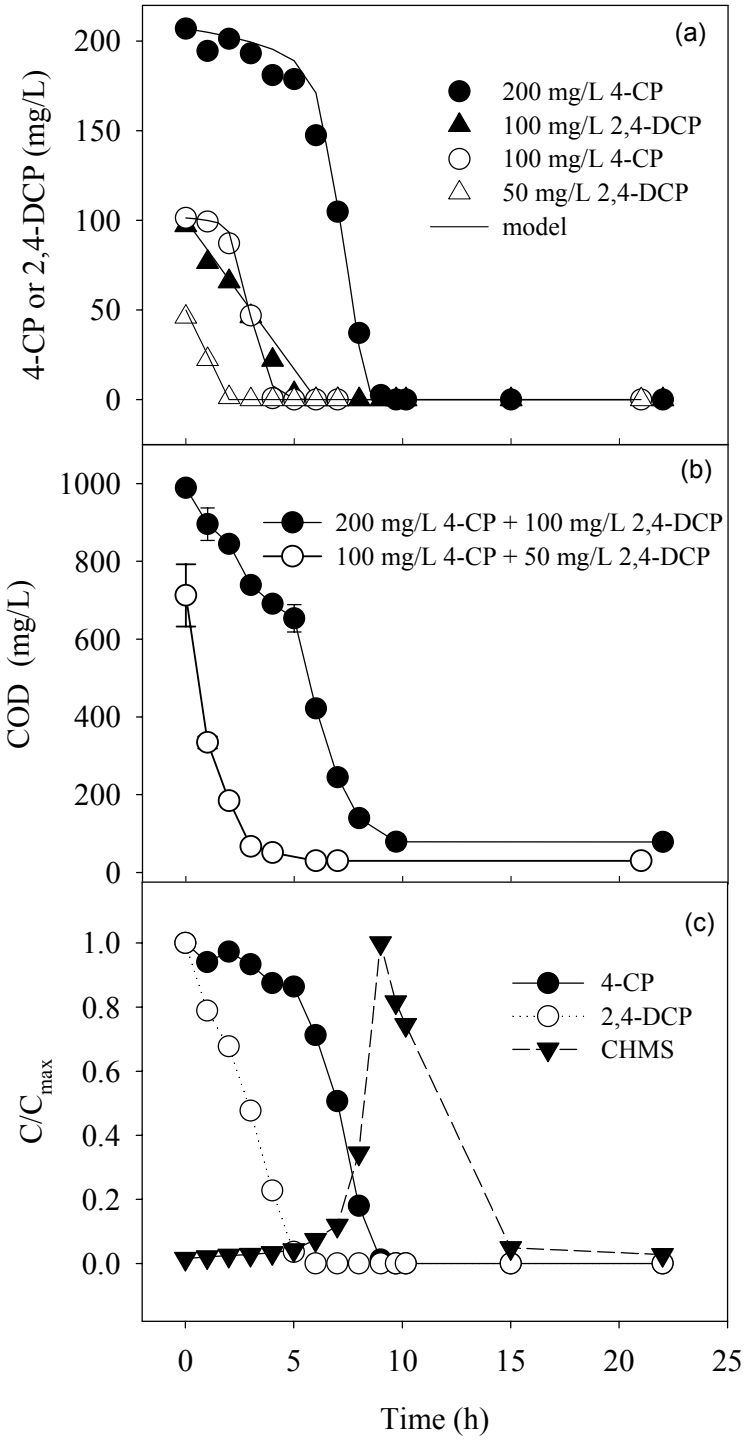

Figure 3.7. Experimental data and model results for FEDM

The average specific degradation rates on the basis of total biomass concentrations (SDR) are depicted in Figure 3.8. The SDR values in FEDM were calculated after 2,4-DCP were completely removed from the medium as negligible removal occurred in the presence of 2,4-DCP due to competitive inhibition. The SDR of chlorophenols increased linearly with increasing chlorophenol fraction in the feed. The reason of this observation, as noted before, is the increase in the fraction of chlorophenol degrading specialized biomass in the community. A better linear relationship was observed for 4-CP in the absence of 2,4-DCP (FED4). The possible reason of this observation is the complex substrate interaction in multi-substrate environments (FEDM). Another interesting point is that SDR values for FEDM were higher compared to those for FED4. Therefore, although presence of 2,4-DCP competitively inhibited 4-CP degradation, its presence increased the SDR of 4-CP after 2,4-DCP disappeared in the medium. There may three possible reasons of such observation. First, the culture community is different in two reactors (see Table 3.1) as the manner in which the culture has been developed determines the types of dominant species (Grady et al, 1996). Second, the cell's macromolecular composition (cell-envelope, protein, RNA and DNA) may be different in two reactors, which determines how rapidly enzymes are synthesized and those enzymes react (Grady et al., 1996). As noted previously, the same enzymes are utilized in initiation of 4-CP and 2,4-DCP degradation. Therefore, presence of 2,4-DCP may increase the amount of enzymes utilized in 4-CP breakdown. Third, the fraction of specialists in the sludge of FEDM may be higher compared to that in FED4 as 2,4-DCP degraders may also have the ability to degrade 4-CP.

Table 3.1. Characteristics of isolated and identified species

Source of sample	Isolated and identified specie	Gram reaction	Colony characteristic	Relative amount
FED4	*Pseudomonas stutzeri*	G-	Yellow to transparent	+++
	Pseudomonas vesicularis	G-	Orange	+
FEDM	*Pseudomonas stutzeri*	G-	Yellow to transparent	+++
	Pseudomonas sp.	G-	Brown	++

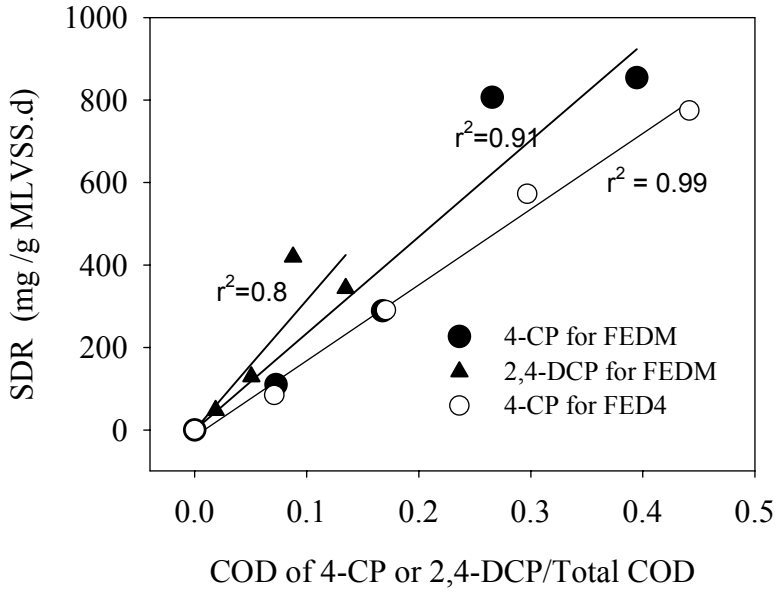

Figure 3.8. Variation of SDRs with the fraction of chlorophenols in the feed on COD basis

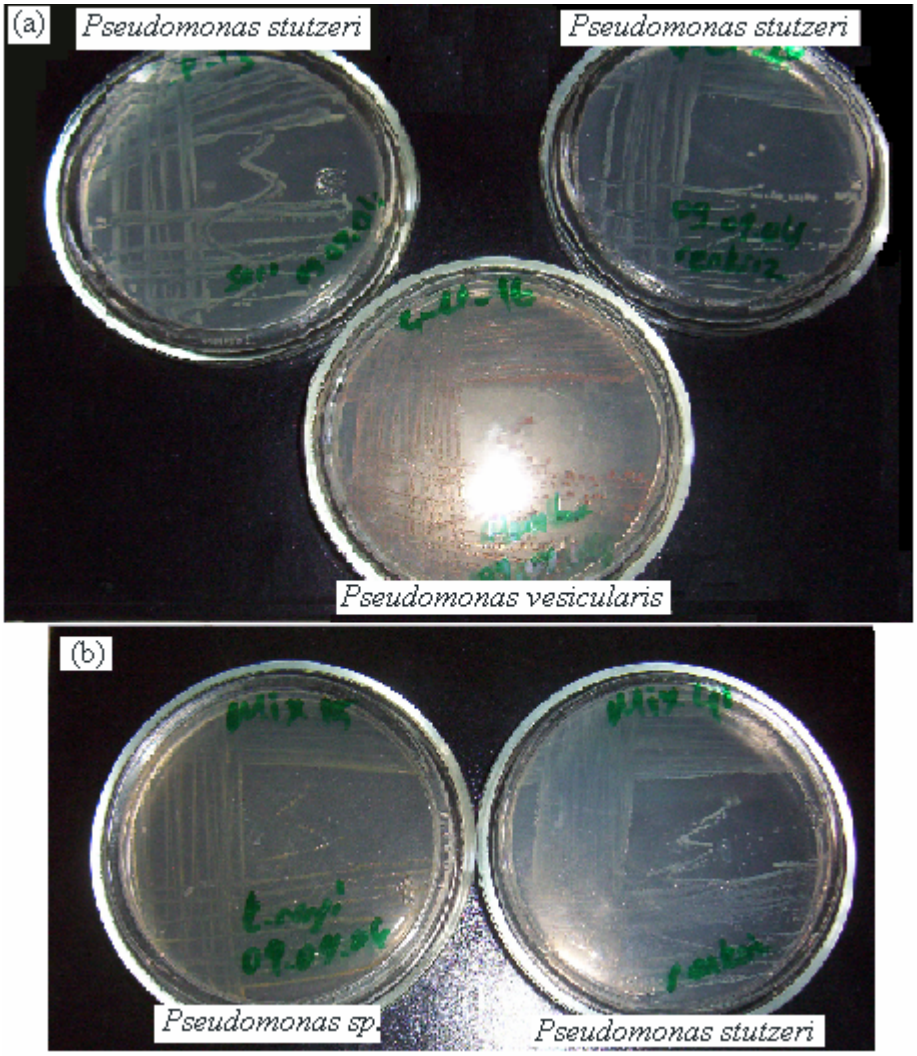

Figure 3.9. A view of isolated and identified species grown on agar plates (a) species from FED4, (b) species from FEDM

The list and photos of isolated and identified species are given in Table 3.1. and Figure 3.9, respectively.

Although the specific degradation rate of chlorophenols calculated on the basis of competent biomass concentration (SDRc) is constant, the specific degradation rate calculated on the basis of total biomass concentration (SDR) shows variation depending on feed chlorophenols concentrations due to the change of specialist biomass fraction in the community. Therefore, the dependence of SDR on SDRc

can be shown as;

$$SDR = aSDRc \qquad (3.7)$$

As noted previously, total chlorophenol (4-CP+2,4-DCP) fraction in the feed was considered in FEDM.

SDRc values for 4-CP degradation were calculated from the experimental results when the reactors received 200 mg/L 4-CP and 100 mg/L 2,4-DCP (FED4 received only 200 mg/L 4-CP). The observed SDRc values for 4-CP degradation were around 1755 and 1615 mg/g MLVSSc.d for FED4 and FEDM, respectively. Although SDR value for FEDM (854.3 mg/g MLVSS.d) is higher than that for FED4 (774.43 mg/g MLVSS.d) (Figure 3.8), the observed SDRc values for both reactors are almost equal (difference is only around 8%). Therefore, the observed higher SDR values in FEDM are most probably due to higher fraction of specialist in the community. Using the observed SDRc values, the SDR values were computed using Equation 3.7 (Figure 3.10). The predicted values are in good agreement with the experimental data. Therefore, it can be concluded that the competent biomass fraction can be approximated as contribution of chlorophenols to the feed on COD basis.

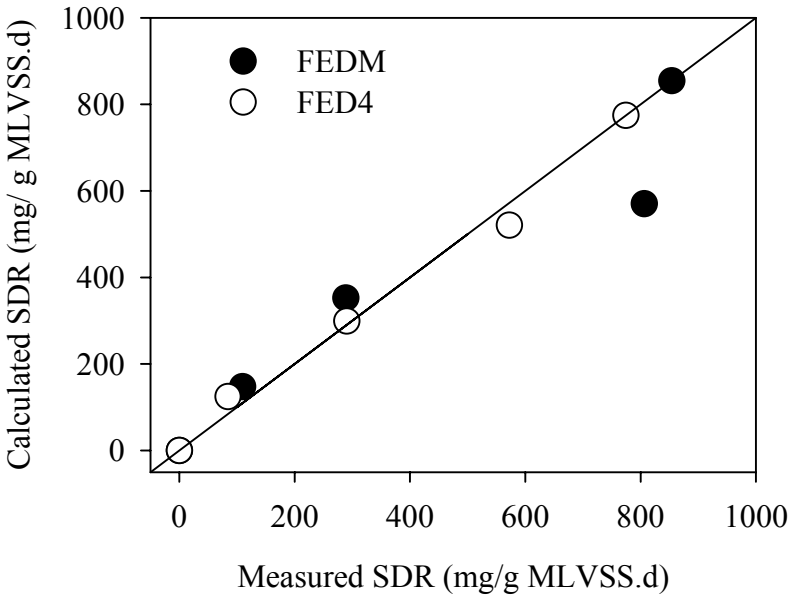

Figure 3.10. Measured and calculated 4-CP SDR values for FEDM and FED4

3.1.2. Effect of Biogenic Substrate Concentration on Chlorophenols Degradation Kinetics

A wastewater treatment plant generally receives influent with a mixture of recalcitrant synthetic organic chemicals (SOCs) and biogenic substrates. This means that SOCs and biogenic compounds often coexist in many wastewater reactors. Interactions among these multiple substrates are complex, partially due to the toxicity, competition for enzymes and cofactors (Hu et al. 2005b). The aim of this set of experiments is to evaluate the effect of biogenic substrate concentration on 4-CP and mixture of 4-CP and 2,4-DCP degradation kinetics using long term operated instantaneously fed SBRs. In this context, biogenic substrate (peptone) concentrations in FED4 and FEDM were decreased gradually from its initial value of 400 mg/L and eventually chlorophenols were fed to the reactors as sole carbon sources.

3.1.2.1. Effect of Biogenic Substrate Concentration on 4-CP Degradation

In this set of experiment, the effect of biogenic substrate concentration on 4-CP degradation kinetics was investigated in FED4. In this context, peptone concentration in the feed of the reactor was gradually decreased and following the observation of steady state conditions, samples were drawn at different time intervals from the reactor for the analyses of 4-CP, COD, MLVSS and CHMS. Figure 3.11 shows 4-CP and COD degradation profiles at different initial biogenic substrate concentrations. Complete 4-CP removal with no significant variation in the degradation patterns and high COD removals (90-95%) were observed for all studied conditions (Figure 3.11). The effluent COD concentration was about 28 mg/L in the absence of 4-CP and it changed between 30-50 mg/L in the presence of 4-CP with varying peptone concentrations. Low effluent COD concentrations were the first evidence of complete 4-CP mineralization, which was supported by HPLC analyses. Also, released chloride ion concentrations indicated 101±7% 4-CP mineralization (data not shown).

After complete elimination of peptone from the feed, 400 mg/L peptone was readded to the feed of the reactor. In Figure 3.11(b), data corresponding to 400 mg/L peptone 1 was observed at the first peptone addition, whereas, data belonging to 400 mg/L peptone 2 was observed when peptone was readded after its complete elimination. Variation in the 4-CP degradation patterns was not significant (Figure 3.11(b)) and the observed small change can be attributed to the natural variability of culture over time. Ellis et al. (1996) reported that biodegradation kinetics might show variation (standard deviation of ± 50%) even in long-term operated steady state reactor due to natural variability of culture. In another study, Kaewpipat and Grady (2002) observed significant difference over time in microbial communities of even two identically operated activated sludge reactors. Similar to those findings, isolation and identification studies in FED4 revealed change in dominant species during the operation of the reactor. *Pseudomonas vesicularis* and *Pseudomonas stutzeri* were dominant species when the reactor fed with 400 mg/L peptone,

whereas, *Stenotrophomonas maltophilia* and *Pseudomonas fluorescens* were the dominant species when peptone was completely eliminated from the feed, i.e., chlorophenols were the sole carbon sources.

During the degradation of 4-CP, the concentration of CHMS increased concomitantly, which reached its maximum when 4-CP was just completely removed from the medium (Figure 3.12). After reaching peak value, its concentration decreased and complete removal of the intermediate has been observed. Similar patterns have also been observed for other peptone concentrations (data not shown). Using the extinction coefficient of CHMS (Farrel and Quilty, 1999), its highest concentration was calculated between 7-10 mg/L for all studied conditions. Therefore, CHMS accumulation was only 2.5-3.6% of its theoretical value assuming complete transformation of 4-CP to CHMS. The measured COD values were also in good agreement with this observation as the COD concentration when CHMS reached its maximum was almost equal to the effluent COD value due to the low concentration of CHMS. Therefore, simultaneous removal of 4-CP and CHMS occurred in the reactor although CHMS removal rate was slightly lower, which caused a slight accumulation in the medium.

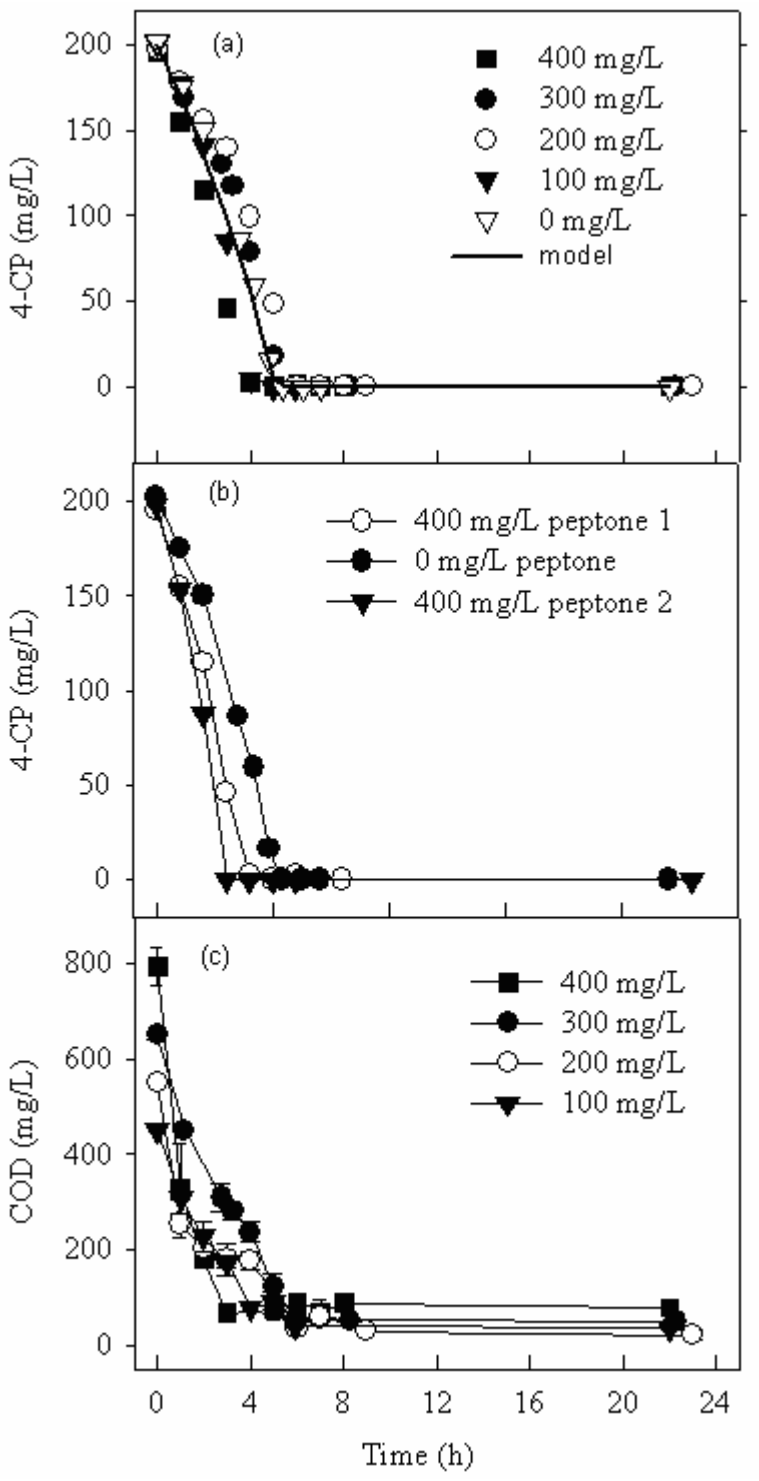

Figure 3.11. Time course variations of 4-CP (a, b) and COD (c) at variable peptone concentrations in FED4

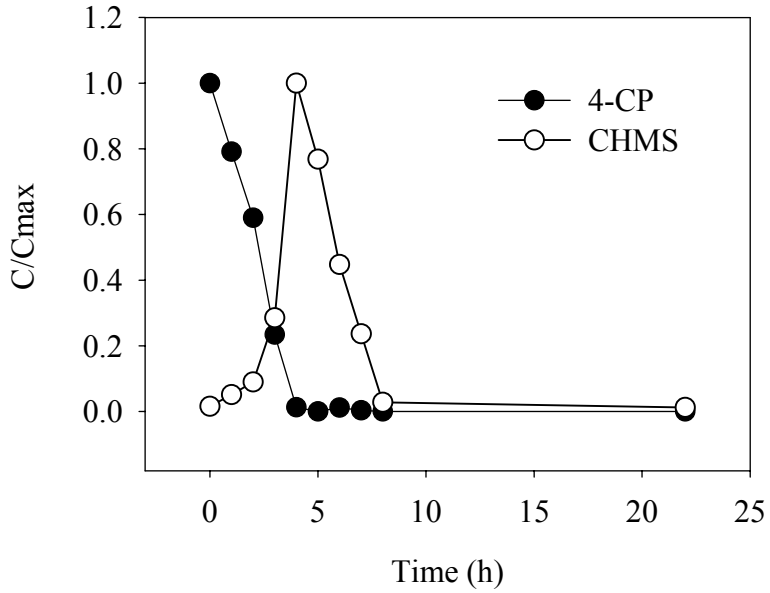

Figure 3.12. Time course variations of normalized 4-CP and CHMS in FED4 receiving 400 mg/L peptone

Almost identical Y values (0.220±0.009 (CV = 4.25%) mg MLVSS/mgCOD) were observed when peptone concentration was between 400 and 100 mg/L and it decreased slightly (0.154 ±0.005 mg MLVSS/mgCOD) with the complete elimination of peptone from the feed (Figure 3.13(a)). The average Y value was 0.207±0.03 (CV = 14.49%) mg MLVSS/mgCOD for whole set of experiments. Although biomass concentration decreased with decreasing peptone concentration, degradation rates (DRs) of 4-CP did not correlate with peptone concentration (Figure 3.13(b)) and the average value was 40.53±7.9 mg/L.h (CV=19.5%). Unlike to DRs, specific 4-CP degradation rates (SDRs) increased when peptone concentration was below 200 mg/L and the highest value was reached in the absence of peptone.

The concentration of specialist biomass having ability to degrade 4-CP (competent biomass) might remain constant, as 4-CP concentration in the feed was kept constant. If only competent biomass is responsible for 4-CP degradation, DRs of 4-CP should not change with peptone concentration, as competent biomass

concentration was constant, whereas, SDRs should increase as the fraction of competent biomass increased in the community with decreasing peptone concentrations. Our observations (Figure 3.13) partially agreed with this hypothesis and 4-CP degradation patterns are very similar at different peptone concentrations (Figure 3.11(a) and (b)). Therefore, the increase in SDR values may be attributed to increased fraction of competent biomass in the community with decreasing peptone concentration (Figure 3.13(b)).

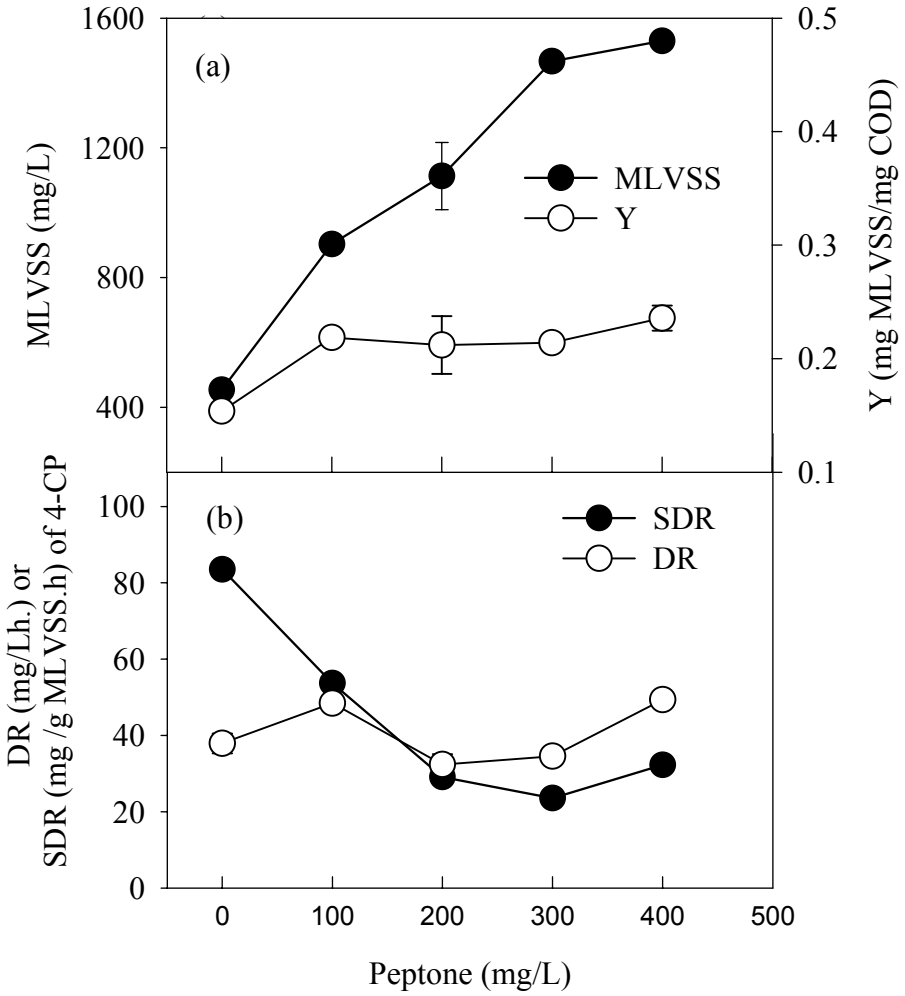

Figure 3.13. Effect of feed peptone concentration on FED4

In order to understand effect of SRT on the kinetics of 4-CP degradation, another reactor with 20 d of SRT (FED4-2) was also operated parallel to the FED4. After

any change in operational parameters, two SRTs were allowed for FED4-2 to reach steady state conditions. Similar to FED4, complete 4-CP and high COD removals were observed even in the absence of peptone as the influent COD decreased from 330 to around 21 mg/L and it was thought that the remaining COD was due to biomass lyses products. Figure 3.14 depicts time course variations of 4-CP concentrations at different initial peptone concentrations for FED4-2. Similar to FED4, peptone did not affect the 4-CP degradation profile in FED4-2.

The effect of peptone concentration on the performance of FED4-2 was also summarized in Figure 3.15. MLVSS concentrations decreased with decreasing peptone concentration in the feed. SDR of 4-CP was observed to be almost constant in the presence of peptone and it significantly increased with the complete exclusion of peptone from the feed. Similar to FED4, almost identical Y values (0.263±0.009 (CV=3.4%) mg MLVSS/mg COD) were observed when the feed included peptone and the Y decreased to a value of 0.175±0.005 mg MLVSS/mg COD with the complete elimination of peptone. The average Y value for whole set of experiments was observed to be 0.234±0.04 (CV=18%) mg MLVSS/mg COD in FED4-2. Although slightly lower values were observed in FED4, the Y values were quite similar for both reactors (Figures 3.13 and 3.15).

The volumetric removal rate of 4-CP was around two times higher in FED4-2 than FED4. It is known that a high steady state biomass concentration in the treatment system usually exhibits high volumetric conversion capacity and the flexibility to the fluctuated loading rates (Tomei et al., 2004).

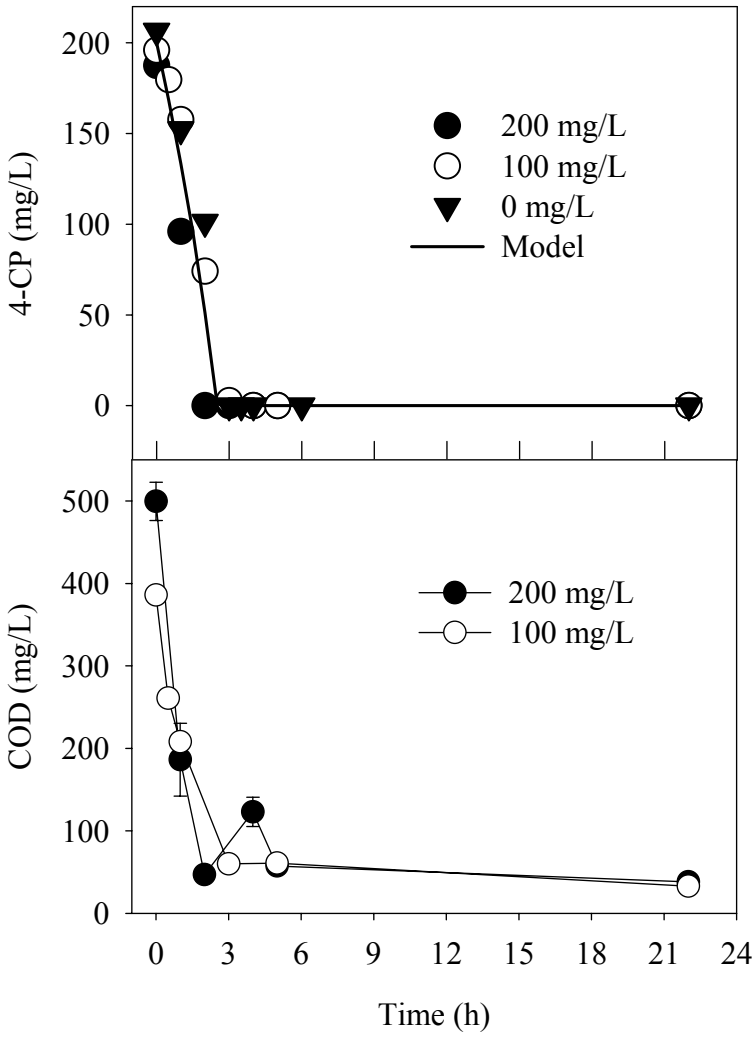

Figure 3.14. Effect of peptone concentration on 4-CP degradation pattern in FED4-2

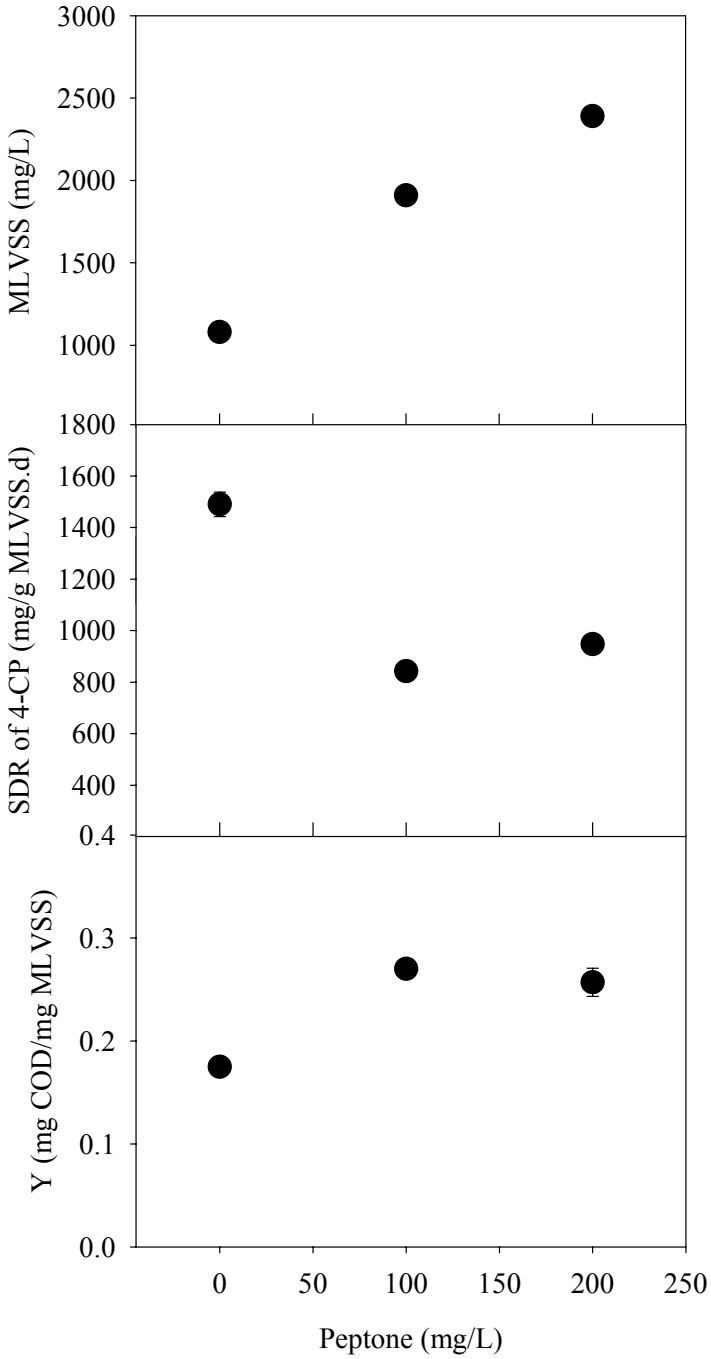

Figure 3.15. Effect of peptone concentration on the performance of FED4-2

3.1.2.2. Effect of Biogenic Substrate Concentration on Degradation of 4-CP and 2,4-DCP Mixture

Similar experiments were also conducted on FEDM in order to evaluate effect of peptone concentration on biodegradation of chlorophenols mixture. In this context, biogenic substrate (peptone) concentration in the instantaneously fed SBR was decreased gradually from its initial value of 400 mg/L and eventually chlorophenols have been fed to the reactors as sole carbon sources.

Figure 3.16 shows 4-CP, 2,4-DCP and COD degradation profiles at different initial biogenic substrate concentrations. As can be seen from the figure, complete chlorophenols removal and high COD removal efficiencies (90-95%) were observed for all studied conditions and no significant change in degradation patterns of 4-CP and 2,4-DCP were observed. In the absence of chlorophenols, the effluent COD concentration was around 28 mg/L. In the presence of chlorophenols with varying peptone concentrations, the effluent COD concentrations changed between 30-50 mg/L suggesting that the remaining COD in the effluent of reactor was due only to biomass lyses products, which may have increased slightly in the presence of chlorophenols. The similar attribution was also done by Tomei (2005), in a study investigating 4-NP degradation, who stated that residual COD in the effluent was in the range of 10–20 mg/L, values reasonably attributable to biomass lysis product. Therefore, low effluent COD concentrations were the first evidence of complete removal of chlorophenols without any remaining by-product, which was supported by HPLC analyses. Also, released chloride ion concentrations indicated complete (101±7%) mineralization of chlorophenols (data not shown).

Figure 3.16 shows that 2,4-DCP was degraded first and 4-CP degradation began only after complete removal of 2,4-DCP. It took 4-6 h for 2,4-DCP to be removed completely, whereas it was 8-10 h for 4-CP. These results suggest that degradation rates (DRs) of chlorophenols were independent of biogenic substrate concentration.

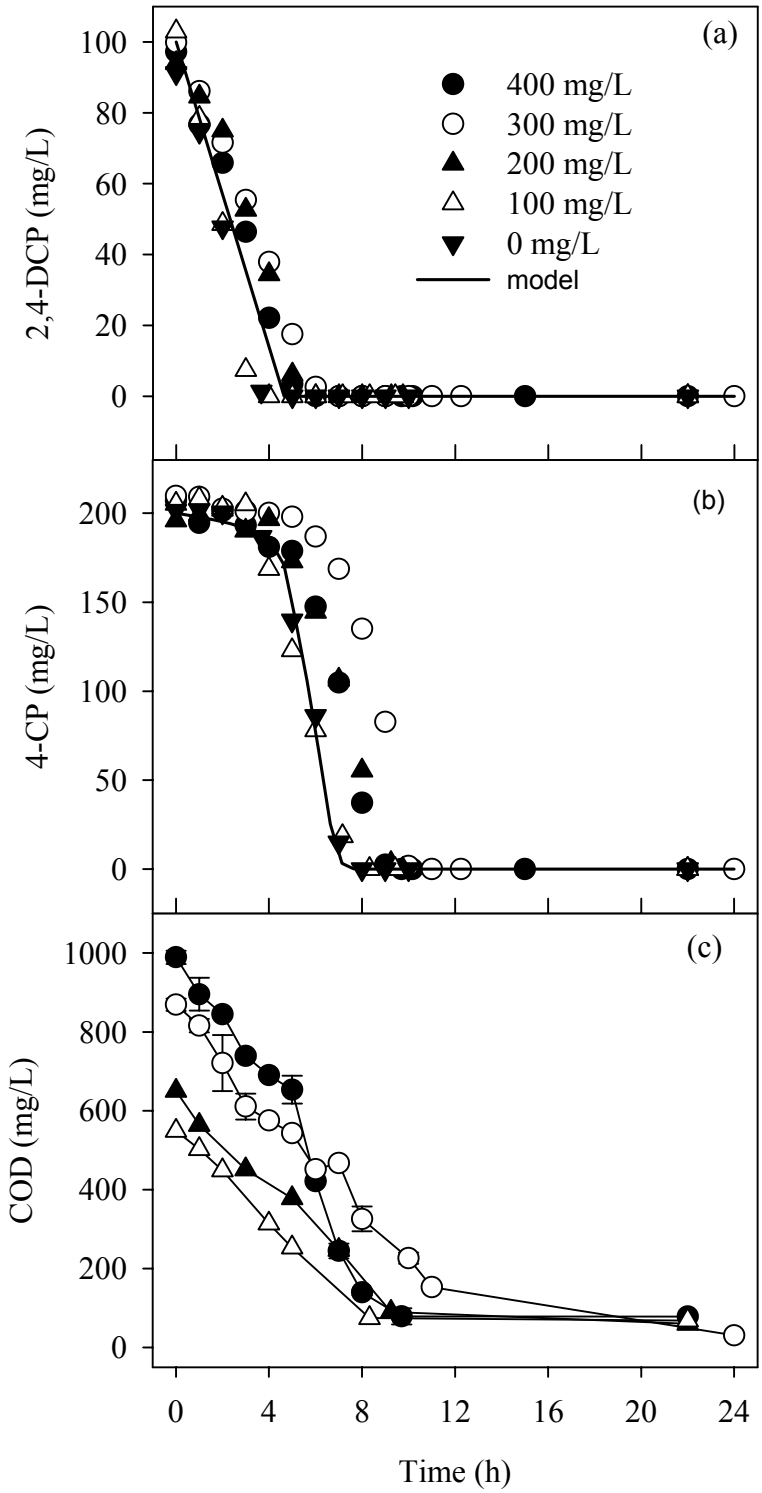

Figure 3.16. Time course variations of 2,4-DCP (a), 4-CP (b) and COD (c) at variable peptone concentrations in FEDM

The effect of peptone concentration on the steady state data observed for FEDM (Figure 3.17) allows several conclusions. First, the MLVSS concentration decreased with decreasing peptone concentration as the fraction of biogenic substrate in the feed decreased. Second, the degradation rate of 4-CP did not correlate with peptone concentration and remained almost constant (average value of 42.3±6.88 (CV=16.26%) mg 4-CP/L.h). This can be attributed to fact that concentration of biomass responsible for degradation of 4-CP was relatively constant, as chlorophenols concentrations were kept constant. It should be noted that in the calculation of 4-CP degradation rate, the time period in which 4-CP degradation occurred was considered. The removal rate of 2,4-DCP increased slightly with decreasing peptone concentration. Unlike for degradation rates, the SDR of 4-CP increased as the decreased ratio of peptone in feed. Also, there was a slight increase in Y value as the peptone concentration decreased to 100 mg/L or less. In the absence of peptone, the Y value for the mixture of 4-CP and 2,4-DCP was observed to be 0.24±0.01 mg MLVSS/mg COD, which was 0.15±0.01 mg MLVSS/mg COD in the presence of 300 mg/L peptone.

It is also important to note that the sludge settleability is very important to obtain a stable performance in real scale applications. It was observed that the produced sludge in both FED4 and FED4-2 has excellent settleability characteristics for the whole experimental period. The SVI of the sludge changed between 40 and 75 mL/g for both reactors. Similarly, in the study of Moreno-Andrade and Buitron (2004) good settling ability of sludge during the chlorophenols treatment was reported.

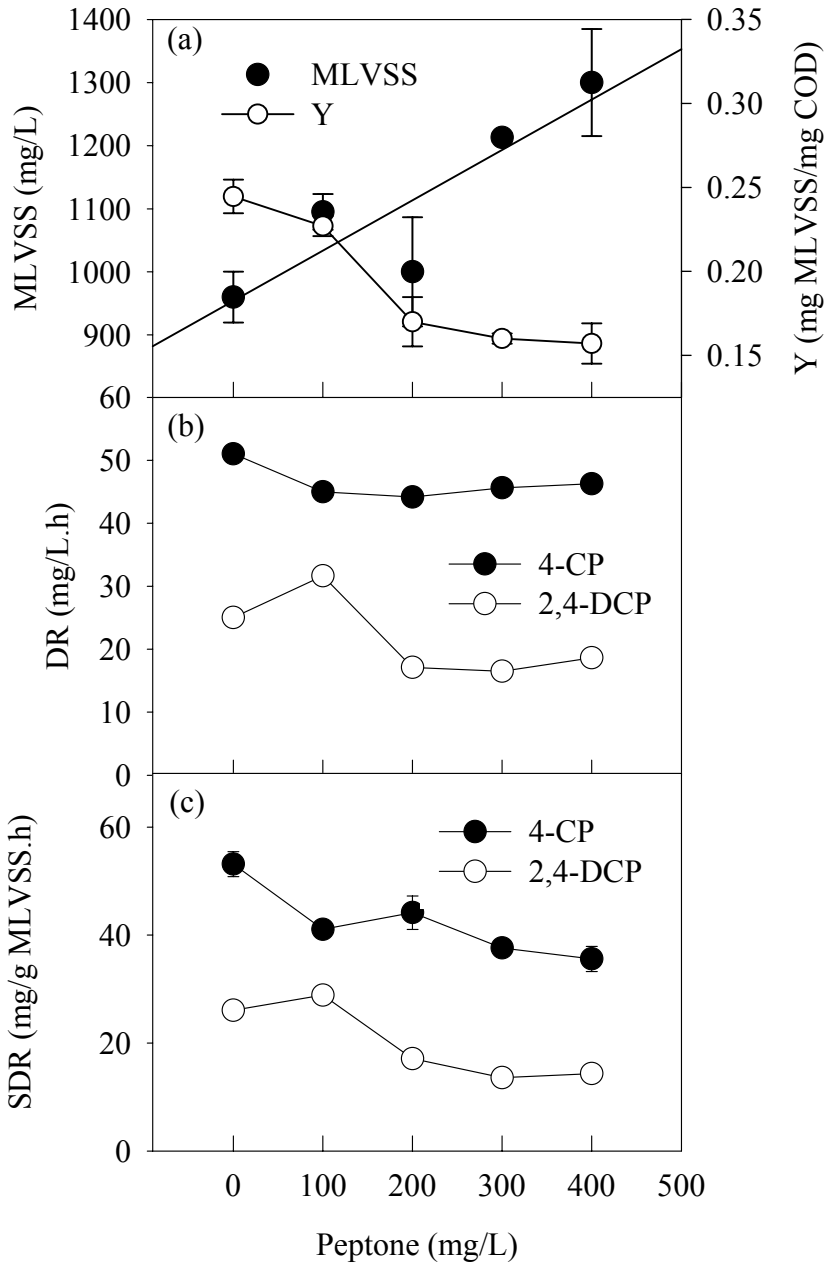

Figure 3.17. Effect of feed peptone concentration on MLVSS,Y (a), DR (b) and SDR (c) in FEDM

3.1.2.3. Kinetics of Chlorophenols Degradation at Varying Biogenic Substrate Concentrations

In the development of the model it was assumed that only competent biomass was responsible for chlorophenols degradation, as explained before. Therefore, the competent biomass can be estimated as:

$$X_c = X_T.a \qquad (3.8)$$

Where, X_c is competent biomass concentration (mg $MLVSS_c$/L), X_T is total biomass concentration (mg MLVSS/L), a is fraction of chlorophenol in the feed on COD basis.

It should be noted that in the determination of competent biomass fraction having ability to degrade 4-CP, total fraction of 4-CP and 2,4-DCP in the feed of FEDM was taken into consideration as explained above. Based on this assumption, the DR of 4-CP should be constant and independent on peptone concentration, whereas SDR should increase with decreasing peptone concentration due to increase in competent biomass fraction. Figure 3.18 depicts the change of SDRs of chlorophenols for FEDM (Figure 3.18(a)) and FED4 (Figure 3.18(b)). SDRs increased linearly with decreasing peptone concentration or increasing chlorophenols fraction in the feed.

Figure 3.19 depicts the measured and estimated SDR values. In the estimation, the specific degradation rate of competent biomass (SDRc) was first calculated using experimental data obtained for 400 mg/L peptone and SDR values were calculated using the following equation, assuming that SDRc does not depended on feed peptone concentration:

$$SDR = SDR_c a \qquad (3.9)$$

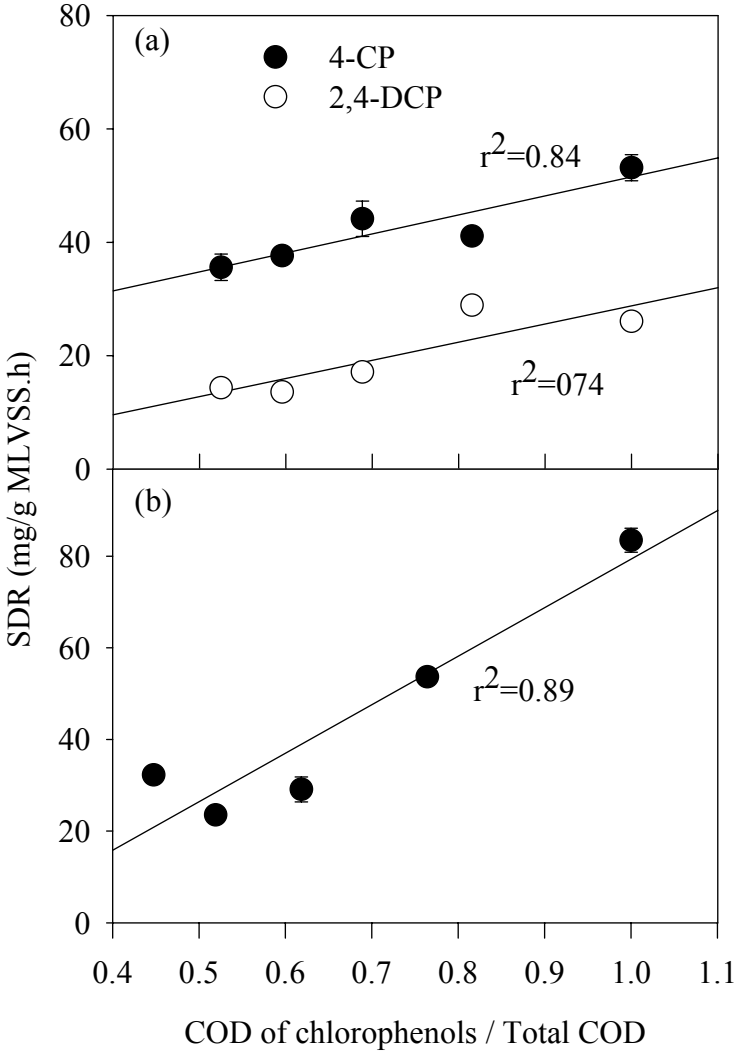

Figure 3.18. SDRs of chlorophenols in FEDM (a) and FED4 (b) at different chlorophenols fractions in the feed on COD basis

Data obtained for FEDM reactor gave better fit than FED4 to the experimental data. The two data points below the predicted values in Figure 3.19 correspond to 100 and 0 mg/L of peptone concentrations. Although the measured SDRs are lower than the predicted ones, the DRs at these peptone concentrations were higher compared to those of higher peptone concentrations (Figure 3.17(b)). This may be due to increased Y values and higher biomass concentrations being greater than expected; on the basis of assumption, a constant competent biomass concentration was

anticipated. Competent biomass concentrations calculated using feed COD fraction of chlorophenols when peptone concentrations were 100 and 0 mg/L (893 and 960 mg MLVSSc/L, in respective order) were observed to be slightly higher compared to higher feed peptone concentrations (688-723 mg MLVSSc/L). This caused a slight increase in chlorophenol removals at lower peptone concentrations (Figure 3.16).

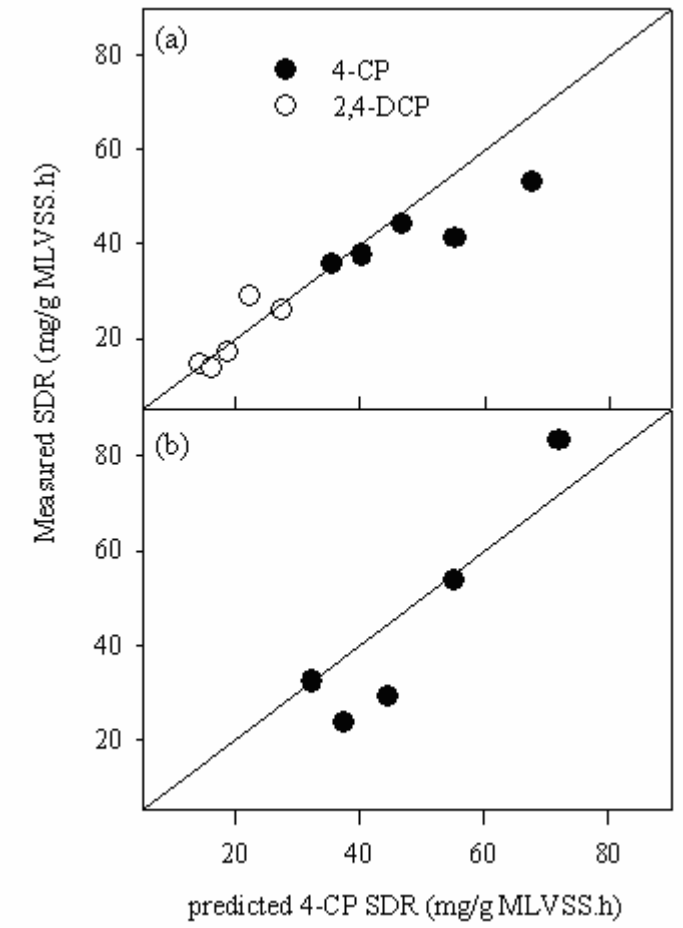

Figure 3.19. Calculated versus measured SDR values of chlorophenols in FEDM (a) and FED4 (b)

In FED4, the average X_c (or X.a) at varying peptone concentrations was observed to be 655.76 ± 117.27 (CV=17.88%) mg $MLVSS_c$/L. Using this value and taking initial 4-CP concentration as 200 mg/L, 4-CP degradation profile was estimated using Equation 3.2 and the model solution was compared with experimental data in

Figure 3.11 (a). The predicted 4-CP degradation profile agreed well with the experimental data, which shows that peptone concentration may not have significant effect on 4-CP degradation profile. Similarly, in FED4-2, only competent biomass was assumed to be responsible for 4-CP degradation and its average concentration (aX) was calculated to be 1339±183 (CV=13.7%) mg MLVSSc/L. It was observed that the competent biomass concentration in FED4-2 was almost two times of that observed for FED4. The model parameters developed using the sludge from FED4 was used to predict time course 4-CP degradation profile and the model gave good fit to the experimental data (Figure 3.14).

Table 3.2. Average competent biomass and DRs in FED4 and FEDM

Reactor	**Competent Biomass (mg MLVSSc/l)**	**DR (mg/l.h)**
FED4	655.76±117.27	40.53±7.9
FED4-2	1339±183	77.91±14

Using the average DRs and competent biomass concentrations (Table 3.2), the SDR values on the basis of competent biomass (SDRc) were calculated as 58.11 and 61.8 mg/g.h for FED4-2 and FED4, respectively. It can be concluded that although DR value observed in FED4-2 was around two times higher, SDRc values are quite similar for both reactors. Therefore, the observed higher degradation rate value for FEDM was only due to observed higher competent biomass concentration.

In the case of FEDM, the average competent biomass fraction (a.X or X_c) was 789.28±128.38 (CV=16.26%) mg $MLVSS_c$/L. The average SDRc value for 2,4-DCP was observed to be 27.3 ± 4.83 (CV=17.7%) mg/g$MLVSS_C$.h. Using average SDRc and X_c values, the average DR value was calculated as 21.54 mg/L.h. Using the calculated average values of X_c and average DR of 2,4-DCP (k in Equation 3.6), the 4-CP and 2,4-DCP degradation profiles were predicted using Equation 3.6 and the model simulation was compared with the experimental data (Figure 3.16(a) and

(b)). The model well predicted 2,4-DCP degradation profile and experimental results showed that peptone concentration did not have a significant effect on 2,4-DCP degradation profile. In the case of 4-CP, although degradation profiles at different feed peptone concentrations differed especially for feed peptone concentration of 300 mg/L, the predicted 4-CP degradation profile gave acceptable fit to the overall data. The change in degradation profiles may partially be due to change in microbial community in the reactors over time. Ellis et al. (1996) reported that biodegradation kinetics might show variation (standard deviation of ± 50%) even in long-term operated steady state reactor. In another study, Kaewpipat and Grady (2002) observed significant differences over time in microbial community of even two identically operated activated sludge reactors.

In summary, the effect of biogenic substrate concentration on 4-CP and mixture of 4-CP and 2,4-DCP degradation kinetics were extensively investigated in instantaneously fed SBRs. High COD removal efficiencies and complete chlorophenols removals were observed even in the absence of peptone. Strong competitive inhibition of 2,4-DCP on 4-CP degradation was observed in FEDM. Results showed that decreasing peptone concentration, when implemented gradually, did not cause significant change in chlorophenol degradation patterns. The reason of this observation is that only a fraction of biomass grown on chlorophenols (competent biomass) was responsible for chlorophenols degradation. Models developed based on this assumption gave good fit to the experimental data.

To our knowledge, this is the first study extensively evaluating the addition of 2,4-DCP on the kinetics of 4-CP degradation at different biogenic substrate concentrations in instantaneously fed SBRs operating in steady-state conditions. A wastewater treatment plant generally receives influent with a mixture of SOCs and biogenic substrates. Therefore, the results of the study can be used to design a bioremediation system as well as to optimize operational conditions.

3.2. Kinetics of Chlorophenols Degradation in Batch Reactors

The kinetic parameters used during the modeling of chlorophenols degradation in instantaneously fed SBRs (presented in previous sections) were obtained from batch experiments. Therefore, this part presents the experimental data obtained from the batch experiments and the development of the model using the experimental data is discussed in detail.

3.2.1. Kinetics of 4-CP Degradation by Culture (Culture 4) Acclimated to 4-CP

In this set of experiments, 4-CP biodegradation kinetics was investigated in the absence of peptone in batch reactors. The biomass used throughout the experiments was obtained from the FED4 operated with feed composed of 4-CP and peptone with respective concentrations of 200 and 400 mg/L. Experiments were conducted keeping initial biomass concentration high enough (around 200 mg/L) to accelerate degradation rate and avoid further acclimation of culture during lag phase, which may affect the observed biokinetic parameters. Adaptation of cell at prolonged lag phase during the phenol degradation was reported in the study of Sae´z and Rittmann (1993). They reported that the acclimation of cell to high concentrations of phenol during assays caused some change in kinetic parameters due to reduced inhibitory effect. Hence, in order to avoid the change of culture history and correctly estimate kinetic parameters, experiments were conducted at an initial biomass concentration of 200 ± 10 mg/L, which gave 4-CP/biomass ratios between 0.05-0.94 on COD basis. After inoculation of biomass, reactors were operated in an orbital shaking incubator at 200 rpm and 25±1°C. The reason of excluding peptone is to avoid high amount of biomass production, which may change the original culture taken from FED4.

Another significant point is that peptone was only used as a biogenic carbon source and its addition is not necessary to initiate 4-CP degradation. Therefore, the

production of biomass on 4-CP would be low enough to assume that the fraction of specialist in the sludge did not change during the assays.

Biomass used for batch assays was taken from the parent bioreactor when it was in steady state operation to supply biomass with constant characteristics to different batch assays.

Figure 3.20 shows time course variation of 4-CP and SDRs at different initial 4-CP concentrations. Complete 4-CP removal was achieved for all studied concentrations. Also, time required to observe complete degradation increased exponentially (r^2=0.97) with increasing initial 4-CP concentrations (Figure 3.21).

The decreased degradation rate with increasing initial 4-CP concentration (Figure 3.20) implies that 4-CP acts as an inhibitor. Therefore, the Andrews substrate inhibition (Equation 3.10) was used to model the inhibitory effect of 4-CP on its own transformation. Andrews equation fitted well to the experimental data (Figure 3.20) as r^2 was observed to be 0.986. The Andrews parameters for culture 4 were obtained as q_m=41.17 mg 4-CP/(g MLVSS.h), K_s=1.104 mg/L, and K_i= 194.4 mg/L. Non-linear least square method with the help of MATLAB 6.5 was used to obtain kinetic parameters.

$$-\frac{d(s)}{d(t)X} = q = \frac{q_m S}{K_s + S + S^2/K_i} \qquad (3.10)$$

where t is time (h), S is substrate concentration (mg/L), q_m is the maximum specific transformation rate (mg/(g MLVSS.h)), Ks and Ki are half saturation coefficient and the self inhibition constant (mg/L), respectively, moreover, X is biomass concentration as g MLVSS/L.

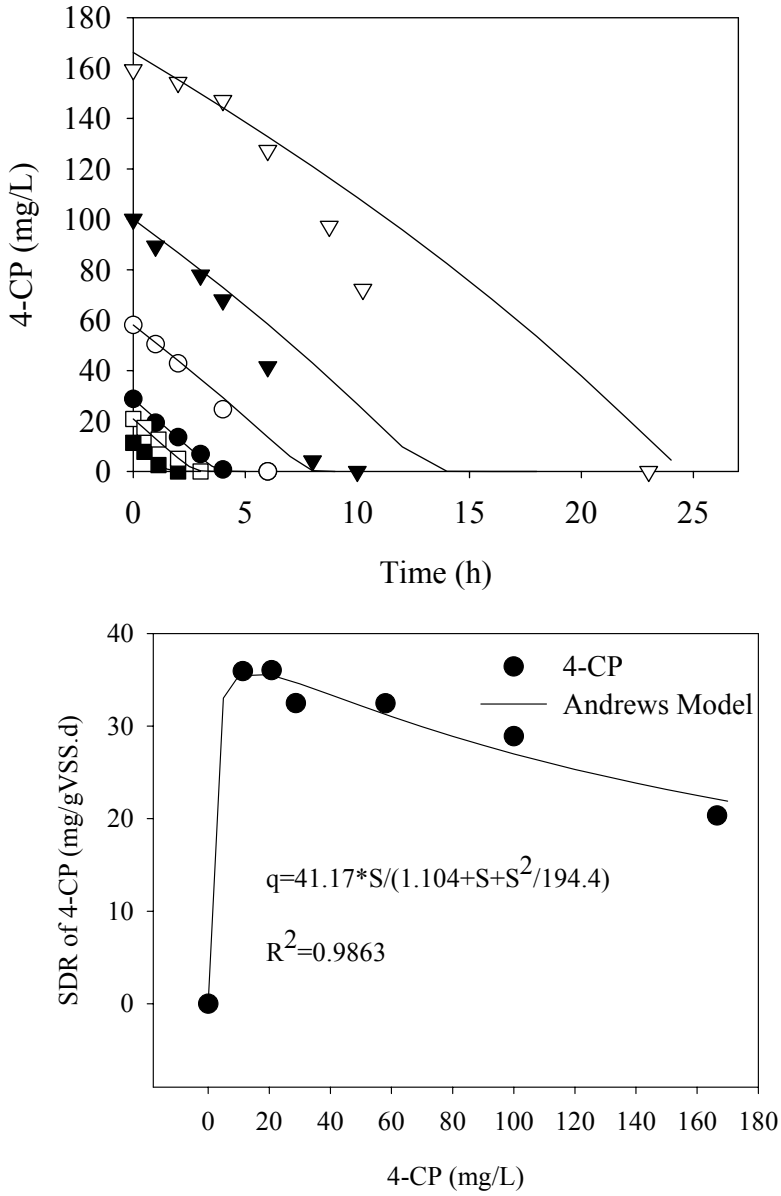

Figure 3.20. Time course variation of 4-CP concentrations (a) and SDRs (b) of culture 4 at different initial 4-CP concentrations

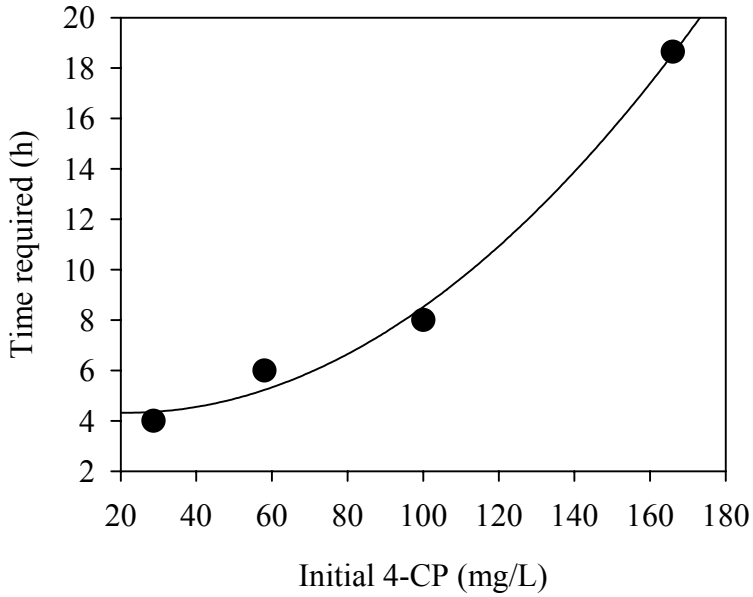

Figure 3.21. Time required for complete degradation at different initial 4-CP concentrations

As it was previously discussed deeply, only a fraction of biomass was responsible for 4-CP degradation and this fraction can be roughly approximated as 4-CP fraction in the feed of parent reactor (FED4) on COD basis.

Considering only specialist biomass, the value of observed q_m can also be expressed as 92 mg 4-CP/gMLVSSc.h. Therefore, Equation 3.2 used in the modeling of 4-CP degradation can be obtained.

Time course variation of 4-CP was also predicted solving the differential equation (Equation 3.2) using the Runge-Kutta-Fehlberg (RFK) numerical integration routine with the help POLYMATH 4.02. In solving the equation, it was assumed that biomass concentration remained constant through out the operation of the reactors. The biomass yield on 4-CP was around 0.15 mg MLVSS/mg COD in FED4, hence the possible increase in biomass concentration considering the studied range of 4-CP concentration can be calculated between 2.5-40 mg/L. Therefore, this calculation also shows that growth on 4-CP can be neglected considering much

higher initial biomass concentration (around 200 mg/L). The model fitted well to the experimental results only if reactors received 4-CP below 100 mg/L (Figure 3.20). When initial concentration of 4-CP was 100 mg/L or higher, the model could not predicted the experimental results satisfactorily. It was observed that applied model over predicted 4-CP concentrations and 4-CP degradation rates were higher than those predicted by the model. Although relatively high initial biomass concentration was in batch reactors, a further acclimation of culture during the degradation caused decrease in toxicity effect of 4-CP on biomass, which lead to increase in the observed degradation rates. The q_m value in Andrews equation was observed to be 145.27 mg 4-CP/(g MLVSSc.h) for 100 mg/L 4-CP (Equation 3.11).

$$\frac{d(S)}{d(t)} = -\frac{145.27SXa}{1.104 + S + S^2/194.4} \qquad (3.11)$$

The model fitted well to the experimental data (Figure 3.22)

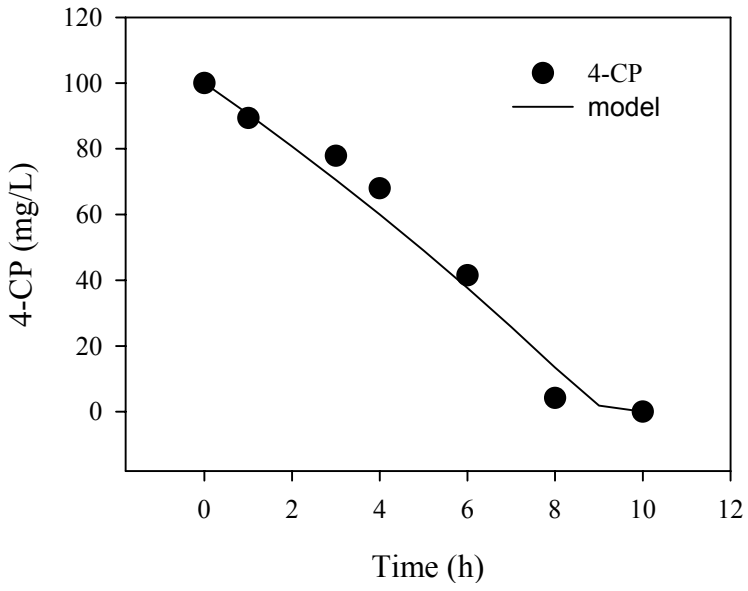

Figure 3.22. Application of Andrews model (q_m = 145.27 mg/ g MLVSSc.h) to 100 mg/L 4-CP

During the biodegradation, pH decreased accompanied with 4-CP degradation (Figure 3.23). For the reactors receiving 4-CP below 100 mg/L, pH values did not decreased drastically, however, for the others receiving 100 mg/L or higher 4-CP concentrations, pH values decreased remarkably and it decreased from 7.2 to around 6.4 for the studied highest 4-CP concentration, 170 mg/L. Although, a significant decrease in pH was observed, no attempt was put on the regulation of it as even the reached lowest pH value was not low enough to cause any adverse effect on biomass activity. Decrease in pH values during the 4-CP degradation was also reported in the studies of Farrell and Quilty (1999) and Wang and Loh (1999).

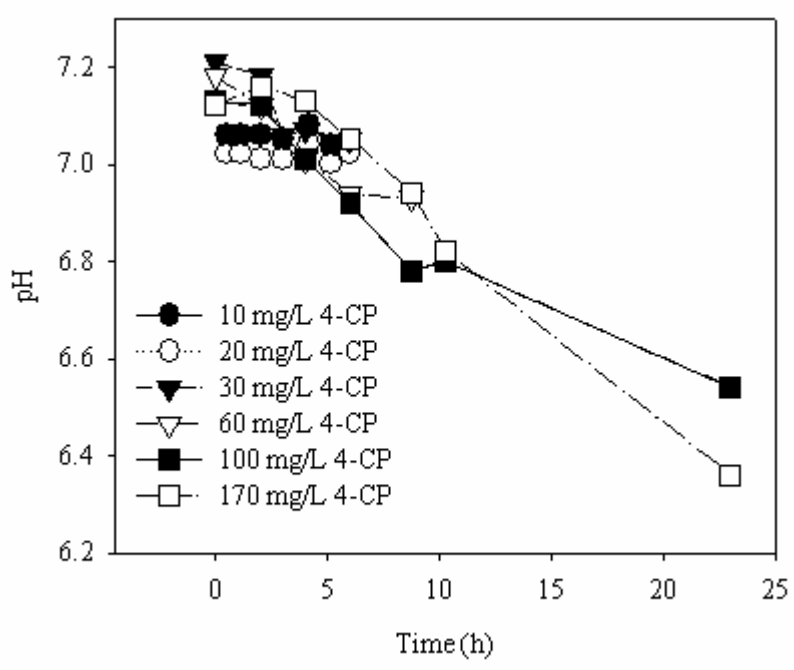

Figure 3.23. Time course variations of pH at different initial 4-CP concentrations

CHMS concentration, measured by OD at 380 nm (Farrell and Quilty, 1999), increased steadily accompanied with 4-CP degradation, and CHMS concentration reached its maximum when 4-CP was just removed completely from the medium as in the case of instantaneously fed SBRs. Figure 3.24 shows the change of relative concentrations of 4-CP and CHMS with time for the reactor receiving 30 mg/L 4-CP. Similar trend was also observed for other studied 4-CP concentrations. Also,

change of CHMS concentrations with time was given for reactors receiving 10, 20 and 30 mg/L 4-CP (Figure 3.25). Analyses of the Figure 3.25 yielded that the observed maximum concentration of CHMS concentration increased with increasing initial 4-CP concentration. Another important point is that after reaching its maximum point, CHMS concentration decreased sharply and reached its initial value, which indicated complete removal of produced intermediate. Therefore, production and then removal of CHMS indicated that 4-CP was degraded completely via *–meta* cleavage pathway. Also, HPLC result of reactor's effluent validated complete 4-CP degradation as no intermediate was observed. For one batch experiment, HPLC results of influent and effluent are given in Figure 3.26.

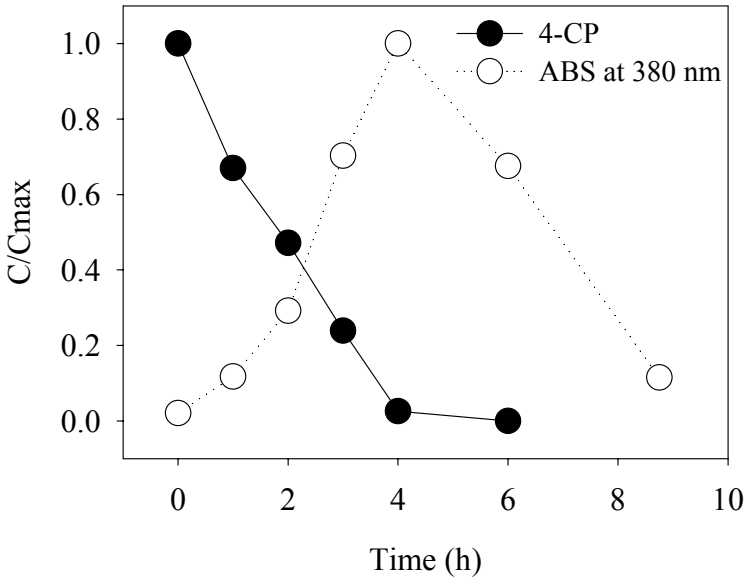

Figure 3.24. Relative concentrations of 4-CP and CHMS

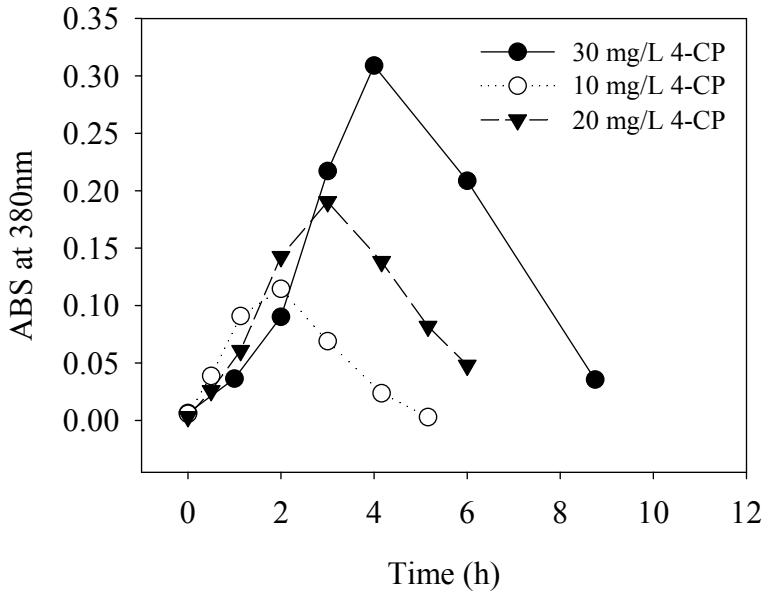

Figure 3.25. Change of CHMS at different initial 4-CP concentrations

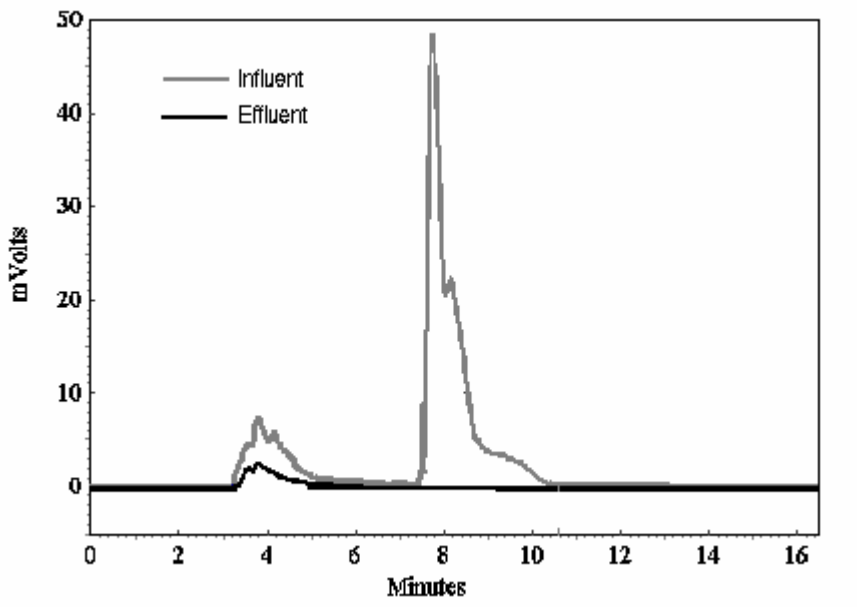

Figure 3.26. HPLC diagrams of the batch reactor receiving 100 mg/L 4-CP

3.2.2. Kinetics of 4-CP Degradation Using Culture (Culture M) Acclimated to Mixture of 4-CP and 2,4-DCP

Another set of batch experiments was conducted to determine 4-CP degradation kinetics using the culture acclimated to the mixture of 4-CP and 2,4-DCP (Culture M). Biomass was drawn from the FEDM fed with 4-CP, 2,4-DCP and peptone with the concentrations of 200, 100 and 400 mg/L, respectively.

Batch kinetic assays were run similar to those given in previous section. Initial biomass concentrations were adjusted to around 200±10 mg MLVSS/L and 4-CP was added as a sole carbon source. After inoculation of biomass, reactors were operated in an orbital shaking incubator at 200 rpm and 25±1°C. During the operation, samples were drawn at different time intervals for the analysis of pH, CHMS and 4-CP.

Figure 3.27 shows time course variation of 4-CP and SDR at different initial 4-CP concentrations. Biodegradation of 4-CP was complete up to 200 mg/L, whereas, only partial removal was observed within around 1d when initial concentration was increased to 270 mg/L. The variation of SDR with initial 4-CP concentration showed that 4-CP inhibited its own transformation at high concentrations. Hence, Andrews model was applied to the experimental data to define the kinetics of 4-CP degradation. Andrews equation gave excellent fit to the experimental data with r^2 of 0.988. The observed kinetic parameters were q_m= 130.3 mg 4-CP/(g MLVSS.h), K_s= 34.98 mg/L, and K_i= 79.74 mg/L. The fraction of biomass having ability to degraded 4-CP was assumed to be fraction of chlorophenols (4-CP+2,4-DCP) in the feed on COD basis. The parent bioreactor, which was FEDM receive chlorophenols in the feed with a fraction of 0.524. If it is considered that the specialists are only responsible for 4-CP degradation, the equation takes the form of;

$$\frac{d(S)}{d(t)} = -\frac{248.49SXa}{1.104 + S + S^2/194.4} \qquad (3.12)$$

Where, *a* is fraction of chlorophenols in feed on COD basis. As it was noted above, for this set of experiments, the value of *a* was 0.524. The observed kinetic parameters for two different mixed cultures were summarized in Table 3.3. It will be useful to note again, the q_m values were calculated considering only specialist biomass in the sludge.

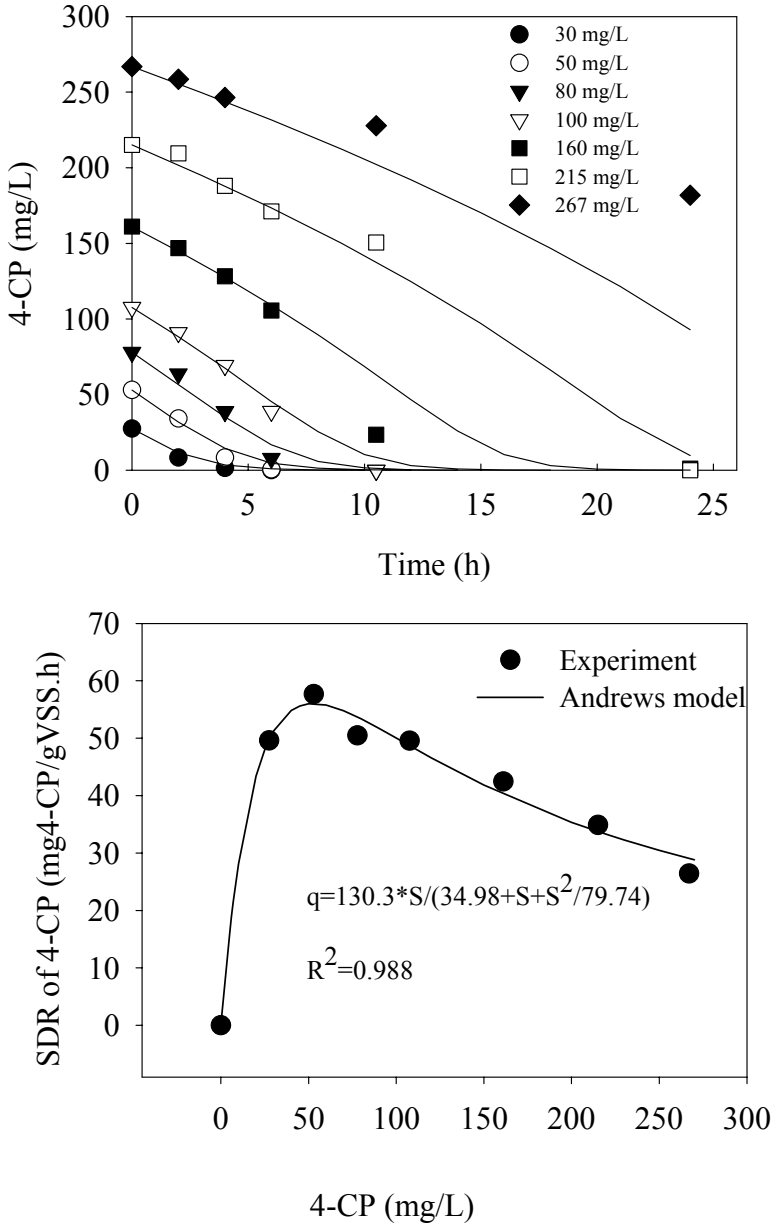

Figure 3.27. Time course variation of 4-CP concentrations and SDRs of culture M at different initial 4-CP concentrations

Table 3.3. Andrews kinetic parameters of 4-CP degradation

Culture	q_m (mg 4-CP/(g MLVSSc.h))	K_s (mg/L)	K_i (mg/L)	R^2
Culture 4	92	1.104	194.4	0.986
Culture M	248.49	34.98	79.74	0.988

Analyses of the table can yield some significant conclusions. First of all, 4-CP degradation can be sufficiently well described using Andrews equation for both cultures as r^2 values were higher than 0.98. Second conclusion is that the maximum specific degradation rate of 4-CP with culture M is almost three times higher compared to that of culture 4. Third, although culture M has higher maximum SDR, Culture 4 has the higher sensitivity due to lower K_s value.

For self-inhibitory compounds, there is a critical substrate concentration, $S_{crt} = \sqrt{K_s K_I}$, above which the substrate removal rate falls (Tomei et al., 2004). Therefore, the critical 4-CP concentrations for culture M and culture 4 were observed to be 52.81 and 14.65 mg/L. Attained maximum degradation rates at critical substrate concentrations can, therefore, be calculated as around 56 and 36 mg 4-CP/(g MLVSS.h) for culture M and 4, respectively. Therefore, the degradation rate of 4-CP at critical concentration was around 1.5 times higher in the case of culture M. During the incubation of the reactors, pH of the medium decreased slightly, especially at high initial 4-CP concentrations (Figure 3.28). The lowest pH at the end of the incubation was observed to be around 6.5 and pH adjustment was not required.

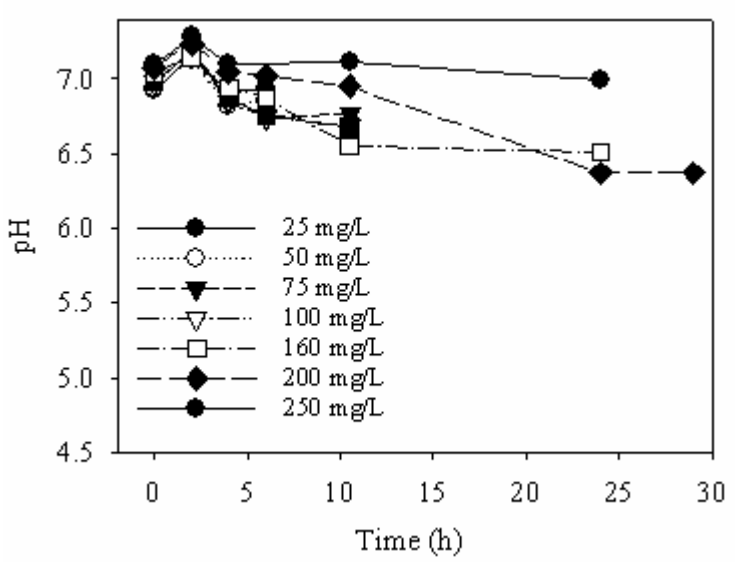

Figure 3.28. Time course variations of pH at different initial 4-CP concentrations

In addition, variation of CHMS concentrations was followed at different initial 4-CP concentrations. Similar to the results obtained in the case of culture 4, CHMS concentration increased to a peak value and then it decreased to its original value, which indicated complete mineralization of 4-CP. Also, peak value of CHMS increased with increasing initial 4-CP concentration (Figure 3.29).

3.2.3. Effect of 2,4-DCP on 4-CP Degradation Kinetics of 4-CP Acclimated culture (Culture 4)

It is known that chlorophenols are generally found in mixtures in the contaminated sites or industrial wastewaters, hence, studying the ability of the culture towards the degradation of chlorophenols mixtures, rather than a single compound, will be more meaningful and informative. In this respect, the effect of a sudden appearance of a compound (2,4-DCP), which has similar structure to the target compound, on the 4-CP degradation ability of culture was deeply investigated.

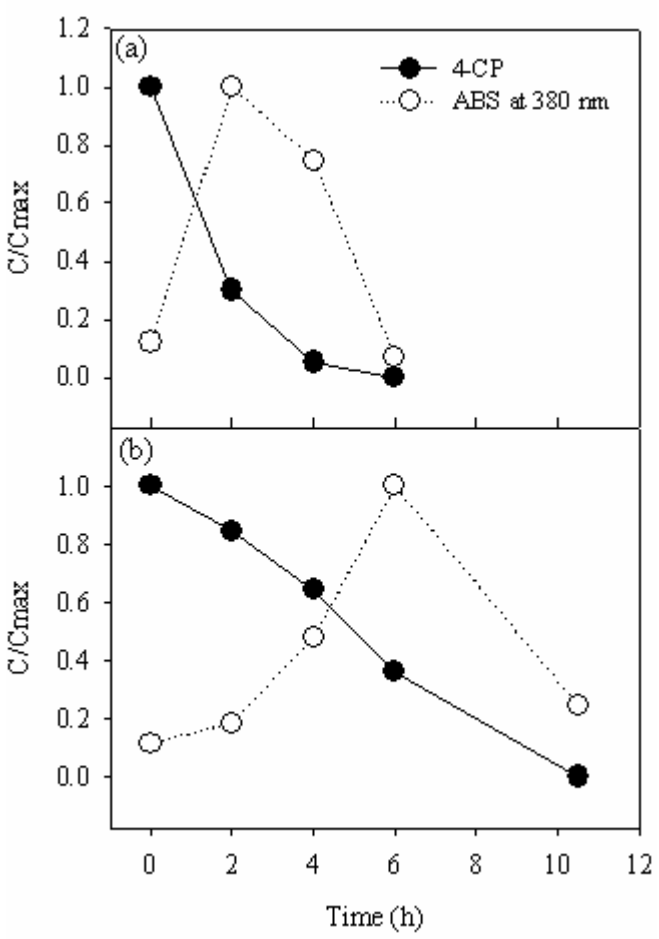

Figure 3.29. Time course variations of normalized 4-CP and CHMS concentrations for 50 and 100 mg/L initial 4-CP concentrations

Acclimated culture (culture 4) for batch assays was obtained from the instantaneously fed SBR receiving 220 mg/L 4-CP and 400 mg/L peptone (FED4). Chlorophenols were supplied to batch reactors as a sole carbon source. To explore the presence of 2,4-DCP on 4-CP degradation kinetics, 4-CP concentration was kept constant at 100 mg/L and 2,4-DCP concentrations were varied. Biomass taken from FED4 was washed two times and then batch reactors were inoculated to have an initial MLVSS concentration close to 250 mg/L.

Control reactor results showed that the removal of 4-CP and 2,4-DCP via volatilization within the incubation period was negligible (data not shown). Also,

the removal of chlorophenol via adsorption on the biomass was not detected. Time course variations of 4-CP and 2,4-DCP were presented in Figure 3.30.

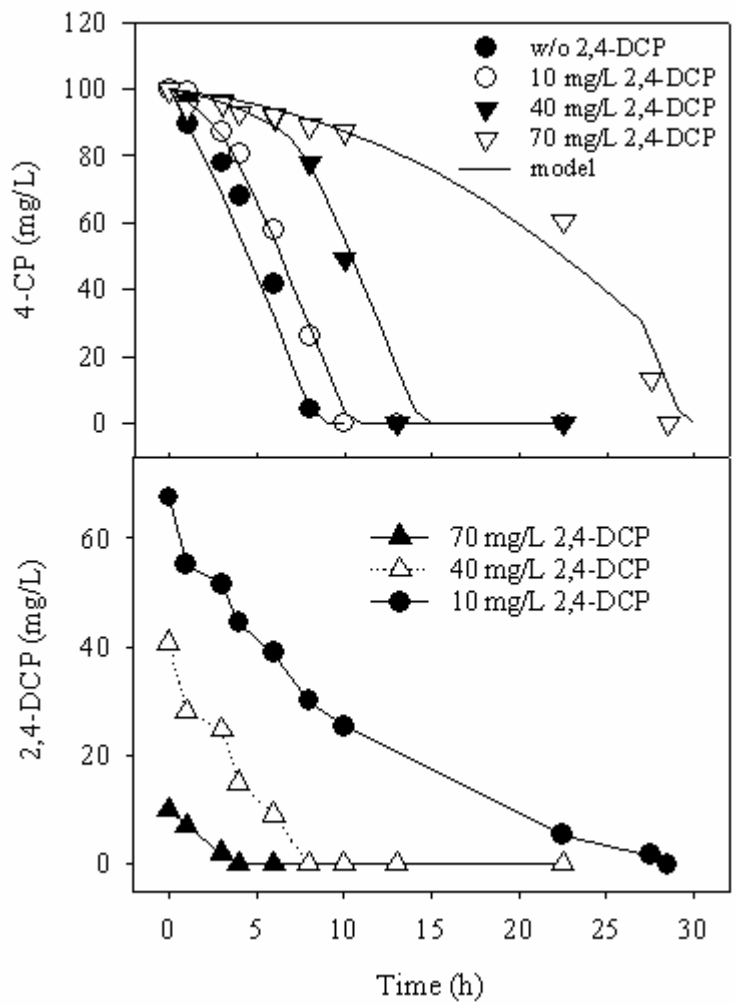

Figure 3.30. Time course variations of 4-CP and 2,4-DCP

As it can be seen from the figure, although 4-CP was degraded completely in all reactors, time required to have complete removal of 4-CP increased exponentially with increasing 2,4-DCP concentrations (Figure 3.31). The reason of such observation can be attributed to the linear decrease in the initial 4-CP degradation rate with increasing initial 2,4-DCP concentrations in culture medium (Figure 3.32). Therefore, the addition of 2,4-DCP have an adverse effect on the degradation of 4-CP. Beside the inhibitory effect of 2,4-DCP addition on 4-CP degradation, it is interesting to observe complete 2,4-DCP degradation as the culture had been

acclimated to 4-CP only. Another interesting point is that similar to instantaneously fed SBR experiments, 4-CP degradation was almost completely inhibited by 2,4-DCP and rapid removal of 4-CP started only after complete removal of 2,4-DCP.

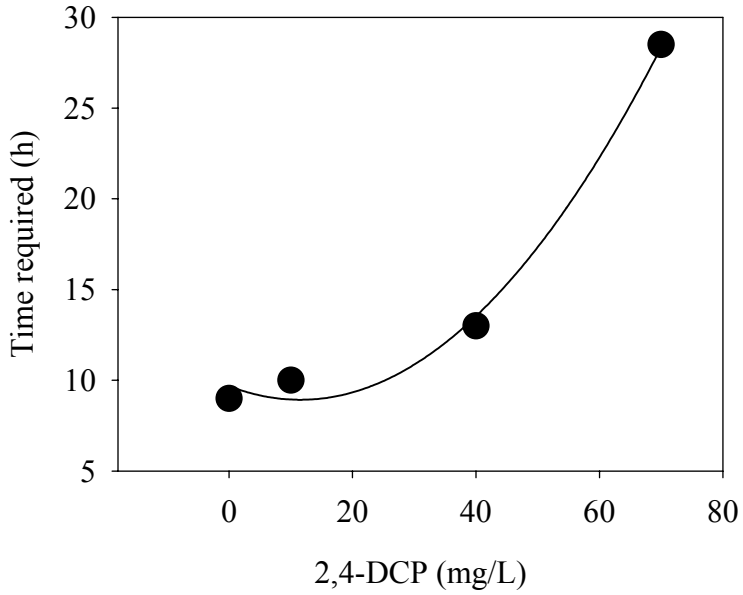

Figure 3.31. Time required to have complete 4-CP degradation at different 2,4-DCP concentrations

During the degradation, the pH of the culture medium decreased from its initial value of around 7.2 to 6.6-6.4 due to HCI production from chlorophenol degradation (Figure 3.33). Although a noteworthy decrease in pH was observed, no attempt was made to regulate pH as it was not low enough to create suboptimal environmental conditions for biomass.

Equation 3.11 was used to predict time course variation of 4-CP in the absence of 2,4-DCP. Similar to FEDM results, competitive inhibition of 2,4-DPC on 4-CP degradation was observed with culture 4. Therefore, Equation 3.3 was used to predict time course variation of 4-CP in the presence of 2,4-DCP.

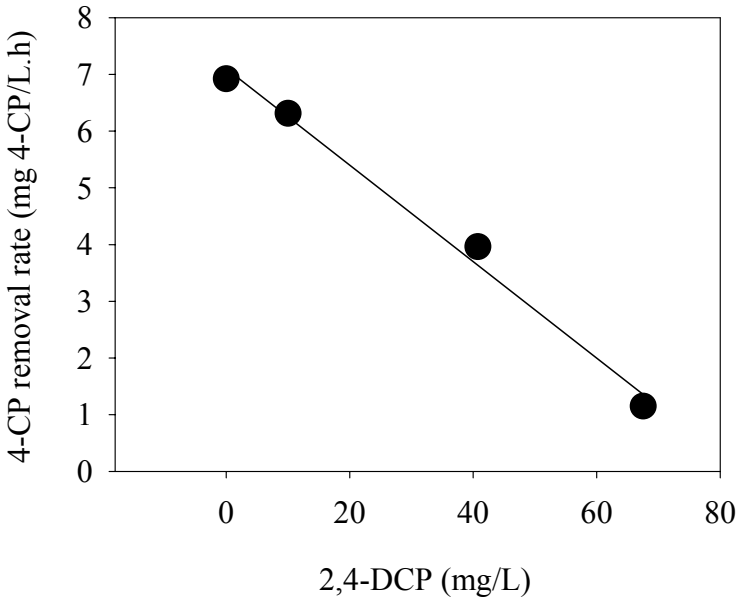

Figure 3.32. Initial degradation rate of 4-CP at different initial 2,4-DCP concentrations

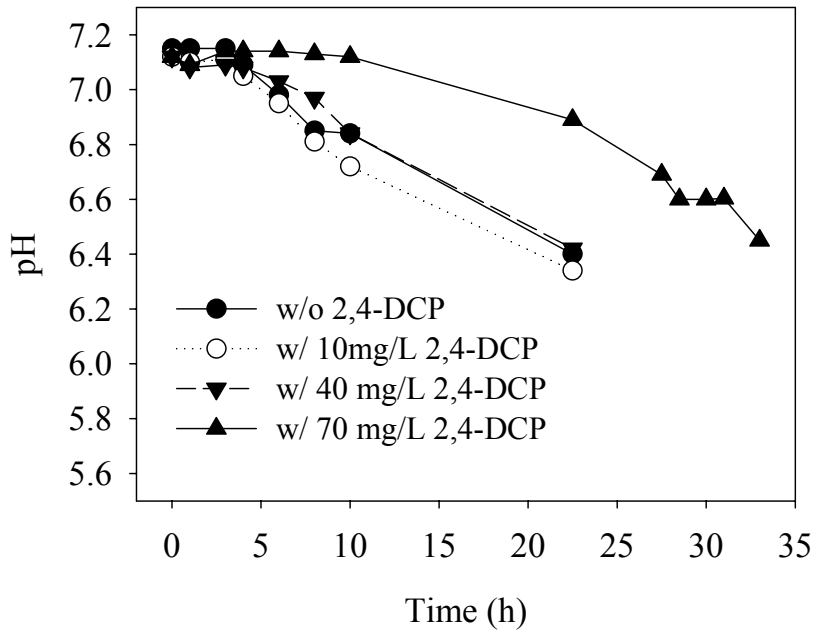

Figure 3.33. pH variations at different initial 2,4-DCP concentrations

The values of qm, K_s and K_i were previously estimated (Equation 3.11) in the absence of 2,4-DCP. Therefore, K_{ii} value should be predicted to model time course variations of 4-CP concentrations. To minimize the errors in the estimation of K_{ii} value, which determines the toxic effect of 2,4-DCP on 4-CP degradation, degradation of 2,4-DCP was not modeled. Hence, the best fitting equation to the experimental data of 2,4-DCP was used in model equation. The value of K_{ii} was determined as 0.038 mg/L (Equation 3.13) using the data obtained for 10 mg/L 2,4-DCP and the model was validated using data obtained for other 2,4-DCP concentrations. Observed differential equations were numerically solved using POLYMATH 4.02. The program used the Runge-Kutta-Fehlberg (RFK) numerical integration routine.

$$\frac{d(S)}{d(t)} = -\frac{145.27SXa}{(1+\frac{I}{0.038})1.104 + S + S^2/194.4} \qquad (3.13)$$

It was observed that the developed model gave excellent fit to the experimental data as model tracked experimental data well (Figure 3.30). Hence, the effect of 2,4-DCP on the 4-CP degradation by culture 4 was modeled and the time course variation of 4-CP concentrations was estimated accurately even in the presence of 2,4-DCP. It is important to note that the value of K_{ii} for culture M was 0.17 mg/L (Equation 3.6), which is almost one order of magnitude higher than that for culture 4 (0.038 mg/L). Therefore, it can be concluded that 2,4-DCP is more toxic to culture 4 compared to culture M, as culture 4 was acclimated to 4-CP only.

In addition to chlorophenol concentrations, time course variations of CHMS were followed and it was observed that as 4-CP was removed from the medium, concentration of CHMS increased (Figure 3.34), which reached the peak value when 4-CP was just completely removed. After reaching a peak value, CHMS concentrations began to decrease sharply and finally complete CHMS removal was observed, which means that chlorophenols degraded without remaining any

byproduct in the medium. Similar trend of CHMS was also observed in instantaneously fed SBR (FED4) from which biomass taken for batch assays.

Although there are some studies on the degradation kinetics of 4-CP (Tarighian et al., 2003; Hao et al., 2002; Hill et al., 1996; Sae´z and Rittmann, 1991, 1993; Wang and Loh, 1999, 2001; Sahinkaya and Dilek, (2005); Farrell and Quilty, 1999), so far little or no work has been conducted to describe the effect of 2,4-DCP, which may appear in the effluent of industrial wastewater together with 4-CP, on 4-CP degradation kinetics of acclimated mixed culture. Therefore, the results of this work may help operation engineer to minimize the adverse effect of a sudden 2,4-DCP appearance in the influent of a treatment plant receiving 4-CP containing wastewater.

The type of environmental conditions imposed is one of the major factor affecting measured kinetic parameters, especially when the examined is a mixed culture. Continued cultivation of mixed culture results in selection of some members of the community and this change may have great impact on the kinetic parameters measured (Grady et al., 1996). Therefore, after a long period cultivation of a mixed culture under stress conditions, the knowledge on the dominant member of community may lead to a better understanding of the system. In this context, isolation and identification studies showed that two gram negative species, *Pseudomonas vesicularis* and *Pseudomonas stutzeri*, were dominant in the 4-CP acclimated mixed culture (Culture 4) as shown in Table 3.1.

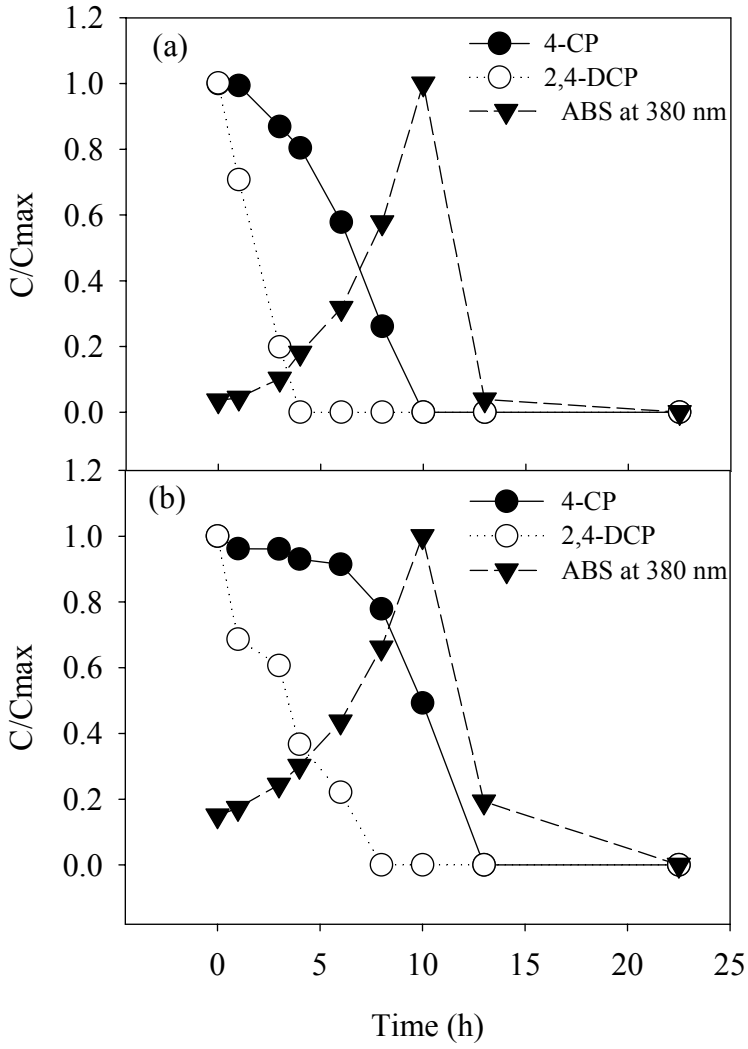

Figure 3.34. Relative concentrations of 4-CP, 4-CP and CHMS for reactors receiving 10 mg/L 2,4-DCP (a) and 40 mg/L 2,4-DCP (b)

3.2.4. Degradation of 4-CP by Pure Cultures Isolated From Acclimated Mixed Cultures

Colonies isolated from FED4 aseptically taken from solidified agar plates were inoculated in a growth medium containing 1000 mg/L peptone and 50 mg/L 4-CP. The colonies isolated from FEDM were incubated in a medium containing 1000 mg/L peptone and mixture of 50 mg/L 4-CP and 25 mg/L 2,4-DCP. Peptone

concentration was kept at high concentration to obtain a dense culture. After observation of a dense culture, the color of the medium was the same with the colonies on agar plates (Figures 3.9 and 3.35). When microorganisms were in their logarithmic phase of growth, biomass samples taken from the reactors were centrifuged and washed two times with distilled water. The batch reactors containing 70 or 120 mg/L 4-CP as a sole carbon source were inoculated to have around 50 mg/L of biomass. Figure 3.36 shows time course variation of remaining 4-CP in the reactors. Although almost no degradation was observed for the three cultures, only *Pseudomonas sp.* was able to degrade 4-CP. Complete degradation of 70 mg/L 4-CP took around 100 h. When the initial 4-CP concentration was around 120 mg/L, again only *Pseudomonas sp.* showed measurable degradation ability (data were not shown), which was around 22% within 150 h. Therefore, increasing the initial 4-CP concentration caused decrease in 4-CP degradation efficiency, most probably due to self inhibitory effect of 4-CP.

Figure 3.35. Isolated pure species grown in erlenmeyers

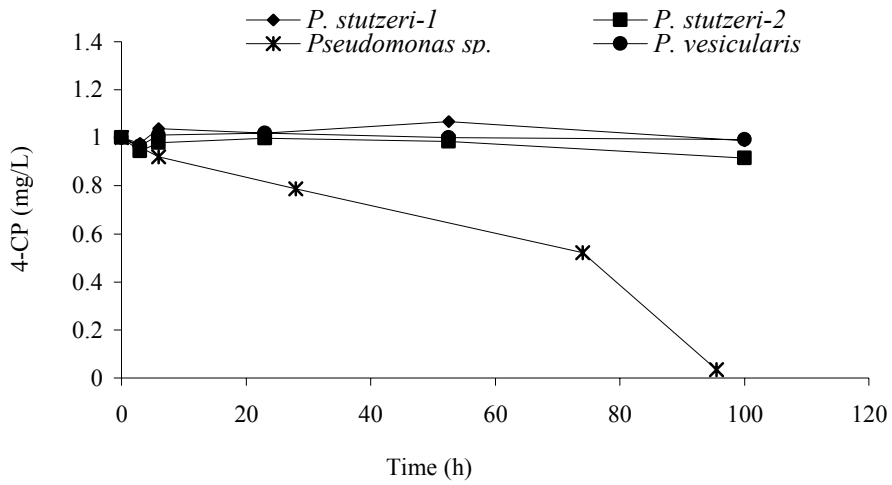

Figure 3.36. Degradation of 4-CP by isolated pure strains

3.2.5. Biodegradation Kinetics of 2,4-DCP by acclimated mixed cultures

The main goal of this set of experiments was to investigate the biodegradation kinetics of 2,4-DCP using two different mixed cultures; one (culture M) was previously acclimated to mixture of 4-CP and 2,4-DCP, the other (culture 4) was acclimated to 4-CP only. Also, degradation ability of pure strains isolated from acclimated mixed cultures was investigated.

The growth conditions of biomass used for batch degradation assay can greatly influence the observed kinetic parameters, even with pure cultures (Ellis et al., 1996a and b; Ellis and Anselm, 1999; Grady et al., 1996). Thus, the kinetic parameters for a particular culture and substrate are not necessarily constant and may reflect previous growth conditions (Ellis et al., 1996). In this context, parent bioreactors used for biomass source for batch experiments should be operated within the limits of real full-scale treatment processes. Therefore, in our study, SRT of parent bioreactor was selected as 10 d to represent a real full-scale application. Also, biomass for batch tests was drawn when parent reactors were in steady-state operation to supply biomass with constant characteristics for different batch assays as suggested by Sae´z PB and Rittmann, (1993).

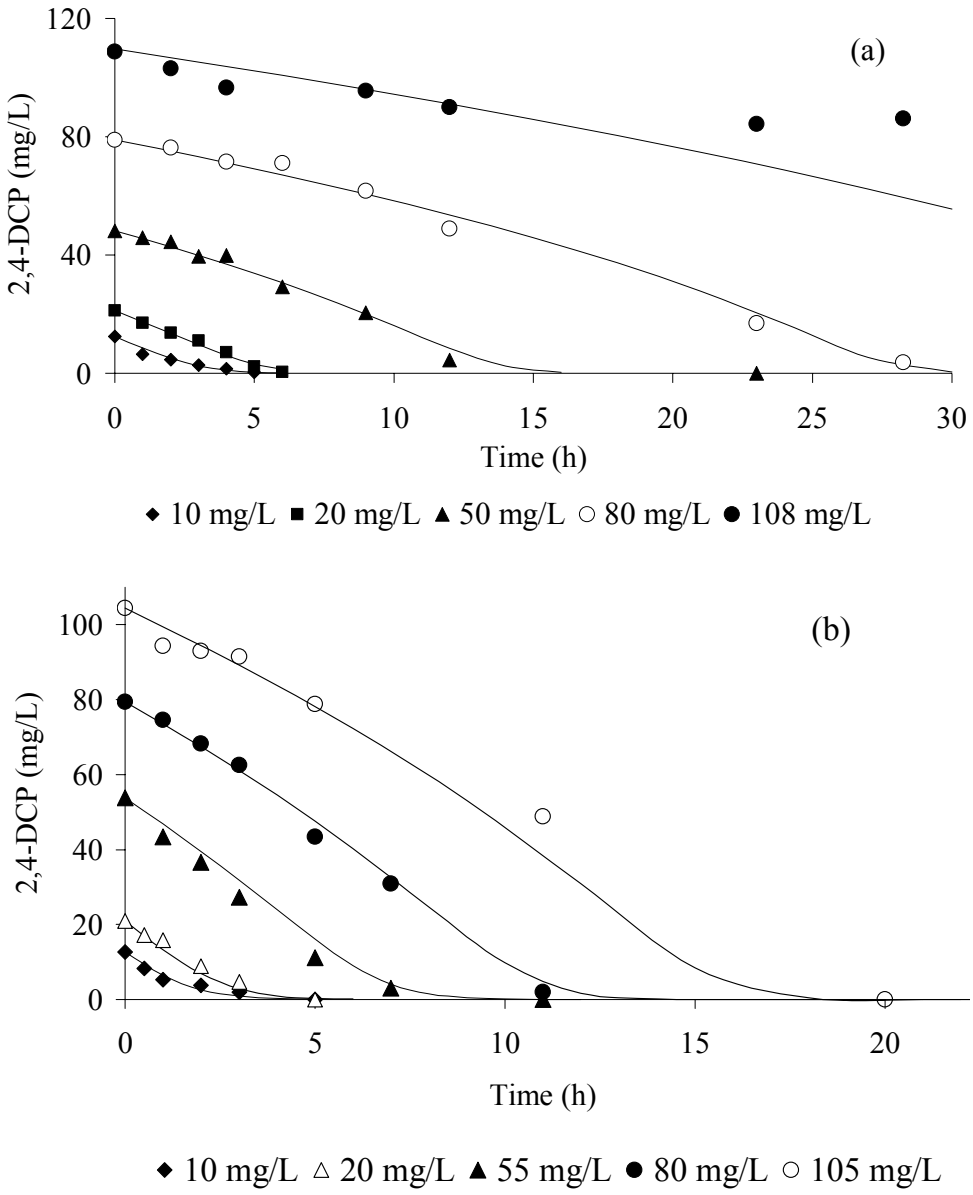

Figure 3.37. Time course variation of 2,4-DCP for culture 4 (a) and culture M (b) (data points represent experimental data, while, smooth curves are optimized fit of Andrews equation)

In order to avoid the change of culture history and correctly estimate kinetic parameters, experiments were carried out at an initial biomass concentration of 220±10 and 150±10 mg/L for reactors inoculated with culture 4 and culture M,

respectively. Therefore, 2,4-DCP/biomass ratios ranged between 0.05-0.55 on COD basis.

Batch experiments were conducted in a media containing 2,4-DCP, ranging from 12.5-108.8 mg/L. Samples taken from the reactors at various time intervals were analyzed for 2,4-DCP (Figure 3.37). Control reactor results showed that the removal of 2,4-DCP via volatilization within the incubation period was negligible. Degradation of 2,4-DCP started without lag phase. Similarly, Sahinkaya and Dilek (2002) reported that lag phase observed for unacclimated culture disappeared after acclimation. Except the studied highest concentration of 108.8 mg/L, removal of 2,4-DCP was almost complete for all concentrations for culture 4 (Figure 3.37(a)). Culture M was able to degrade 2,4-DCP more efficiently than culture 4 (Figure 3.37). Although only 21% removal (Figure 3.37(a)) was observed for 108.8 mg/L of 2,4-DCP by the culture 4, culture M was able to remove 104.4 mg/L 2,4-DCP completely (Figure 3.37(b)). Time required to achieve complete degradation of 2,4-DCP increased linearly with initial 2,4-DCP concentration (Figure 3.38). For culture 4, when the initial concentration of 2,4-DCP was 12.5 mg/L, time required for complete degradation was only 5 h, which increased to 16 and 30 h when the initial 2,4-DCP concentration was increased to 50 and 80 mg/L, respectively. Compared to culture 4, culture M required much less time to remove the same amount of 2,4-DCP as complete removal of 80 mg/L 2,4-DCP took around 30 h for the culture 4, whereas, it was only 13 h for the culture M. Therefore, time required for complete degradation of 2,4-DCP at any concentration is about two times shorter for the culture M compared to culture 4 (Figure 3.38).

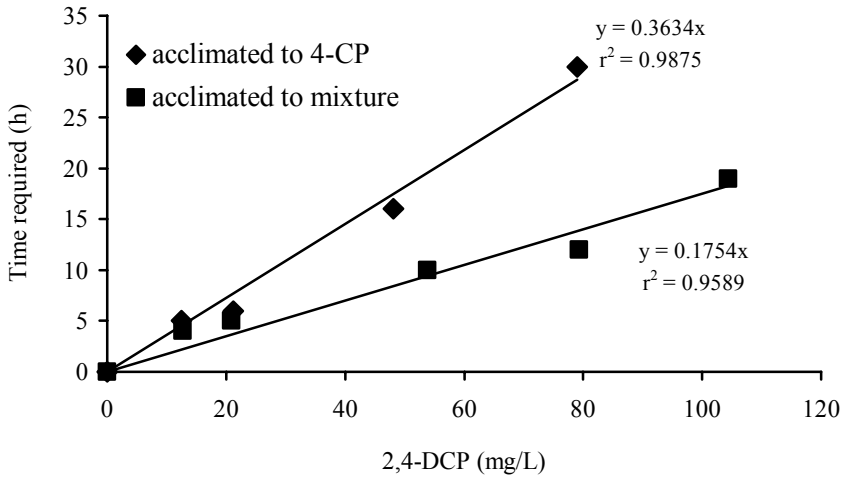

Figure 3.38. Required time to achieve complete degradation of 2,4-DCP

Culture pH is an important parameter in chlorophenol degradation (Wang and Loh, 1999). Although slightly higher decreases were observed at higher 2,4-DCP concentrations, the monitored values were always between 7.2-6.8 and pH adjustment was not required.

Figure 3.39 shows relation between specific degradation rate (SDR) and initial concentration of 2,4-DCP. The decreased SDR with increasing initial 2,4-DCP concentration implies that 2,4-DCP acts as an inhibitor. Therefore, the Andrews substrate inhibition was used to model the inhibition of 2,4-DCP on its own transformation. In modeling, only the substrate utilization term was considered and the biomass production from the utilization of 2,4-DCP was neglected due to the relatively higher initial concentration of biomass, which was 220±10 and 150±10 mg/L for reactors inoculated with culture 4 and culture M, respectively. The accuracy of the assumption was also validated with the measurements of MLVSS concentrations at the end of the degradation assays as the biomass increase remained within the range of 0-16% depending on initial 2,4-DCP concentrations. Similarly, Rutgers et al. (1997) reported that the growth yield coefficients on chlorinated phenols are lower than those of heterotrophic growth on non-chlorinated compounds. They studied growth yield coefficient of *Sphingomonas* sp.

strain P5 on various chlorophenols in chemostat culture and reported the growth yield coefficients within the range of 0.255-0.11 molC/molC. Using the given range of yield coefficient and taking average biomass weight 113 g/mol (Rittmann and McCarty, 2001), the possible increase in biomass concentration even at 100 mg/L of 2,4-DCP, which is the studied highest concentration, can be calculated in the range of 10-20 mg/L, which can be neglected considering much higher initial biomass concentrations in the batch assays. It should also be emphasized that the community structure of our original mixed culture may be relatively constant during the batch experiments due to lower biomass growth and, the obtained kinetic parameters will be representative for the original mixed culture taken out from the parent bioreactor. Chudoba et al. (1992) also reported this fact as substrate to biomass concentration should be selected not to allow substantial cell multiplications and, the kinetic constants obtained only under these conditions can be safely used for the activated sludge plant design and operation.

Application of experimental data to the Andrews equation (Equation 3.10) gave excellent fit (Figure 3.39) as r^2 was observed to be higher than 0.99 for both cultures (Table 3.4). The kinetic parameters for culture 4 were obtained as q_m=38.83 mg 2,4-DCP/(g MLVSS.h), K_s=7.72 mg/L, and K_i= 22.81 mg/L. Therefore, the observed kinetic equation is;

$$\frac{d(S)}{d(t)} = \frac{-38.83SX}{7.72 + S + S^2/22.81} \qquad (3.14)$$

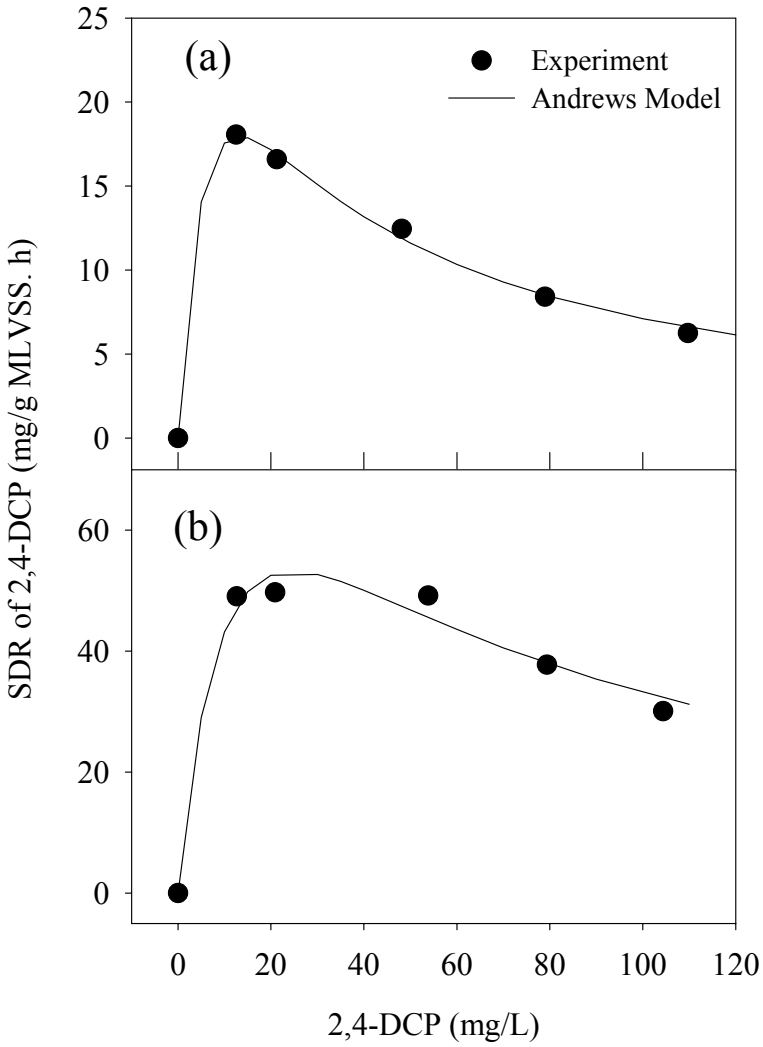

Figure 3.39. Specific initial degradation rate of 2,4-DCP by culture 4 (a), and culture M (b)

By solving the model established, the 2,4-DCP concentration profiles can be simulated for different initial 2,4-DCP concentrations. Although predicted values using Andrews equation gave good fit to the experimental data (Figure 3.37(a)), a slight discrepancy was observed at the highest concentration studied (108.8 mg/L). The similar discrepancy of Andrews equation was also noted by Hao et al. (2002) during the degradation of phenol at high concentrations using a pure culture of *Acinetobacter sp.* Moreover, only a partial removal of 2,4-DCP (21%) (Figure

3.37(a)) was observed at the end of 30 h incubation due to toxic effect of 2,4-DCP at the highest studied concentration (108.8 mg/L).

Table 3.4. Andrews Model parameters of 2,4-DCP biodegradation

Sludge	q_m (mg/(g MLVSS.h))	K_s (mg/L)	K_i (mg/L)	C_{crt} (mg/L)	r^2
Culture 4	38.83	7.72	22.81	13.27	0.997
Culture M	112.4	13.77	44.46	24.74	0.996

Meanwhile, in the case of culture M, the dependence of SDR on the initial 2,4-DCP concentration displayed a similar trend (Figure 3.39(b)) to that of the culture 4 and Andrews model was used to describe the experimental data. Andrews equation (Equation 3.10) gave good fit to the experimental results and kinetic constants were observed as q_m=112.4 mg 2,4-DCP/(gMLVSS.h), K_s=13.77 mg/L, and K_i= 44.46 mg/L. Therefore, the model equation is;

$$\frac{d(S)}{d(t)} = \frac{-112.4SX}{13.77 + S + S^2/44.46} \qquad (3.15)$$

The model well fitted to the experimental data (Figure 3.37(b)) and it can be concluded that the model could be used to estimate variations of 2,4-DCP concentrations with time.

Critical 2,4-DCP concentrations, $S_{crt} = \sqrt{K_s K_I}$, were calculated as 13.27 and 24.74 mg/L for culture 4 and culture M, respectively. The corresponding removal rates were 17.95 and 53.19 mg/(g.MLVSS.h). Therefore, the obtained maximum removal rate for culture M is around three times higher compared to culture 4.

The parameters of the model estimated from the experimental data are listed in Table 3.4. The observed maximum specific degradation rate constant for culture M is around 3 times higher than that for culture 4. Small K_s values were obtained for both cultures, which means that maximum observed degradation rate could be reached at very low 2,4-DCP concentrations if substrate inhibition has not been a factor. Observed K_i values were also low, which means that 2,4-DCP is toxic to the cells even at low concentrations.

Although unacclimated sludge cannot degrade 2,4-DCP (Sahinkaya and Dilek, 2002), 4-CP acclimated culture was able to degrade 2,4-DCP without any lag phase due to structural similarity between 4-CP and 2,4-DCP. Culture acclimated to a specific chlorophenol may have ability to degrade other types of chlorophenols due to structural similarity (Sahinkaya and Dilek, 2002; Quan et al., 2004c); however, there is no information in the literature on kinetics of such ability. Therefore, in the present study, Andrews equation parameters were estimated for two different acclimated activated sludge cultures. Also, the proposed model can be helpful for understanding the behavior of biological processes and predicting the 2,4-DCP concentrations in the treatment systems.

Table 3.5. SDR of 2,4-DCP for different cultures

2,4-DCP (mg/L)	Culture	SDR (mg/(gMLVSS.h))	SDR[a] (mg/(gMLVSS.h))	Reference
75	Culture 4	8.84	19.64	This study
100		7.11	15.80	
75	Culture M	39.15	75.28	This study
100		33.18	63.80	
75	Culture 24	9.54	63.60	Sahinkaya and Dilek (2002)
100		8.68	57.87	

[a] When the specialist biomass concentrations were taken into account

The inhibition of 2,4-DCP on its own degradation was also reported in our previous work (Sahinkaya and Dilek, 2002). In this previous study, degradation of 2,4-DCP was investigated with a culture previously acclimated to 2,4-DCP (Culture 24) and it was observed that the degradation rate of 2,4-DCP decreased linearly with increasing concentrations. Using the data in our previous work (Sahinkaya and Dilek, 2002) and the present study, the calculated SDRs of 2,4-DCP for two different inlet concentrations are given in Table 3.5. The analyses of the table could yield two important conclusions. First, SDRs of culture 4 and culture 24 are similar, whereas, SDR of culture M is almost four times higher compared to those of culture 4 and culture 24. Although there may be several reasons of this, two of them seem to be particularly important. First, the feed compositions of the parent bioreactors used to supply biomass to the batch assays were different. Hence, the type of species dominant in the cultures may be different (Grady et al., 1996). Second, even the dominant species were similar; their enzyme systems may be different, as adaptation by enzyme regulation does not require any change in community structure (Rittmann and McCarty, 2001). The fraction of total chlorophenol in the feed of SBR receiving both 4-CP and 2,4-DCP was the highest. Therefore, one

reason of observing higher substrate removal in the case of culture M may be the presence of comparably higher fraction of specialist biomass within the community. The COD fraction of total chlorophenols in the feed of the parent bioreactor (FEDM) used as the source of culture M was around 0.52, and it was 0.45 and 0.15 for the culture 4 and culture 24, respectively. Considering these fractions, SDRc values for 100 mg/L 2,4-DCP can be computed as 63.8, 15.8 and 57.87 (mg/(gMLVSSc.h)) for culture M, culture 4 and culture 24, respectively. Therefore, when the specialist biomass concentrations were considered, there is no so much difference between SDRc values of culture M and culture 24, and both cultures have the higher SDRc values than culture 4. The observation of low SDR value for culture 4 is reasonable, as the culture was not previously exposed to 2,4-DCP, but only to another structurally similar compound, 4-CP.

HPLC results of influent and effluent samples indicated complete removal of 2,4-DCP without any by-product. As an example, Figure 3.40 shows HPLC diagrams of selected one batch experiment.

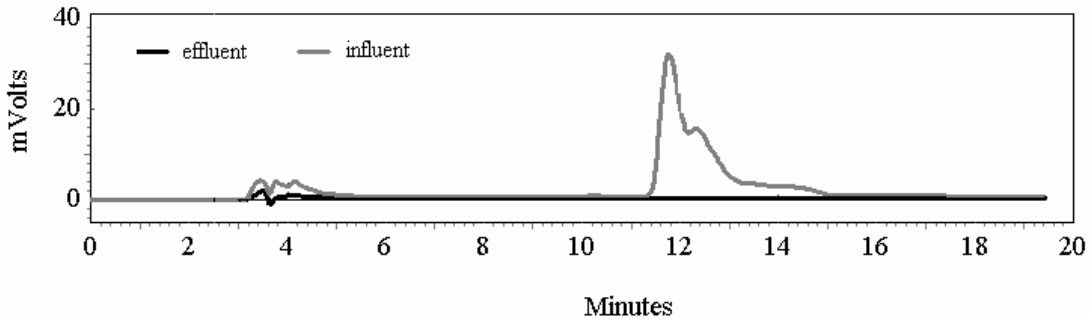

Figure 3.40. HPLC diagram of influent and effluent of batch experiments inoculated with culture M receiving 104.4 mg/L 2,4-DCP

Although complete mineralization 2,4-DCP was observed in our study, accumulation of dichlorocatechols was detected (based on HPLC results) during the degradation of 2,4-DCP by *Micrococcus A* (Gallizia et al., 2003). Therefore, mixed

culture was also important to achieve complete mineralization of toxic compounds to CO_2.

In the degradation of toxic chemicals, the ability of microbes to form good settling flocs is especially important. Flock forming microbes have distinct advantages over the nonflocculating ones as they remain within system for longer period of time through biomass recycling (Farrell and Quilty, 2002). Both of the cultures used in our study had good settling and flock formation ability. A clear supernatant was observed when the culture was allowed 30 minutes to settle at the end of the experiments. The biomass concentration in the supernatant was lower than 25 mg/L, and the index of flocculation (calculated using Equation 2.1) was higher than 86% for all reactors.

3.2.6. Degradation of 2,4-DCP by Pure Cultures Isolated From Acclimated Mixed Cultures

Isolated and identified pure strains (Table 3.1) were inoculated to the reactors in which 2,4-DCP was supplied as a sole carbon and energy source. The stock solution of 2,4-DCP was used to adjust initial 2,4-DCP concentrations as around 30 mg/L. Although almost nil degradation was observed for 4 d of incubation, complete degradation of 2,4-DCP was attained for all strains within 7 d (data not shown). Also, control reactor validated that all removal was biotic as no evaporation loss was detected in control reactor. In the second spike experiment, degradation of 2,4-DCP started without lag period and removal of 2,4-DCP was almost complete within only 3 or 6 h depending on the strain (Figure 3.41). Using the data belonging to the second spike experiment, the specific degradation rates of pure species are comparatively given in Table 3.6.

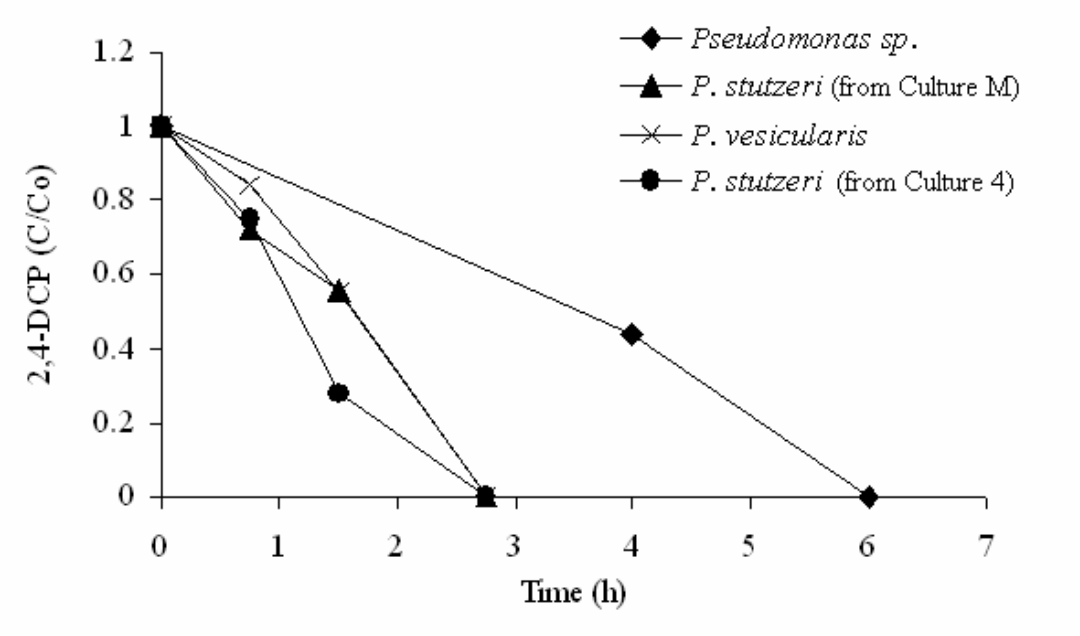

Figure 3.41. Time course variations of 2,4-DCP for reactors inoculated with pure strains isolated from acclimated mixed cultures (second spike)

Taking the initial 2,4-DCP concentration of 30 mg/L, the SDR for culture 4 and M can be calculated using the obtained Andrews Equation constants as 15.1 and 52.68 mg/gMLVSS.h in respective order. Although degradation rates of pure strains are much higher compared to mixed cultures (Table 3.6), pure cultures did not settle under quiescent conditions, and therefore, it would be difficult to use them in suspended type of reactors.

Table 3.6. SDRs of 2,4-DCP using isolated pure bacterial cultures

Pure strain	*Pseudomonas sp.*	*P. stutzeri*[1]	*P. vesicularis*	*P. stutzeri*[2]
SDR of 2,4-DCP (mg/g MLVSS.h)	83.3± 9.35	179.1± 16.88	174.1± 8.4	200.3 ±9.7

[1] isolated from culture 4; [2] isolated from culture M

There are also some other studies in the literature on 2,4-DCP degradation using pure cultures. Gallizia et al. (2003) reported that *Micrococcus A* is capable of

removing 100 mg/L 2,4-DCP within 10 days and the observed SDR was around 3.68 mg/(g biomass h). Kargi and Eker (2004) reported that complete degradation of 2,4-DCP by *Pseudomonas putida* was achieved when its initial concentration was 30 mg/L, and removal efficiency decreased sharply further increasing the concentrations. They also reported that SDR of 2,4-DCP were within 0.7-1.1 mg/(g biomass.h) depending on initial concentrations. The removal efficiency of 10 mg/L 2,4-DCP by *Bacillus insolitus* is only 42% at the end of 16 d incubation (Wang et al., 2000). Similarly, Buitron et al. (1998) pointed that 2,4-DCP-degradation rate of acclimated mixed culture was 25.4 mg/(g MLVSS. h), whereas, it was in the range of 0.67-1.66 mg/(g MLVSS.h) for pure cultures, which were isolated from acclimated activated sludge. Contrary to these findings, we have observed much higher SDRs for the isolated and selected pure strains compared to the acclimated mixed cultures and the literature findings. There are two possible reasons of such an observation; first, strains were isolated from acclimated mixed cultures, which were exposed to chlorophenols for 5 months. Second, the isolated pure cultures were also stored on agar plates containing 4-CP and/or 2,4-DCP and the pure cultures were pre-exposed to 2,4-DCP in first spike experiments.

3.3. Effect of Filling Time on the Performance of Sequencing Batch Reactor (SBR) Treating 4-CP and 2,4-DCP Mixture

SBRs offer an attractive alternative to conventional biological wastewater treatment systems, mainly because of their simple and flexible operation and cost effectiveness for small-scale treatment facilities (Mangat and Elefsiniotis, 1999; Chiavola et al., 2004). The key difference is that the reactor volume of SBRs varies with time but remains constant with traditional continuous flow systems (Mohan et al., 2005). Enforcement of controlled, short-term, non-steady conditions in SBRs may favor induction of enzymes to degrade biorefractory compounds (Tomei et al. 2004). Another significant point for SBRs is that the chlorophenols peak concentration in the reactor, adjustable by changing the feeding time, markedly affects reactor performance. Accumulated compounds may adversely affect both

their own degradation via self-inhibition and the degradation of other compounds in the feed via substrate interaction and/or inhibition of the biomass. Therefore, SBR operational parameters should be optimized especially when chlorophenols are present in the mixture.

The ratio of K_I/K_S determines the how degradation rate changes with increasing substrate concentration. When the ratio approaches infinity, the Andrews equation reduces to Monod equation and the substrate removal rate approaches its maximum. For low K_I/K_S ratio, as the substrate concentration increases there is a critical substrate concentration, $S_{cr} = \sqrt{K_s K_i}$, above which the substrate removal rate falls (Tomei et al., 2004). The ratio of K_I/K_S for 4-CP and 2,4-DCP was calculated as 2.28 and 3.22, respectively using the values given in Sections 3.2.2 and 3.2.5. For both cases, the observed relatively low values of K_I/K_S clearly indicated that both of the substrates significantly inhibit their own transformation above the critical substrate concentration, at which degradation rate is maximal. Based on the results, critical substrate concentrations for 4-CP and 2,4-DCP were observed as 53 and 25 mg/L, respectively. Hence, the critical concentration of 4-CP was 2.5 times that of 2,4-DCP, indicating higher toxicity of 2,4-DCP. At this point, the use of SBR with long filling time gains importance as the concentrated wastewaters can be readily degraded without exceeding critical substrate concentration.

In an SBR, we studied 4-CP and 2,4-DCP degradation using four different feeding periods to determine the effect of feeding duration on reactor performance. During the experiments, peptone, 4-CP, and 2,4-DCP concentrations were kept constant at 300, 220, and 110 mg/L, respectively, and the reactor was operated over four feeding durations of 8, 5.5, 2.2, and 0 h (see Table 3.7 for reactor operating conditions). After each change of feeding regime, the reactor was allowed three SRTs to reach a steady state, determined also by measuring effluent COD and MLVSS at least three successive days.

Table 3.7. SBR operating conditions

Filling Time (h)	**8**	**5.5**	**2.2**	**0**
Hydraulic residence time (d)	1.125	1.125	1.125	1.125
Fill flow rate (l/h)	0.25	0.36	0.91	-
Steady state MLVSS at the end of daily cycle (mg MLVSS/L)	1050±56	1502±29	1373±35	1335±8
Chlorophenols loading rate (mg chlorophenols/gMLVSS.h)	34.92	35.5	97.11	-

Figure 3.42 shows changes in chlorophenol and COD concentrations with time for one cycle at 8-h feeding. As the figure shows, though the influent chlorophenol concentrations were high, their maxima in the reactor did not exceed 2 mg/L for both 4-CP and 2,4-DCP; thus by the end of the cycle, both chlorophenols were completely removed (their concentrations were below the detection limit).

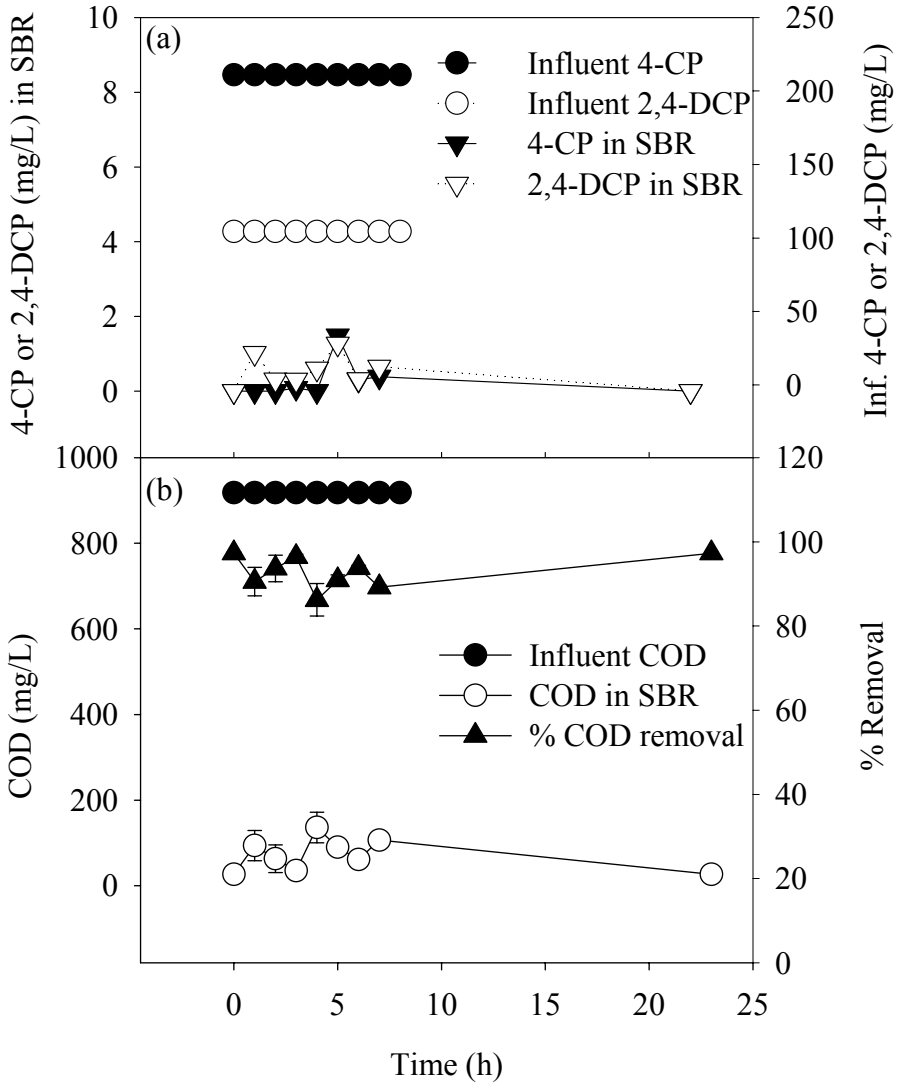

Figure 3.42. Time course variation of COD and chlorophenols for 8 h of filling time

Similarly, the influent COD was close to 900 mg/L and the maximum COD during the operation remained at about 140 mg/L and decreased to 27±3 mg/L, thus corresponding to about 97% removal at the end of the operation. The low COD at the end of operation implies that chlorophenols were fully removed without residual by-products in the medium. In addition, effluent COD remained at about 25 mg/L when the reactor was fed with peptone without chlorophenols. Therefore, adding chlorophenols to the medium caused no significant increase in effluent COD, and the low COD at the end of the cycle may have come from biomass lyses. These

findings coincide with those of Tomei et al. (2004) on 4-nitrophenol degradation. They reported that after complete degradation of 4-nitrophenol, residual COD was 10-20 mg/L, also accounted for by biomass lyses.

Figure 3.43 shows time course variation of chlorophenols and COD for 5.5-h feeding. Decreasing feeding from 8 to 5.5 h increased the maximum 4-CP and 2,4-DCP concentrations to about 70 and 7 mg/L, respectively. Similarly, the maximum COD concentration increased from 140 mg/L for 8-h feeding to about 215 mg/L for 5-h feeding. Both maximum chlorophenol and COD concentrations also occurred at the start of feeding, a finding that allows two possible explanations. First, at the beginning of operation, the low reactor volume prevented incoming wastewater from being diluted. Second, because during settling and idling the reactor was not aerated, biomass activity decreased resulting in increased reactor concentration. Similarly, Tomei et al. (2004) observed a short initial lag phase, which they attributed to time required for the solution to homogenize and the biomass to adapt during the settling and idling of the reactor. Like in 8-h feeding, high COD removal (91±3%) occurred also at the end of the 5.5-h cycle.

Similar to the operation at 8 h filling time, high COD removal efficiency (91±3%) was observed at the end of the cycle.

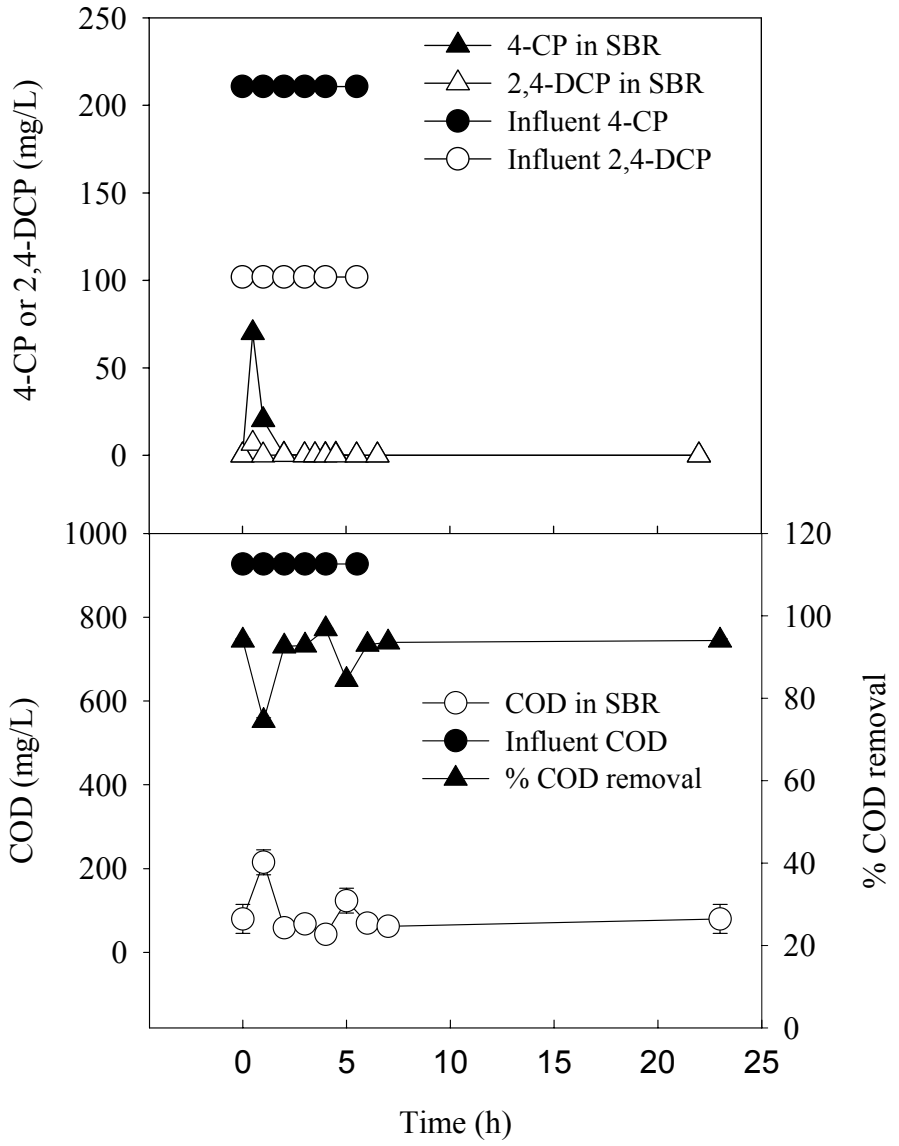

Figure 3.43. Time course variation of COD and chlorophenols for 5.5 h of filling time

Figure 3.44 gives chlorophenol and COD variations over 2.2-h feeding and shows that both concentrations increased during the feeding period and more significantly than during 5.5-h feeding. Therefore, during the former feeding the chlorophenol and COD degradation rates were lower than the loading rate, as also noticed by Chiavola et al. (2004) in their study of 3-CP/phenol degradation via cometabolism. Another significant point, also seen in the figure, is that 4-CP degradation did not start until all 2,4-DCP was consumed in the medium: the 4-CP and 2,4-DCP peak

concentrations were about 170 and 40 mg/L for 4-CP and 2,4-DCP, respectively. Though the 2,4-DCP influent concentration was half that of 4-CP, the peak value of the latter was four times that of the former. We can thus conclude that somewhat high concentrations of 2,4-DCP in the medium competitively inhibited the degradation of 4-CP. Chiavola et al. (2004) observed the same, reporting that 3-CP degradation reached its maximum rate when phenol was removed and 3-CP reached a non-inhibitory concentration in an SBR fed with a mixture of phenol and 3-CP. In addition, the COD concentration increased especially during the first hour of feeding and then decreased rapidly after feeding was completed.

Figure 3.45 shows variations in chlorophenols and COD when the reactor was fed instantaneously (0-h feeding). The 2,4-DCP inhibition of 4-CP degradation observed during 2.2-h feeding became more pronounced when synthetic wastewater was fed into the reactor instantaneously. At the end of the 0-h cycle, about 96% of COD, corresponding to about 30 mg/L of effluent COD, was removed.

Degradation of 4-CP and 2,4-DCP mixture was also modeled using the Andrews Equation constants which were observed in batch reactors. The observed inhibition effect of 2,4-DCP on 4-CP degradation was so strong that it completely ceased 4-CP degradation. After complete removal of 2,4-DCP from the medium, degradation of 4-CP started without any remaining inhibitory effect (Figure 3.45a and c). The degradation of 2,4-DCP in the presence of 4-CP was modeled using a simple zero order model with a rate coefficient of 14.28 mg/L.h. The competitive inhibition coefficient of 2,4-DCP on 4-CP degradation was 0.17 and Equation 3.6 was used in the modeling study. The observed equation was solved with POLYMATH 4.02 using Runge-Kutta-Fehlberg (RFK) numerical integration routine. The developed model proved an excellent fit to the experimental data (Figure 3.45a).

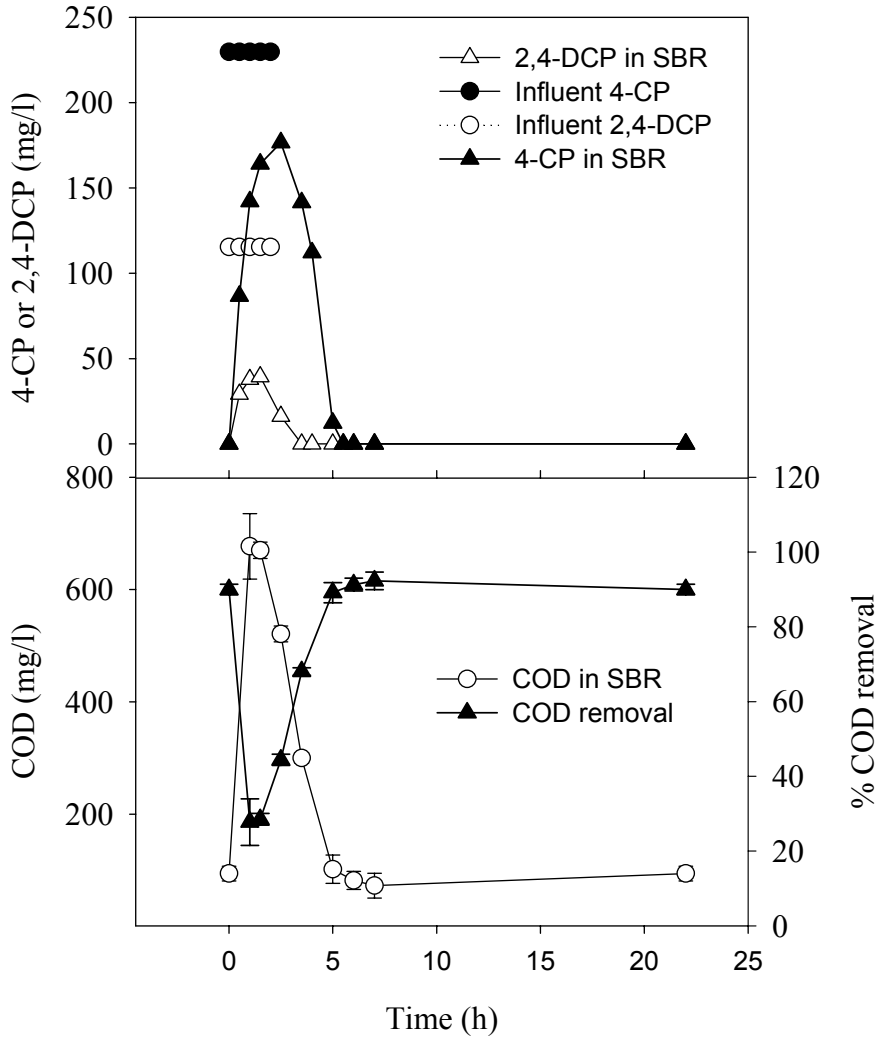

Figure 3.44. Time course variation of COD and chlorophenols for 2.2 h of filling time

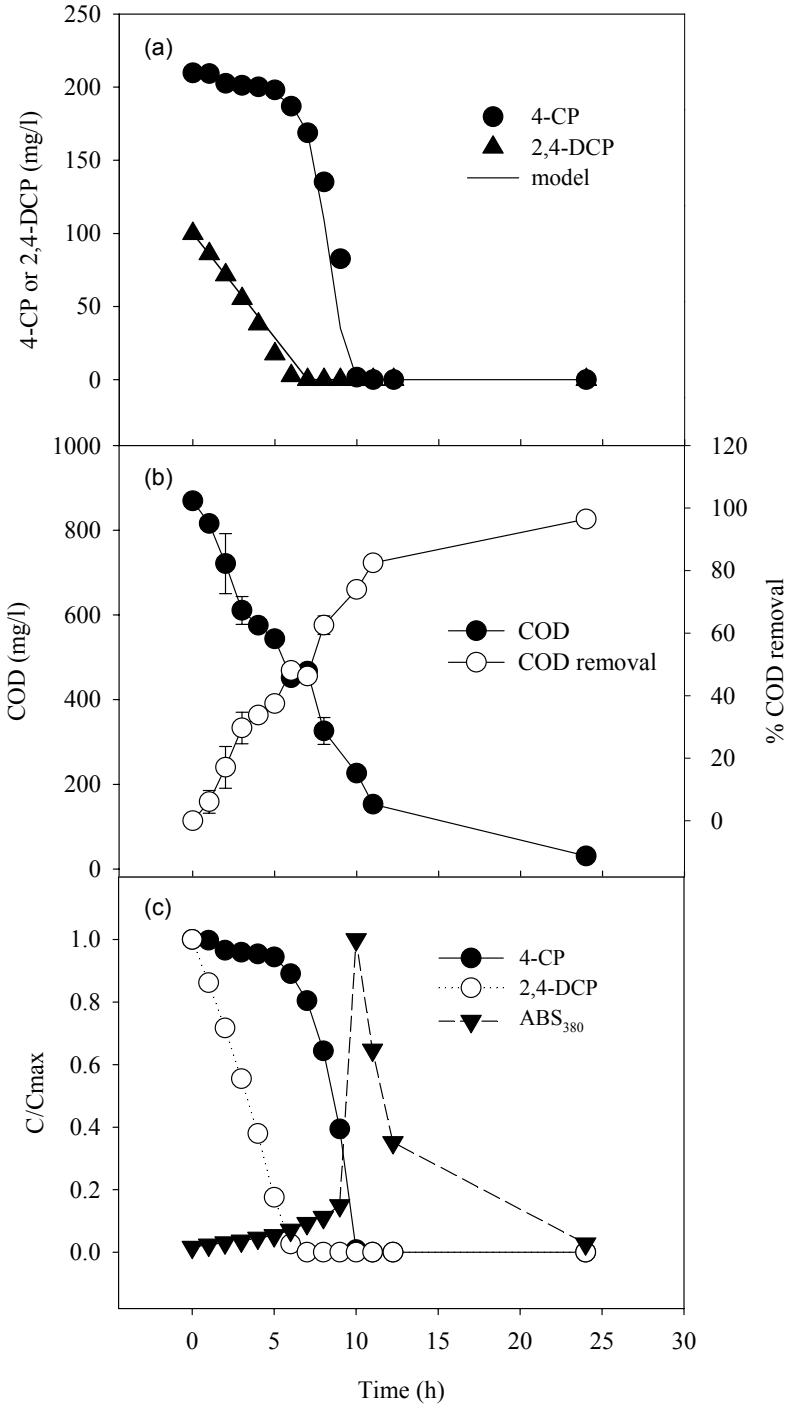

Figure 3.45. Time course variation of COD and chlorophenols when SBR fed instantaneously (filling time is 0 h.)

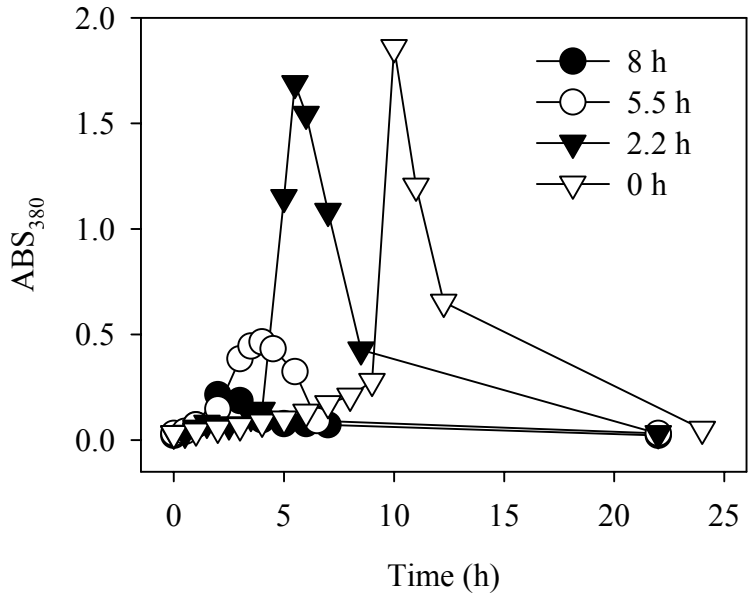

Figure 3.46. Time course variation of CHMS at different filling times

Figure 3.46 depicts time course variation of CHMS concentration as absorbance at 380 nm (ABS_{380}) for different filling periods. The observed maximum concentration of CHMS increased with decreasing filling time. Another significant point is that the maximum CHMS concentration occurred immediately after 4-CP was fully removed from the medium (Figure 3.45c). During the degradation of 4-CP, the concentration of CHMS increased and after complete removal of 4-CP from the culture medium, the concentration of the intermediate decreased sharply.

The production and subsequent complete removal of CHMS imply that 4-CP was degraded completely *via –meta* cleavage pathway without by-product in culture medium. Furthermore, HPLC analysis showed no intermediates in the treated effluent (Figure 3.47). The complete break down of the chlorophenols was also confirmed by the chloride material balance showing a total chlorophenols degradation of 103±6% (data not shown).

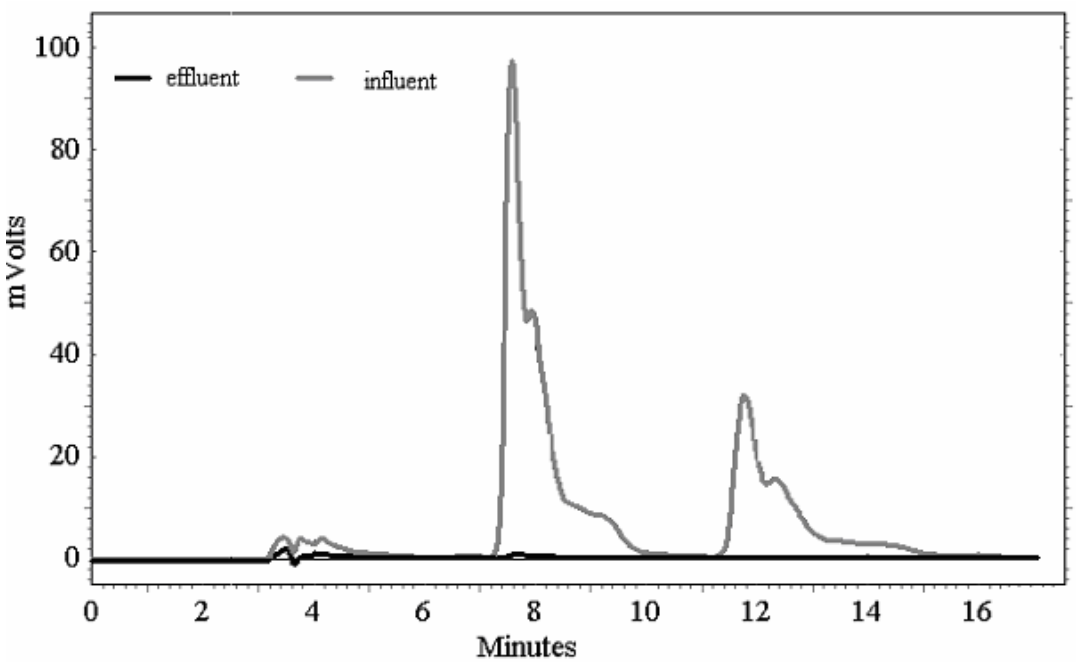

Figure 3.47. HPLC results of influent and effluent of SBR operated at 5.5 h of filling time

Figure 3.48, shows the effects of feeding time on the time required to completely degrade chlorophenols and on the maximum concentrations of COD, chlorophenols, and CHMS. The degradation time and the concentrations both increased linearly with decreasing feeding time, whereas the maximum 2,4-DCP concentration increased exponentially with decreasing feeding time. Consequently, as stated by Tomei et al. (2004), long and aerated feeding contributes to significant substrate removal during feeding and moderates the substrate concentration peak at the end of the feeding period. Figure 3.48 shows also the Y values at different feedings and that Y remained relatively constant between 0- and 5.5-h feedings but dropped markedly, lowering the steady-state MLVSS concentration (Table 3.7) during 8-h feeding. With respective critical substrate concentrations of 53 and 25 mg/L for 4-CP and 2,4-DCP, the feeding time under the studied conditions could have been longer than 5.5 h because the maximum 4-CP and 2,4-DCP concentration were 70 and 7 mg/L, respectively. Therefore, in degrading high strength wastewater, feeding time should be optimized so as not to allow the reactor

substrate to concentrate beyond non-inhibitory levels. Moreover, our sludge settled well, its volume index (SVI) ranging between 30-77 mL/g SS throughout reactor operation. After the settling period, the supernatant (effluent SS concentration always ≤ 20 mg MLSS/L) remained clear throughout the SBR study.

The react time for a sudden fed of chlorophenols was selected as 22 h, which is high enough not to adversely affect the efficiency of the reactor. However, based on the results, it can be said that "filling + react time" can be selected as low as 6.5 h for filling time of 5.5 h as almost complete removal of chlorophenols and CHMS was observed at 6^{th} h. However, for the case when reactor was fed instantaneously, react time should not be less than 15 h to completely remove chlorophenols and CHMS. In this context, it can be concluded that filling time and the react time should be decided based on degradation kinetics and substrate interactions to optimize reactor performance.

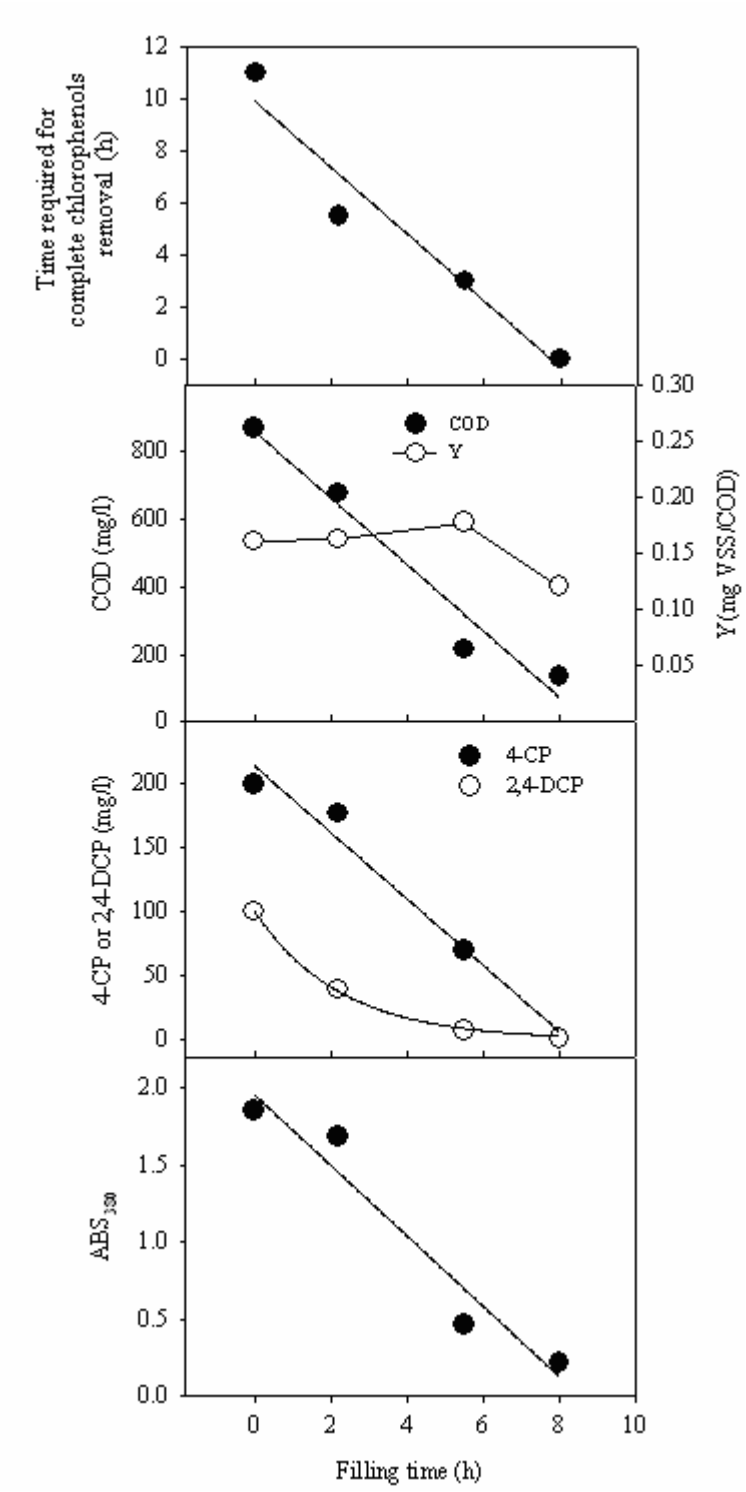

Figure 3.48. Effect of feeding time on the time required to completely degrade chlorophenols and on the maximum concentrations of COD, chlorophenols, and CHMS

As mentioned above, biomass activity slowed down in the first 30 min of feeding after the reactor had settled and idled for about 2 h. Especially during short feeding periods, this lowered activity and caused chlorophenols to accumulate. In addition, because 2,4-DCP competitively inhibited 4-CP degradation, the latter rate was much lower than the loading rate during short feedings (Figure 3.49b). Figure 3.49b shows that the 4-CP removal/loading rate was lower than unity especially for the 1st h of the 2.2-h feeding. Significantly also, the 4-CP degradation rate was highest in for 1st h of the 5.5-h feeding because during the first 30 min reduced biomass activity allowed the 4-CP concentration to reach about 70 mg/L, which was close to the critical substrate concentration (about 53 mg/L). The degradation rates for the 8- and 2.2-h feedings were lower because of 4-CP concentrations, which were, respectively, lower and higher than the critical substrate concentration. With an instantaneously fed reactor, the 4-CP degradation rate was almost zero for the first 5 h because a high 2,4-DCP concentration completely inhibited 4-CP degradation. Thereafter, the 4-CP degradation rate increased steadily because 2,4-DCP was all but removed from the medium. After its maximum, the degradation rate began to drop as the 4-CP concentrations slid below the critical value (Figure 3.49a). Therefore, fed over a long period, the SBR avoided the toxic compounds from self-inhibiting their own degradation and from being inhibited by substrate interaction.

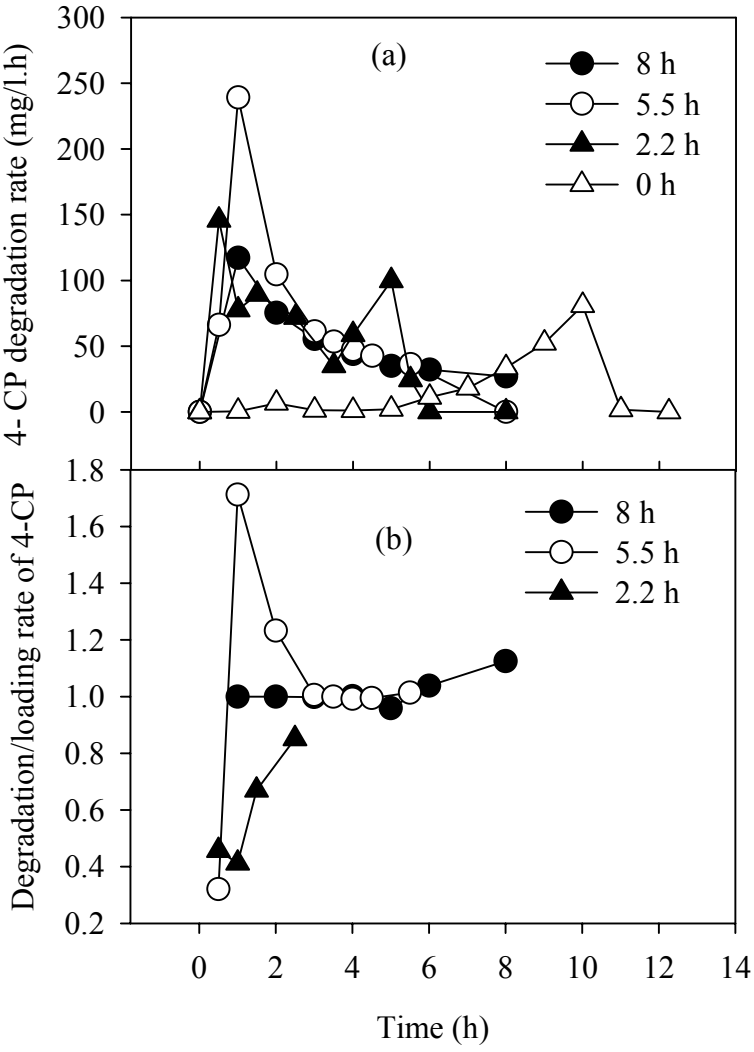

Figure 3.49. Degradation rate of 4-CP during the reactor operation (a) and ratio of degradation to loading rate for different filling times

3.4. Effect of Biogenic Substrate Concentration on the Performance of Sequencing Batch Reactor (SBR) Treating 4-CP and 2,4-DCP Mixture

After determination of the effect of filling time on the reactor performance, the effect of biogenic substrate concentration on the SBR performance was extensively evaluated.

The filling time was kept constant at 8 h throughout the experiments. In order to understand the effect of biogenic substrate concentration on the performance of the reactor, peptone concentration was gradually decreased from its initial value of 300 mg/L, while the chlorophenols concentrations were kept constant at 220 and 110 mg/L for 4-CP and 2,4-DCP, respectively. After any change in reactor operation, three SRTs were allowed to reach steady state conditions, which were also checked measuring MLVSS and COD concentrations at the end of at least three successive cycles. After ensuring the steady-state conditions, samples were drawn from the reactors at predetermined time intervals and analyzed immediately for chlorophenols, COD and CHMS. MLVSS concentrations at the end of the cycles were also determined.

At the start of each cycle, the volume of settled sludge was 0.250 L and at the end of the filling period the reached total volume of the reactor was 2.25 L. Therefore, for each cycle, SBR received 2 L synthetic wastewater (HRT = 1.125 d), which was fed to the reactor within 8 h of filling period.

Figure 3.50 gives time course variations of the 4-CP, 2,4-DCP, CHMD and COD concentrations for each peptone concentration studied. The initial feed COD concentration was around 800 mg/L when reactor received 300 mg/L peptone, 220 mg/L 4-CP and 110 mg/L 2,4-DCP. A 100 mg/L exclusion of peptone caused around 100 mg/L decrease in the COD of reactor influent and the COD concentration was around 500 mg/L when reactor was fed with chlorophenols as a sole carbon and energy sources. Complete degradation of chlorophenols was observed for all studied conditions, although the trend of chlorophenol concentrations showed variation depending on the biogenic substrate concentration. Although reactor was fed with a high concentration of 4-CP and 2,4-DCP mixture, 4-CP and 2,4-DCP concentrations were observed to be below 2 mg/L during the filling period of the SBR for influent peptone concentration of 300 mg/L, which were increased to around 12.5 and 5 mg/L in respective order with decreasing influent peptone concentration to 200 mg/L (Figure 3.50(a)). When the peptone

concentration was decreased to 200 mg/L, chlorophenols started to exhibit a noticeable peak value which corresponds to the very beginning of the cycle. Then, it can be said that decreasing peptone concentration adversely affected chlorophenol removal rate, which led to a slight increase in observed maximum chlorophenol concentrations within the reactor. However, the maximum COD concentration within the cycle was not affected from the decreasing peptone concentration from 300 to 200 mg/L as it was observed to be 135 and 140 mg/L in respective order (Figure 3.50(c)). When peptone concentration decreased further to 100 mg/L, the 4-CP peak concentration increased to 126 mg/L and a slight decrease in the peak 4-CP concentration to 104 mg/L was observed when chlorophenols were fed to the reactor as sole carbon and energy source (Figure 3.50(a)). The decrease in 4-CP peak value when peptone concentration decreased from 100 to 0 mg/L is not known, but the possible reason is thought as change in community structure with the complete elimination of peptone from the influent. This can also be taken into consideration as an effect of acclimation of the culture to the chlorophenol as the slow or gradual exclusion of the peptone from the feed might have allowed the culture to acclimatize better.

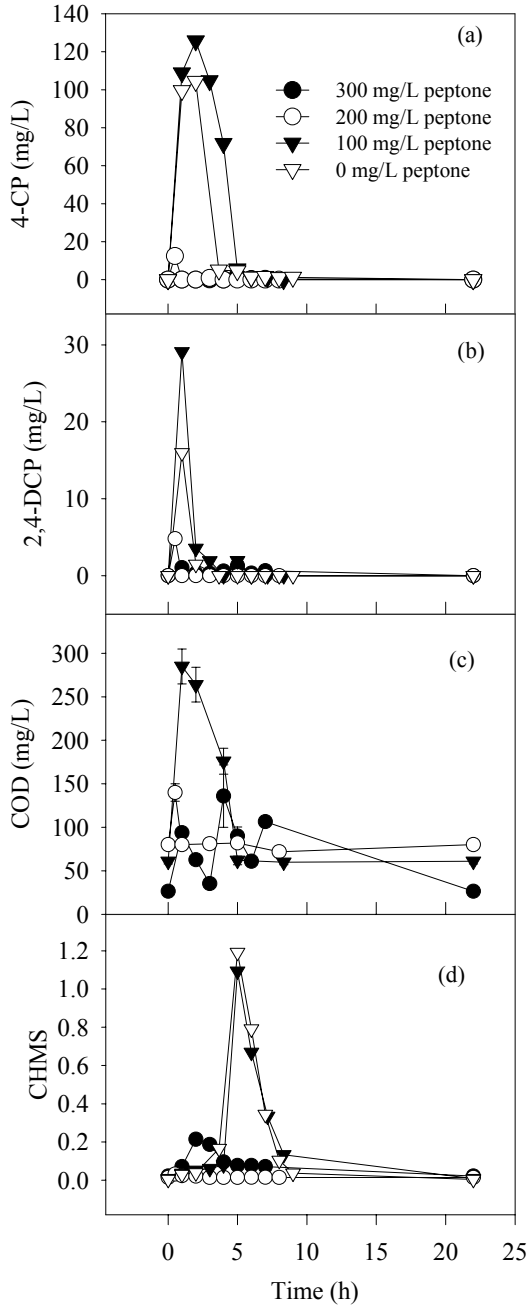

Figure 3.50. Time course variation of 4-CP (a), 2,4-DCP (b), COD (c) and ABS_{380} (d) in SBR at different feed peptone concentrations (Influent 4-CP and 2,4-DCP were 220 and 110 mg/L, respectively)

Similar to 4-CP, increase in 2,4-DCP peak concentrations were observed with the

decreasing peptone concentration from 300 to 100 mg/L (Figure 3.50(b)), whereas, a slight decrease in the peak 2,4-DCP concentration from around 30 to 16 mg/L was observed with the complete elimination of peptone from the feed solution (Figure 3.50(b)). It is important to note that 4-CP is much more accumulated in the medium compared to 2,4-DCP. The reason of this is that 2,4-DCP caused a strong competitive inhibition on 4-CP degradation, the degradation of which started only after complete 2,4-DCP removal. This observation can also be verified from the evaluation of time course variation of chlorophenols concentrations given in Figures 3.50(a) and (b). The accumulation of 2,4-DCP at lower biogenic substrate concentrations decreased the removal of 4-CP further due to competitive inhibitory effect of 2,4-DCP on 4-CP degradation. Therefore, the accumulation of 4-CP in the growth medium is not solely due to the decreasing 4-CP degradation ability of the culture with the elimination peptone from growth medium. Despite the increased peak values of chlorophenols with decreasing readily degradable substrate concentration, 4-CP and 2,4-DCP were effectively degraded even in the absence of a biogenic substrate and biomass can grow on the mixture of 4-CP and 2,4-DCP.

The peak COD concentration increased from around 140 mg/L to 285 mg/L with the decreasing peptone concentration from 200 to 100 mg/L. Although different peak values were observed depending on the initial peptone concentration, high COD removal efficiencies (≥90%) were observed at the end of cycle for all studied conditions. When chlorophenols were fed to the reactor as sole carbon and energy sources, only initial and final concentrations of COD were measured as the COD concentration in the reactor at any time may be calculated using the time course data for 4-CP and 2,4-DCP. The influent COD concentration decreased from 503 mg/L to 53 mg/L, corresponding to around 90% COD removal efficiency, which shows that acclimated mixed culture have ability to use mixture of 4-CP and 2,4-DCP as sole carbon and energy sources. The observed COD concentration at the end of the cycle was thought to be due to biomass lyses products, although it seems to be slightly high (53 mg/L).

The peak value of CHMS increased with decreasing peptone concentration similar to other measured parameters. The CHMS concentration increased steadily with time course decreasing 4-CP concentration and the peak value was reached when 4-CP was just completely removed from the medium (Figure 3.50(a) and (d)). Observing high COD removal efficiencies (≥90%) and complete removal of CHMS confirm the complete chlorophenols mineralization with acclimated mixed cultures for all studied conditions, which was also validated by HPLC data and released chloride ion concentration, which indicated 102±5% chlorophenols removal.

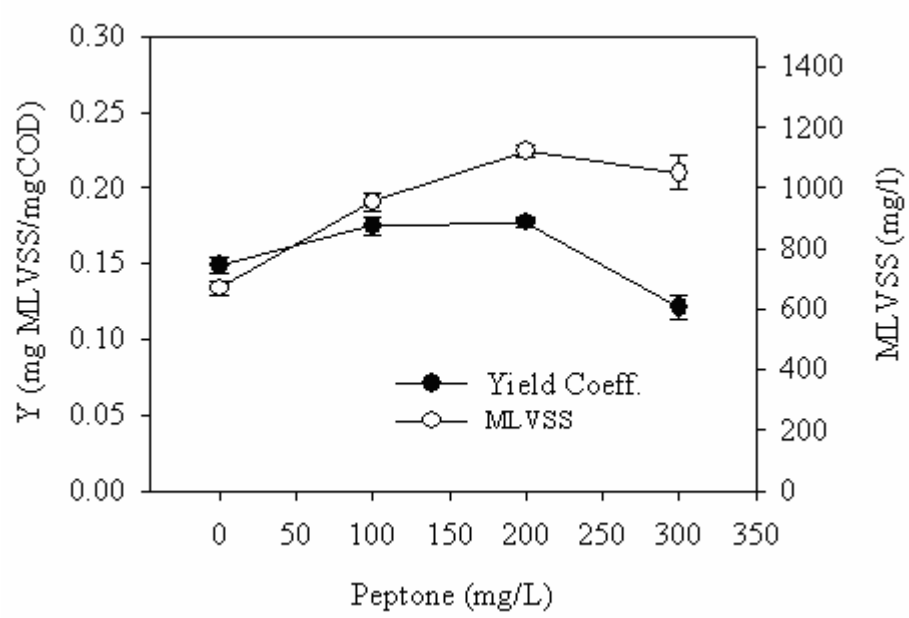

Figure 3.51. Effect of peptone concentration on Y values and biomass concentrations

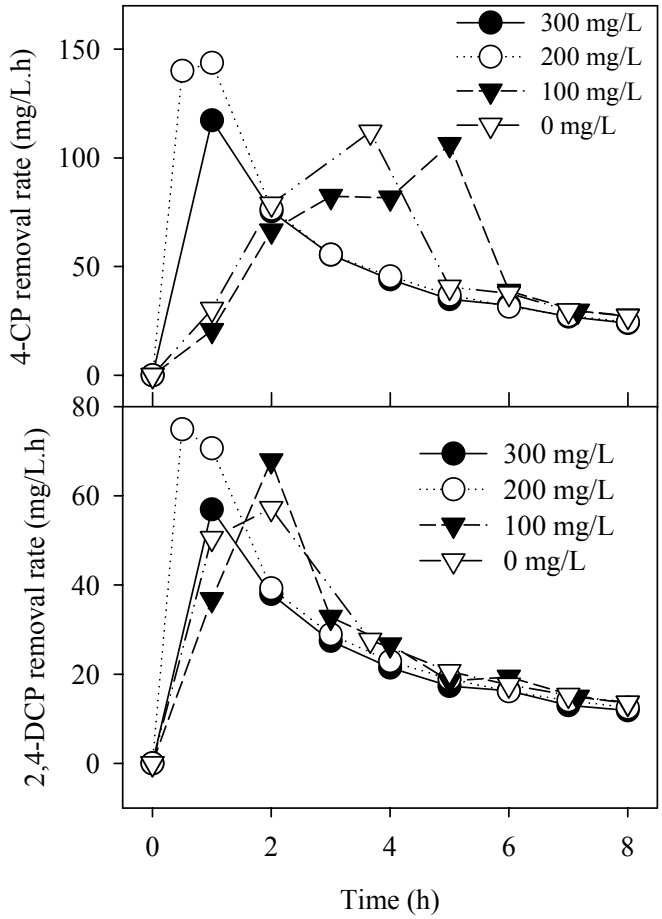

Figure 3.52. Effect of peptone concentration on removal rates of 4-CP and 2,4-DCP

The reason for the increases in peak concentrations of measured parameters with decreasing peptone concentration may be due to decreasing biomass concentration (Figure 3.51). The effect of peptone concentration on the observed yield coefficient (Y) and MLVSS values are depicted in Figure 3.51. Another striking conclusion, which can be drawn from Figure 3.51, is that Y did not correlate with peptone concentration and the average value was observed to be 0.155±0.028 (CV=18%) mg MLVSS/mg COD. The Y value was significantly lower than one expected for a readily degradable substrate (0.4-0.6 MLVSS/mg COD) and it was 0.42±0.008 mg MLVSS/mg COD on only peptone in instantaneously fed SBR.

Effect of peptone concentration on time course degradation rates of 4-CP and 2,4-DCP are given in Figure 3.52. In the calculation of degradation rates the following equation was used and the change in volume of the reactor was taken into account;

$$\frac{dc}{dt}V = QC_0 - rV \qquad (3.16)$$

Where, C and C_0 are chlorophenol concentrations (mg/L) in SBR and feed, respectively. V (L) is total reactor volume and r is chlorophenol removal rate (mg/L.h). Total volume during the filling period of the reactor is;

$$V = V_0 + Qt \qquad (3.17)$$

Where, V_0 is settled sludge volume at the start of the cycle. Hence, degradation rate can be observed as;

$$r = \frac{QC_0}{V_0 + Qt} - \frac{dc}{dt} \; . \qquad (3.18).$$

Degradation rates of 4-CP and 2,4-DCP were very high at the start of the experiments for 300 and 200 mg/L of influent peptone concentrations. The observed initial degradation rates for 4-CP decreased to very low values for peptone concentrations of 100 and 0 mg/L, which caused accumulation of chlorophenols within the reactor. The maximum observed 4-CP degradation rate increased slightly with decreasing peptone concentration from 300 to 200 mg/L. The reason of this increase in degradation rate is due to the slight increase in peak 4-CP concentration from 2 to 12.5 mg/L. Degradation of chlorophenols obeys Andrews kinetics, which means chlorophenol degradation rate increases up to a certain concentration (critical substrate concentration) at which degradation rate is maximum, whereas, after this point degradation rate decreases with increasing 4-CP concentration. 4-CP critical substrate concentration was previously observed to be close to 53 mg/L.

Therefore, up to this point increase in 4-CP concentration results in increase in degradation rate.

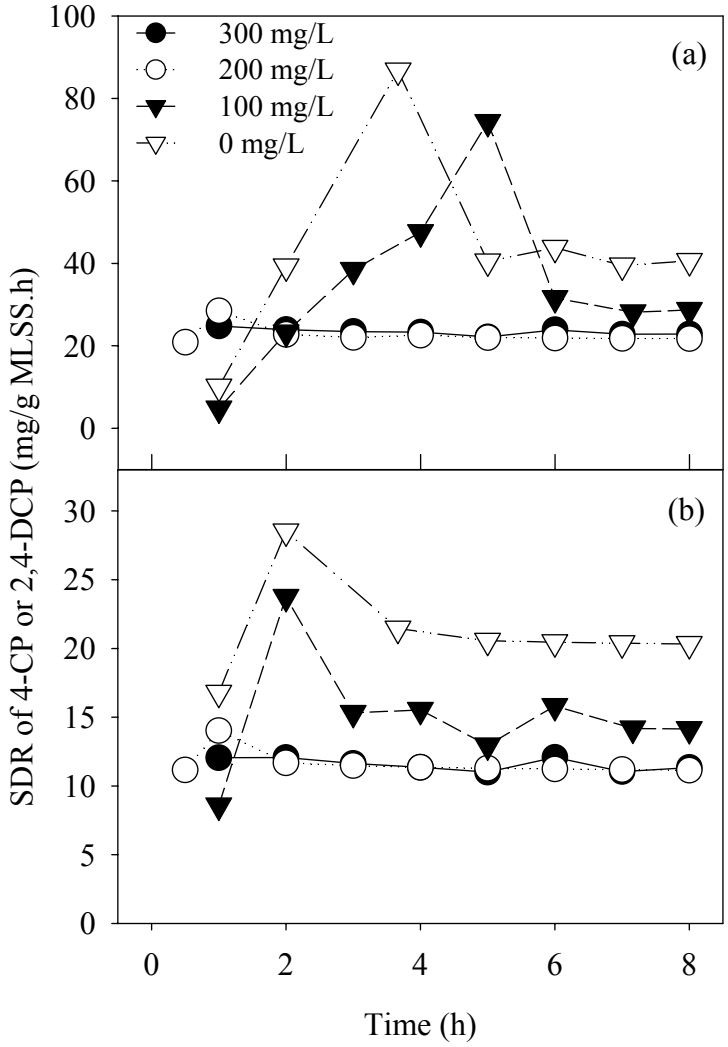

Figure 3.53. Effect of peptone concentration on SDRs of 4-CP (a) and 2,4-DCP (b)

Time course variations of calculated SDRs values using Equation 3.19 is given in Figure 3.53.

$$SDR = \frac{r}{X/V} \qquad (3.19)$$

Where, r is DR of chlorophenol calculated using Equation 3.18. X and V are total biomass concentration (g MLVSS) and reactor volume, respectively. The reactor volume at any time was calculated using Equation 3.17.

Although DR values decreased with time, SDR values were almost constant for 300 and 200 mg/L of peptone. The average SDR values for 4-CP and 2,4-DCP were observed to be 23.4±081 and 11.56±0.45 mg/gMLVSS h for 300 mg/L peptone and 22.67±2.25 and 11.61±0.92 mg/gMLVSS h for 200 mg/L peptone, respectively. Another significant point is that although DR values of 2,4-DCP did not show significant variation with different peptone concentration (Figure 3.52), the SDR values of 2,4-DCP increased with decreasing peptone concentration (Figure 3.53). Therefore, based on the values given in Figure 3.53, it can be concluded that the accumulation of chlorophenols during the fill period is due to low DR and SDR values observed at the beginning of filling period at low biogenic substrate concentrations and the strong 2,4-DCP competitive inhibition on 4-CP degradation.

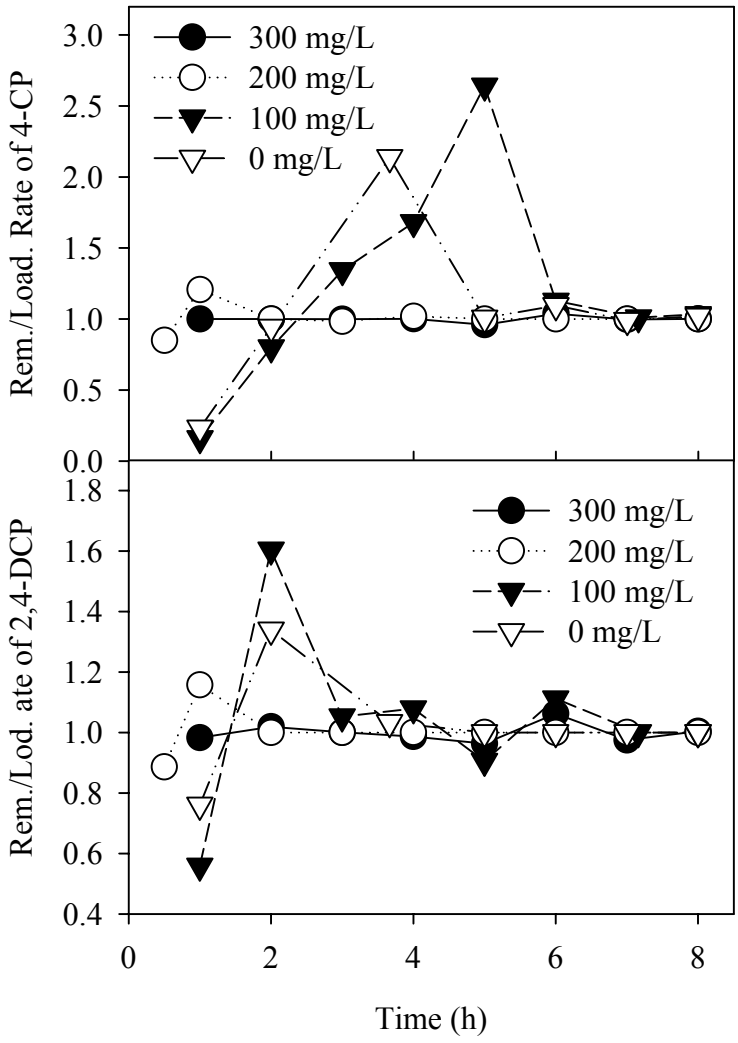

Figure 3.54. Effect of peptone concentration of removal/loading rates of 4-CP and 2,4-DCP

The comparison of loading and degradation rates was also depicted in Figure 3.54. The ratio of removal/loading rates were almost one during the filling of the reactor when the initial peptone concentration was 300 and 200 mg/L. Whereas, for 100 and 0 mg/L of peptone, initial 4-CP removal/loading rates were at very low levels, which led to the accumulation within the reactor.

These observations are contradictory to our previous findings as we observed that decreasing peptone concentration did not affect the chlorophenols degradation profiles in instantaneously fed SBRs possibly due to low chlorophenols removal rates during the filling period of SBR. Also, different community structures may have developed in different types of reactors and the community may have changed in the reactors over time due to highly dynamic nature of bacterial community (Kaewpipat and Grady, 2002). Zaidi and Mehta (1995) have reported that different bacteria may give different response to the presence of alternative carbon source. They reported that addition of glucose did not enhance the p-nitrophenol (PNP) degradation of *Corynebacterium* strain Z4 and the bacterium utilized the two substrates simultaneously. Glucose used at the same concentration, inhibited degradation PNP in wastewater by *Pseudomonas* strain MS. However, glucose increased the extent of degradation of PNP by *Pseudomonas* strain GR. Phenol also enhanced the degradation of PNP in wastewater by *Pseudomonas* strain GR, but had no effect on the degradation of PNP by *Corynebacterium* strain Z4. They also reported that the concentration of toxic compound may also play important role as the addition of glucose increased the PNP degradation by *Corynebacterium* strain Z4 when PNP concentration was too low to support growth. Therefore, difference in the community structures may be one of the reasons of observing different outcomes. For example, Hu et al. (2005a) reported different competent biomass fractions in three different reactor configurations. For example, the fractions of biomass utilizing acetate, benzoate and 4-CP, in respective order, were 3.02, 5.52, 0.02% in anoxic/aerobic SBR; 34.34, 1.65, 0.03% in aerobic biofilm SBR and 53.33, 4.44 and 0.01% in aerobic SBR.

3.5. Degradation of 4-CP and 2,4-DCP Mixture Using Rotating Biological Contactors (RBC)

The performance of a two stage rotating biological contactor (RBC) was evaluated for the treatment of synthetic wastewater containing peptone, 4-CP and 2,4-DCP. The first aim was to determine the effect of disk rotational speed on COD removal efficiency in the absence of chlorophenols. Rotational speed of disks has an important effect on the removal efficiency of the system as limiting oxygen concentration for low rotational speeds and shearing the active biomass off at high rotational speeds may cause deterioration of effluent quality. In this context, COD removal efficiency of the RBC was investigated at different rotational speed of disks (10, 7.5 and 5 rpm) keeping all other operational parameters constant.

Influent COD concentration of the synthetic wastewater (devoid of chlorophenol) was 580±38 mg/L. The pH of the synthetic wastewater was 7.2±0.2 and it was 7.5±0.2 for effluent of the reactor. Reactor was operated at room temperature without temperature adjustment (20-25°C). Figure 3.55 presents the effluent COD variations and corresponding removal efficiencies with operational time at different rotational speeds of the disks. The average COD removal efficiency was around 95% for rotational speeds of 10 and 7.5, and it slightly increased to a value of 97% at 5 rpm (Figure 3.56). Based on the results, it can be concluded that there is no significant effect of disks rotational speed on COD removal performance of the system in the absence of chlorophenols. Hence, it was decided to operate the RBC at a rotational speed of 5 rpm considering that energy expenses constitute the main portion of operational expenses in a full-scale system.

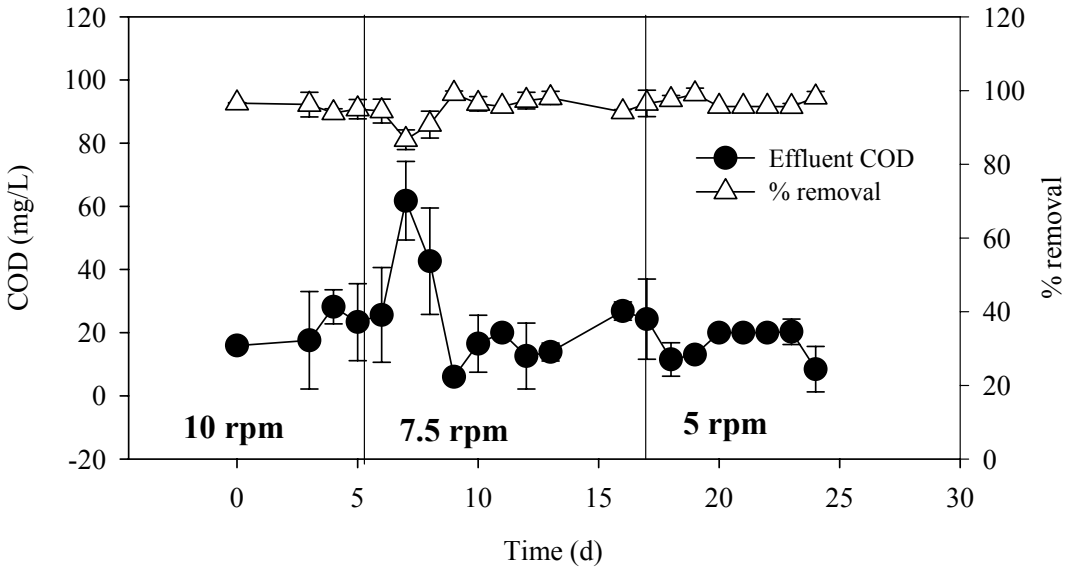

Figure 3.55. Variation of effluent COD and corresponding removal efficiencies in RBC

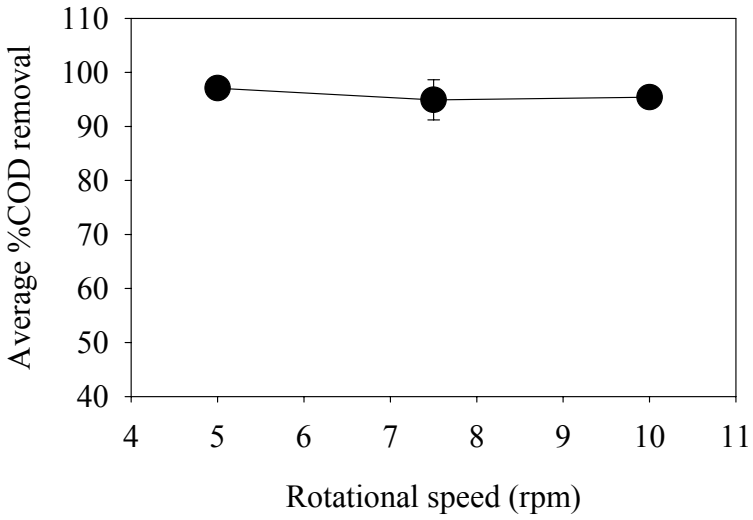

Figure 3.56. Effect of Rotational speed of Disks on the COD removal efficiency

After determination of optimum rotational speed of disks, the reactor was fed with 4-CP and 2,4-DCP mixture. Similar to SBR experiments, the ratio of feed 4-CP to 2,4-DCP was kept constant at a value of 2. In the first step of the RBC operation, the readily degradable substrate, which was peptone, was fed to the reactor with a

concentration of 400 mg/L (around 400 mg/L COD) and chlorophenol concentrations were increased gradually. In the second step of the study, the concentrations of chlorophenols were kept constant (200 mg/L 4-CP and 100 mg/L 2,4-DCP) and peptone concentration was decreased gradually and finally, 4-CP and 2,4-DCP was fed to the system as sole carbon sources. Finally, the feed chlorophenol concentrations were increased to find out the limits of the reactor. Also, after acclimation to 4-CP and 2,4-DCP as a sole carbon sources, the system was deacclimated by feeding only peptone around 2.5 months and then system was fed with chlorophenols again to evaluate whether preacclimation decreases the time needed for acclimation.

During the acclimation, the feeding of 4-CP and 2,4-DCP was started at concentrations of 2 and 1 mg/L, which were gradually increased to around 20 mg/L and 10 mg/L with respective order, within 15 days. Although complete degradation of chlorophenols was observed up to this point without any degradation byproduct, a straw yellow color due to CHMS accumulation appeared at the effluent of the reactor. Although, both 4-CP and 2,4-DCP removals were almost complete, the CHMS was not removed and it appeared without any decrease in its concentration for around 1 month. Accumulation of biomass was observed at the corners of the reactor, which was not observed for the feed devoid of chlorophenols. The accumulation of biomass was due partly to insufficient mixing of the reactor content at low rotational speed and it was observed that produced sludge had a quite well settling ability. In addition, the dissolved oxygen concentration in the first stage was lower than 1 mg/L, which caused a dense filamentous growth (Figure 3.57). When the reactor was fed with peptone in the absence of the chlorophenol, although slightly thick film and filamentous growth was observed, the concentration of oxygen was always higher than 1 mg/L. The decrease of oxygen concentration in the presence of chlorophenol favored the filamentous growth. This situation was more noteworthy in the first stage due to rapid removal of organic matter (Figure 3.58), which was also reported in the study of Surampali and Baumann (1997). The development of filamentous organism such as *Beggiatoa* at

low dissolved oxygen concentrations due to rapid removal of COD in the first stage of the reactor was well known problem in RBC operation (Metcalf and Eddy, 1991). After one month, the rotational speed of the reactor was increased to 10 rpm to increase dissolved oxygen transfer rate. However, the biomass on the disks was almost completely sloughed off and the performance of the reactor further deteriorated. It should be noted that although high COD removal efficiencies were observed when the reactor was operated at a rotational speed of 5-10 rpm in the absence of chlorophenols, the biomass could not attach on the disk surfaces when the reactor was operated at a rotational speed of 10 rpm when the feed contained mixture of 4-CP and 2,4-DCP. This observation leads to state that dominant species in the presence of chlorophenols were more sensitive and they can easily be detached from the surface of the disks. As the biomass on disks was almost completely lost, the reactor was inoculated again with a fresh activated sludge not previously exposed to chlorophenols. After fresh inoculation, the rotational speed was adjusted to 5 rpm and the liquid phase of the reactor was aerated to have around 3 mg/L of dissolved oxygen. Under these conditions, CHMS disappeared in the effluent of RBC within a few days along with a complete removal of chlorophenols. With the supplementation of air, in addition to complete chlorophenols removal along with intermediates, filamentous growth on disks was completely eliminated and development of a thin but dense biofilm was observed (Figure 3.59). Air supplementation was also caused complete mixing of the reactor content, which prevented the accumulation of biomass within the reactor. The beneficial effect of air supplementation was also reported in the study of Surampali and Baumann (1997). In another study, Kargi and Uygur (1997) also reported a remarkable increase in the performance of the RBC unit with the liquid phase aeration in the treatment of saline wastewater.

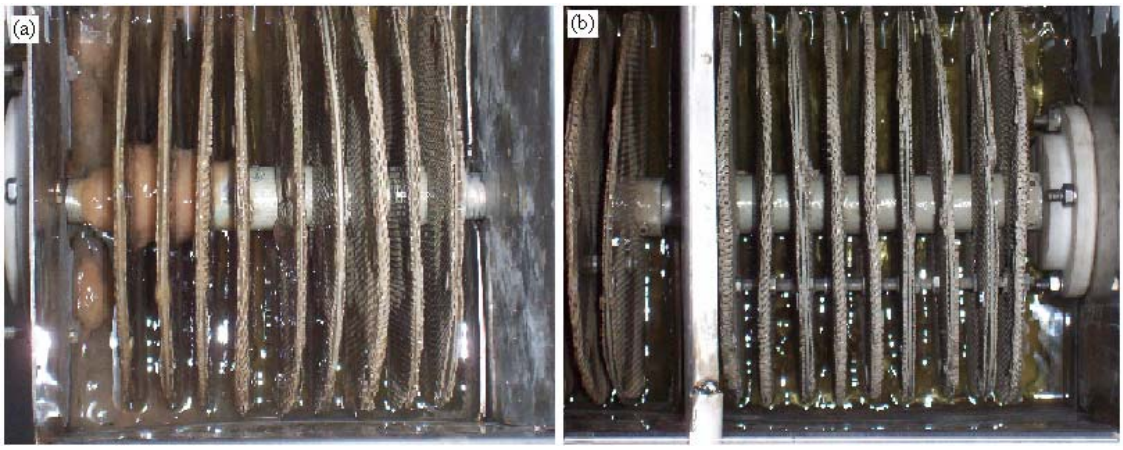

Figure 3.57. First (a) and second (b) stage of RBC discs in the absence of additional aeration

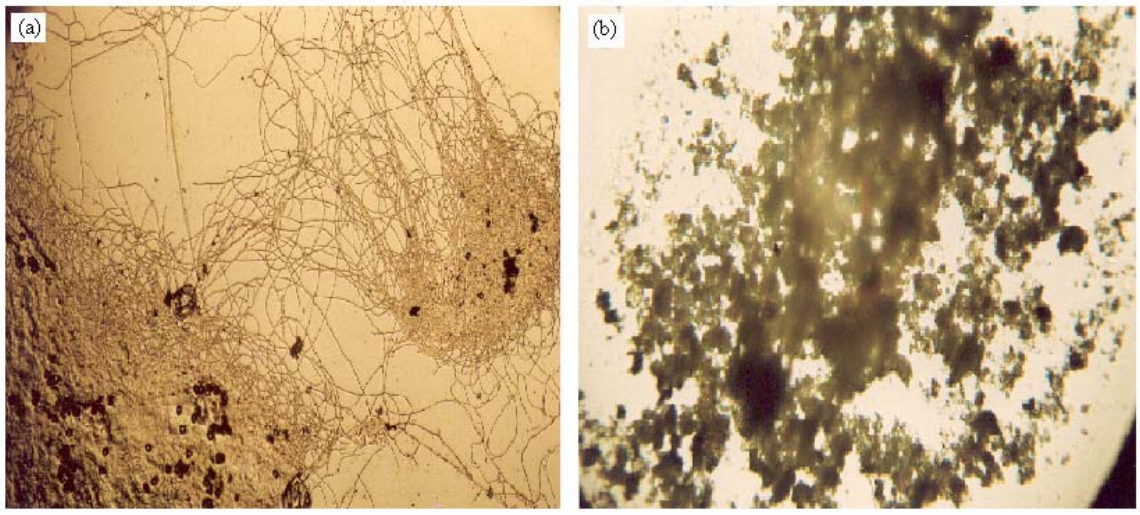

Figure 3.58. Microorganisms in first (a) and second (b) stage of RBC in the absence of additional aeration

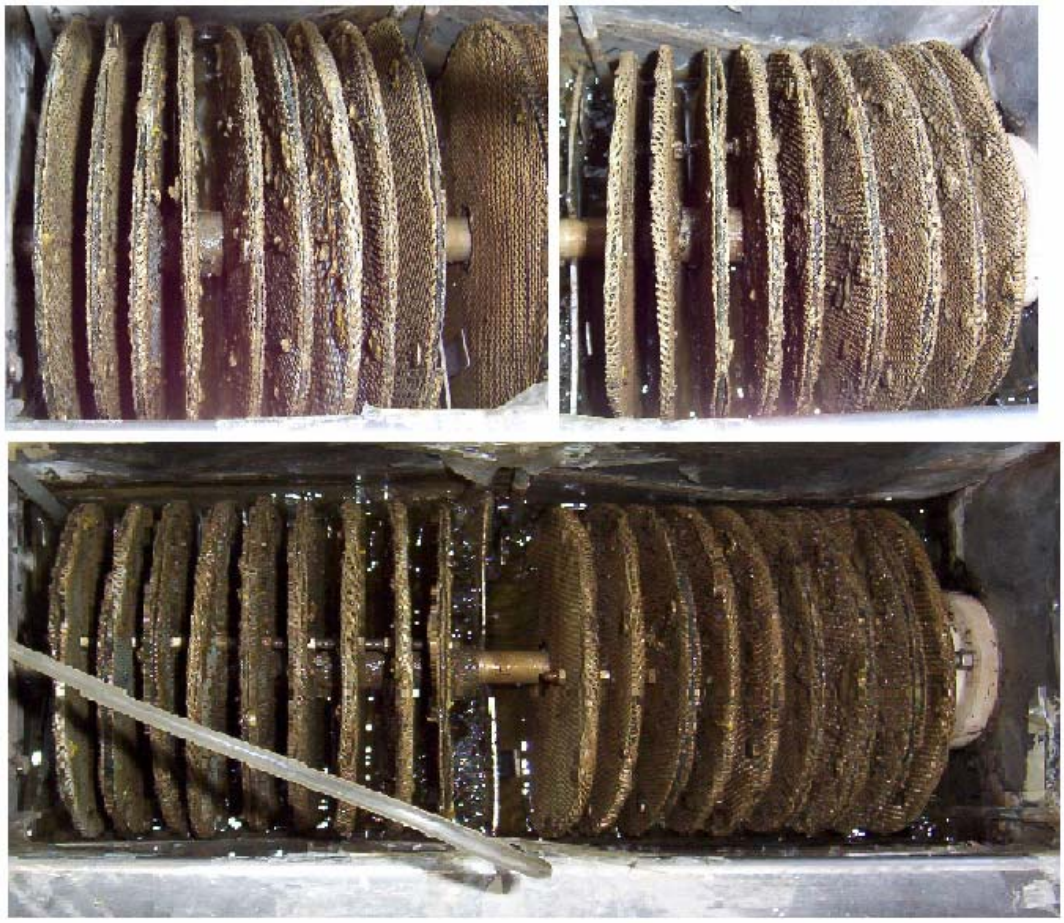

Figure 3.59. RBC discs after additional aeration

Figure 3.60 presents the feed and effluent concentrations of 4-CP and 2,4-DCP. 4-CP and 2,4-DCP concentrations were increased steadily and 220 mg/L 4-CP and 110 mg/L 2,4-DCP (chlorophenol loading rate = 470 mgCP/L.d) were reached at the end of 153 days. Although 2,4-DCP removal was complete, effluent 4-CP concentration in the first stage increased with increasing feed 4-CP concentration. Although 4-CP concentration at the effluent of 1st stage increased steadily, the concentrations were much lower than feed concentrations, as the effluent concentration of 4-CP was lower than 2 mg/L at a feed 4-CP concentration of 220 mg/L. Another conclusion can be drawn from the Figure 3.60 is that most of the chlorophenols were degraded in the 1st stage of the reactor and it is an easy task to completely remove the remaining impurities in the following stage and for whole

experimental period almost complete removal of both 4-CP and 2,4-DCP was observed in 2nd stage of the reactor.

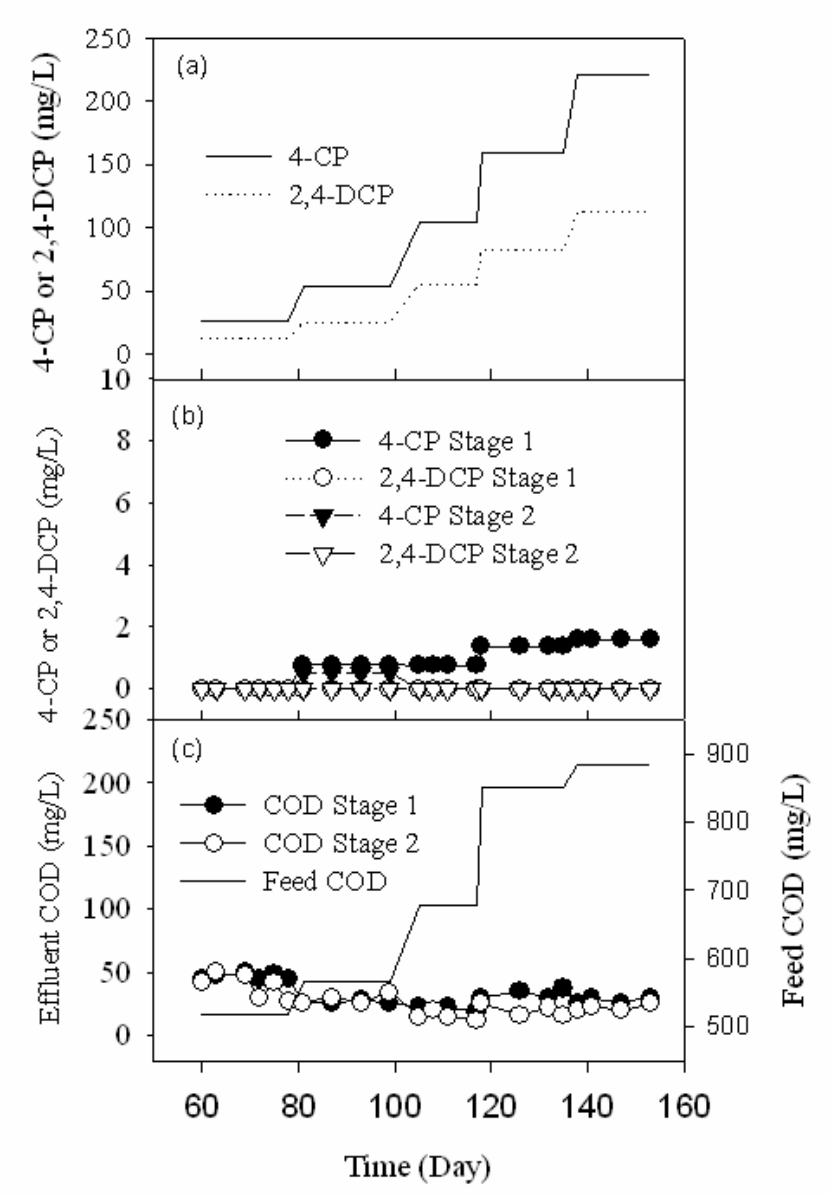

Figure 3.60. Influent and 1st stage effluent of chlorophenol concentrations in RBC

The feed and effluent of COD concentrations were also depicted in Figure 3.60. Although feed COD concentrations increased with increasing chlorophenols concentrations, high COD removal efficiencies were observed throughout the reactor operation. Similar to chlorophenols, most of the COD was degraded in 1st

stage and 2nd stage behaved only as a polishing step. The effluent COD concentrations were between 20-40 mg/L and the average COD removal efficiency was 96±2.36%. The observed low COD concentrations and the absence of CHMS in the effluent is the evidence of complete chlorophenols degradation without accumulation of degradation intermediates.

In the second set of the experiments, the 4-CP and 2,4-DCP concentrations were kept constant at around 220 and 110 mg/L (CP loading rate = 470 mg/L.d) and peptone concentration was gradually decreased from its initial value of 400 mg/L to null (Figure 3.61). Then, 4-CP and 2,4-DCP concentrations were increased to 300 and 150 mg/L, respectively (CP loading rate =643 mg/L.d) (Figure 3.61). The feed COD concentration decreased steadily as the peptone concentration was decreased and it increased with increasing feed chlorophenol concentrations. It was observed that high portion of the chlorophenols was removed in the first stage of the reactor and the second stage was only used as a polishing step. The average 4-CP concentrations in the effluent of first and second stage were 1.64±0.248 and 0.074±0.08 mg/L, respectively. As for 2,4-DCP, the average concentration in the effluent of first stage was 0.324±0.142 mg/L and complete removal of 2,4-DCP was observed in the effluent of second stage (concentrations were below detection limit (0.05 mg/L)).

Similar to chlorophenols, most of the COD was removed in the first stage as the average COD concentrations in the effluent of first and second stage of the reactor were 26.55±5.31 and 20.53±3.13 mg/L, respectively. The corresponding COD removal efficiencies were 96.03±0.717 and 97.14±0.465 mg/L, respectively (Figure 3.61). Hence, peptone presence at different concentrations had not any remarkable effect on chlorophenol removal performance of the system.

Figure 3.62 depicts the effect of peptone concentration on the MLSS and MLVSS concentrations at the effluent of the reactor and sludge production (mg MLVSS/mg COD). Both MLSS and MLVSS concentrations did not correlate with feed peptone

concentration and both were almost constant with the average values of 132±22 and 90.75±22.28 mg/L in respective order. Biomass production in the effluent of reactor per removed COD was also remained almost constant and its average value was observed to be 0.14 ±0.034 mg MLVSS/mg COD.

In the next experimental part, the effect of shock chlorophenols loading on the reactor performance was studied. For this purpose, reactor was fed with 400 mg/L peptone 200 mg/L 4-CP and 110 mg/L 2,4-DCP and HRT was increased from 0.70 to 3 days. The reactor was operated around 2.5 months under this conditions and then 300 mg/L 4-CP and 150 mg/L 2,4-DCP was given to the reactor in the absence of peptone and HRT was decreased to 0.70 d. It was observed that such a shock loading caused strong inhibition that shut down the metabolic activity of the microorganisms, leading to reactor failure. In order to recover the reactor from process failure, the influent stream was cut off and the reactor was operated in batch mode (Figure 3.63), whereas, almost no removal of chlorophenols was observed within 6 days.

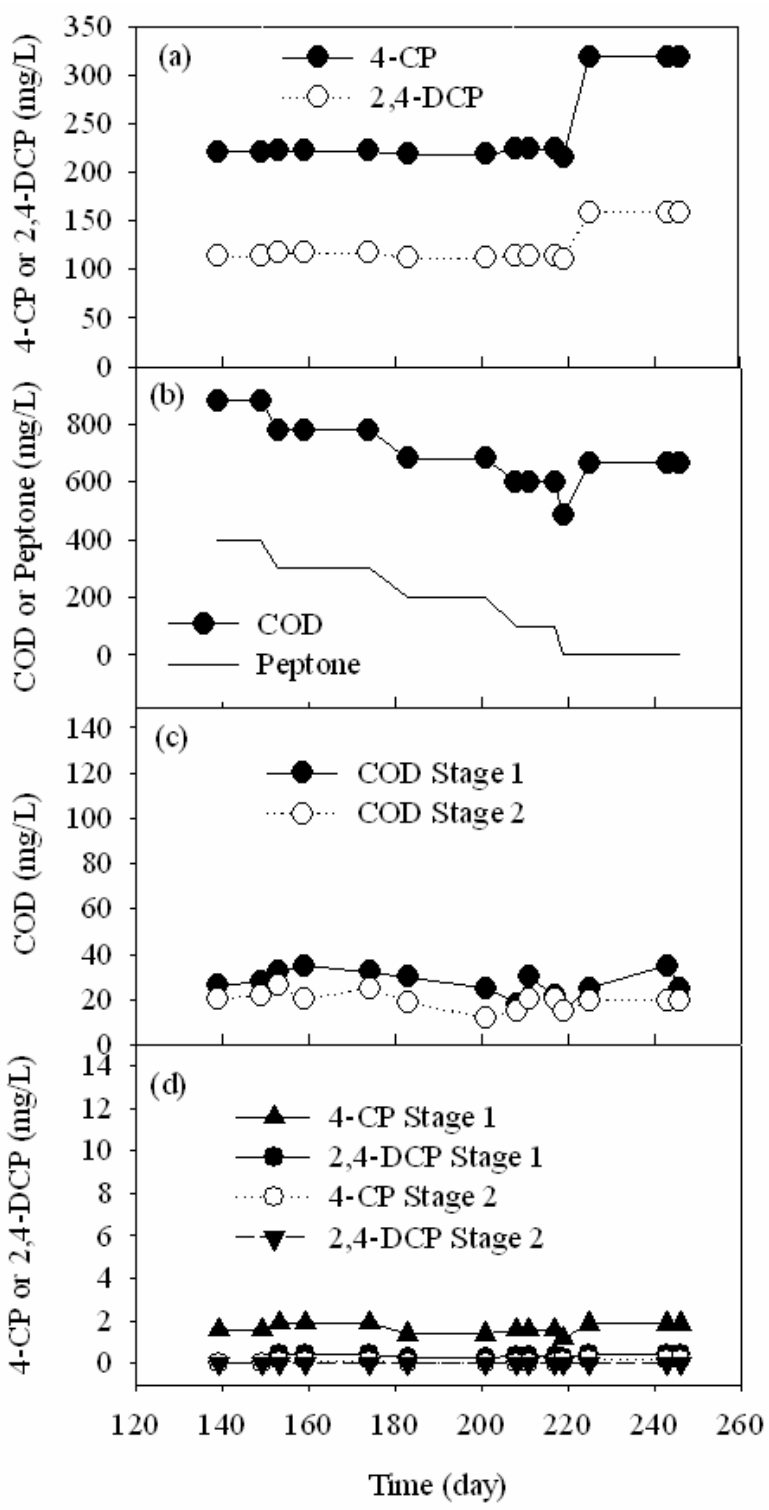

Figure 3.61. Effect of peptone concentration on the performance of RBC

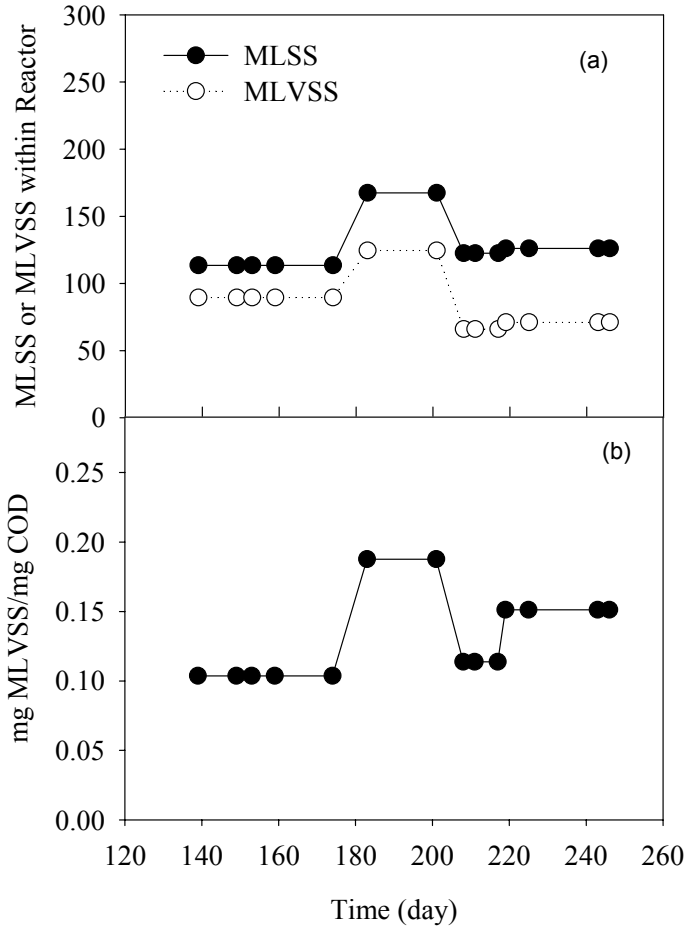

Figure 3.62. Effect of peptone concentration on the MLSS and MLVS concentrations in the effluent of RBC (a) and sludge production per mg COD removal (b)

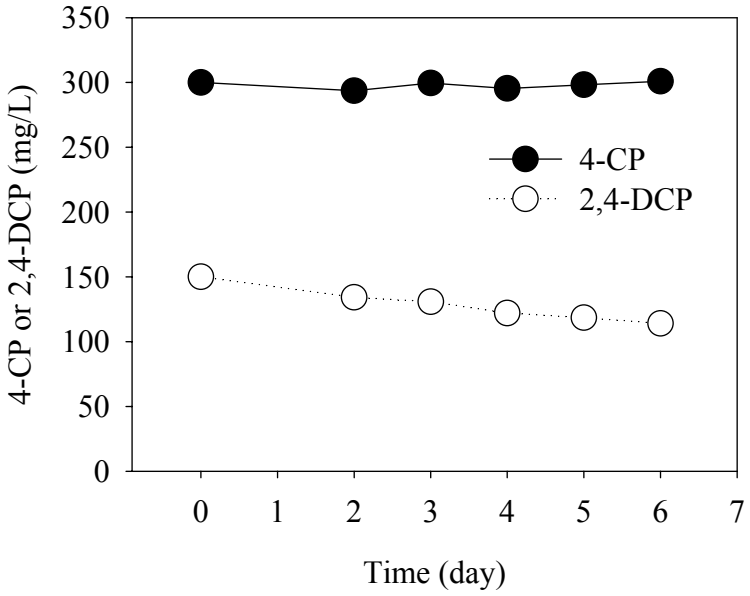

Figure 3.63. Batch wise operation of RBC to recover the reactor from process failure

After this observation, the reactor was fed again continuously with 400 mg/L of peptone in the absence of chlorophenol for 3.5 months. The aim is deacclimate the biomass and then evaluate the need of time to reacclimate the biomass to chlorophenols. Hence, after 3.5 months of operation with peptone only, chlorophenols were added again. Before addition of chlorophenols, it was observed that biomass have a good ability of peptone removal as the 400 mg/L COD in the influent decreased to 12 mg/L in the effluent. Figure 3.64 shows the feed and effluent chlorophenols concentrations. The reactor can directly start with a high removal efficiency when 4-CP and 2,4-DCP were started to be fed at 25 and 12.5 mg/L. Within16 days, 4-CP and 24-DCP concentrations were increased to 200 and 100 mg/L, respectively and no deterioration in the removal efficiency have been observed. When reactor was fed with 400 mg/L peptone, 200 mg/L 4-CP and 100 mg/L 2,4-DCP, high COD removal efficiencies were also observed as the synthetic wastewater COD of 915±13 mg/L decreased to 17±2 mg/L in the effluent. The high COD removal was the indicative of complete removal of chlorophenols without

accumulation of by-products, which was also verified by HPLC measurements. On day 37, peptone concentration was instantaneously decreased from 400 to 0 mg/L and no deterioration in the removal efficiency was observed (Figure 3.64). Also, on day 52, when the feed COD was 473±5 mg/L, 1st and 2nd stage COD of the reactor were 10 and 7 mg/L, respectively. When feed 4-CP and 2,4-DCP concentrations were 200 and 100 mg/L, respectively, the average effluent chlorophenols concentrations between days 37 and 52 are given in Table 3.8.

Table 3.8. Average chlorophenols concentrations within days 37 and 52

Compound	**Influent (mg/L)**	**Stg-1 Effluent (mg/L)**	**Stg-2 Effluent (mg/L)**
4-CP	208.35±5.2	1.07±0.25	0.077±0.031
2,4-DCP	106.86±4.6	0.236±0.082	<0.050

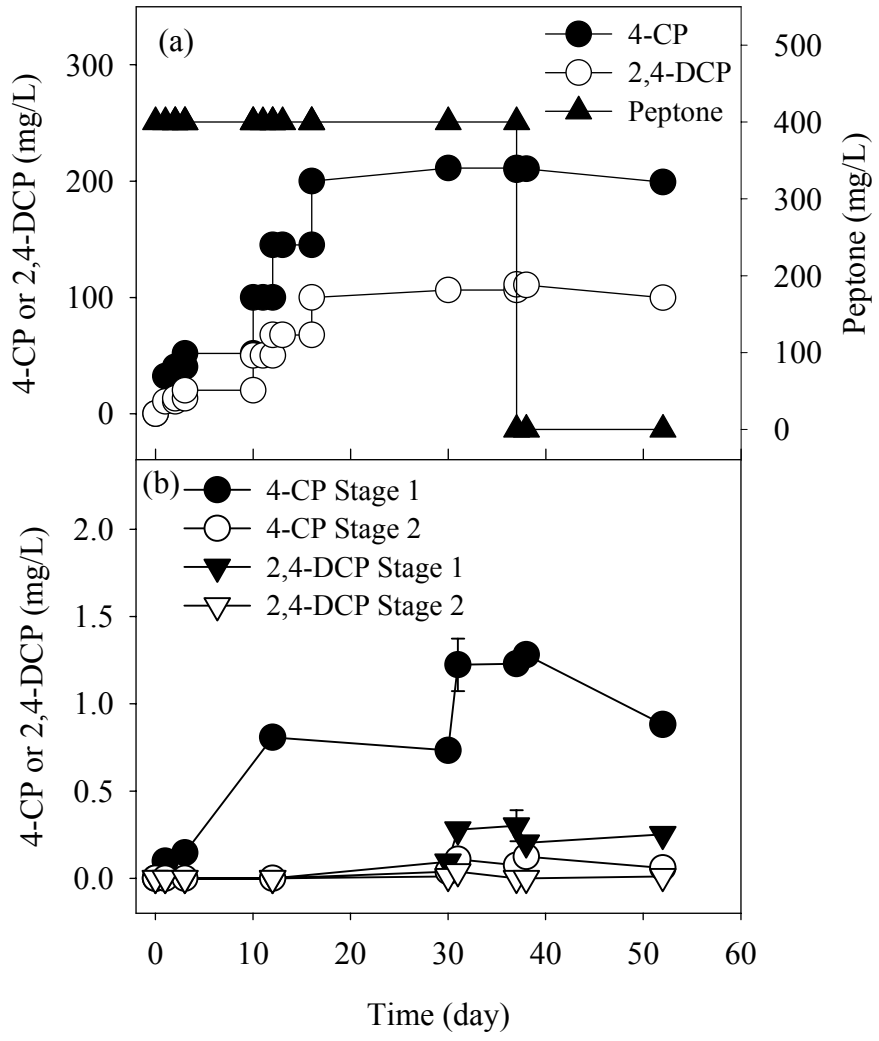

Figure 3.64. Influent (a) and effluent (b) chlorophenols concentrations in RBC

As explained above no adverse effect of peptone exclusion was observed on the performance of the reactor. In order to evaluate the effect of sudden peptone exclusion from the feed of the reactor on chlorophenol removal rate, batch experiments were carried out using sludge drawn from the reactor on day 32 (Figure 3.65). The RBC effluent was directly used to carry out batch experiments in which average MLVSS concentration was around 110 mg/L.

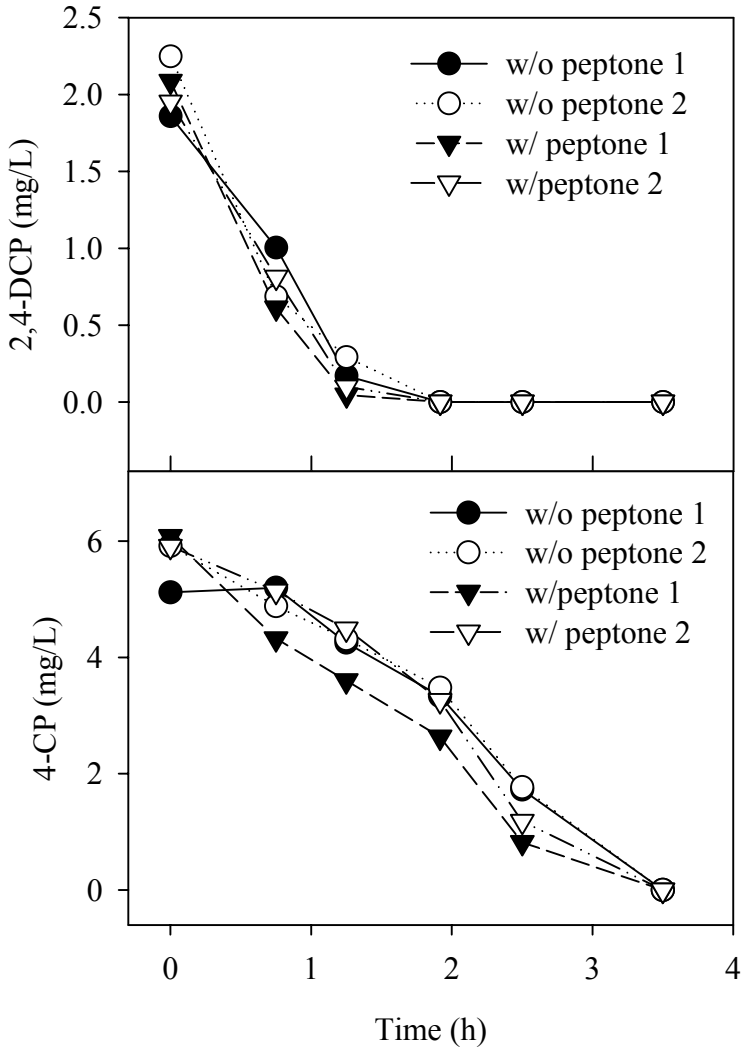

Figure 3.65. Batch experiments conducted in the presence and absence of peptone using sludge from RBC on day 32

No effect of peptone on chlorophenols removal pattern was observed also in batch experiments (Figure 3.65).

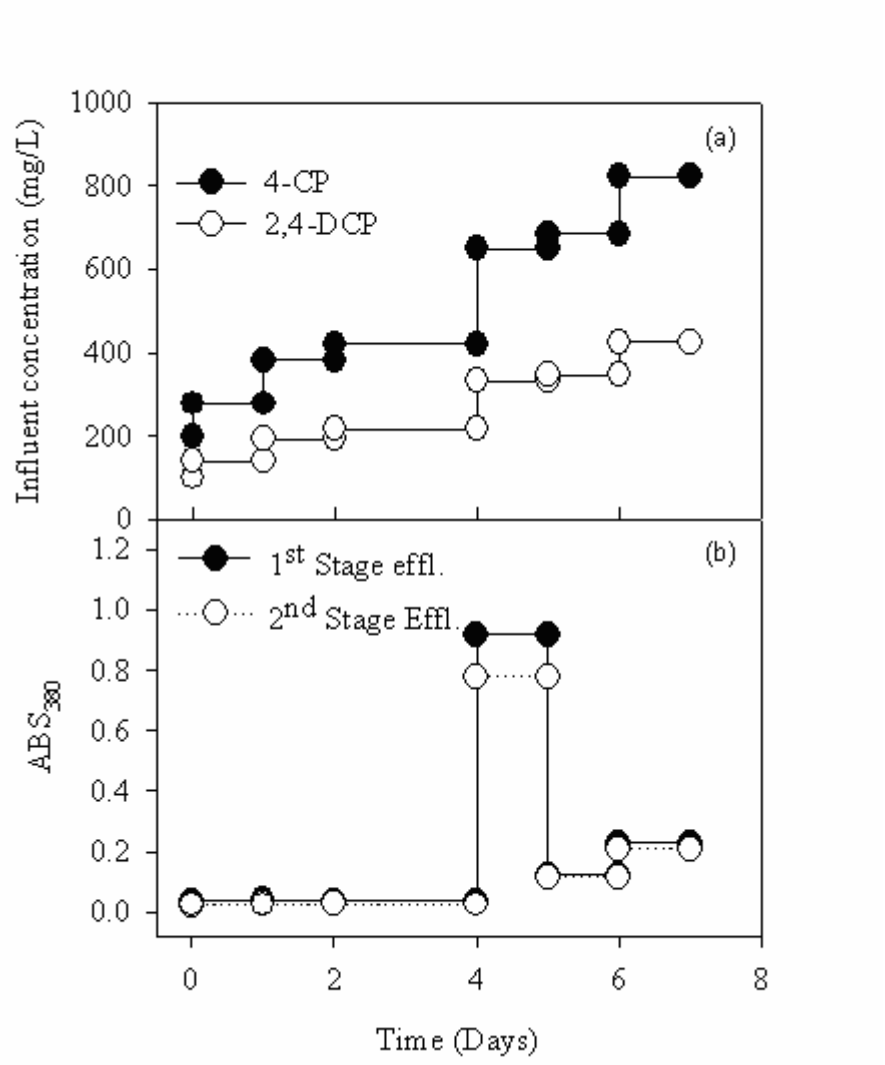

Figure 3.66. Feed chlorophenols concentrations (a) and effluent CHMS as absorbance at 380 nm (ABS380) (b) during the shock loading experiments

In the next stage of the study, effect of shock chlorophenols loading on the reactor performance was deeply investigated. The chlorophenols were fed to the reactor as sole carbon sources and the concentrations were increased as shown in Figure 3.66(a). The initial 4-CP and 2,4-DCP concentrations were around 200 and 100 mg/L, respectively, and the concentrations were increased up to 822.7±1.4 and 424.6±1.9 mg/L (total CP loading = 1781 mg/L.d) in respective order within 7 days. As feed chlorophenols concentrations were increased, the effluent concentrations in both stages increased linearly (Figure 3.67). For both stages, the effluent 4-CP

concentrations were almost 4 times higher than 2,4-DCP, which may be due to strong competitive inhibition of 2,4-DCP on 4-CP biodegradation. When feed 4-CP and 2,4-DCP concentrations were further increased to 1000 and 500 mg/L, respectively, removal efficiency sharply decreased and no removal was attained further due to complete inhibition of biomass (data not shown). The observed maximum 4-CP and 2,4-DCP removal rates in the first stage were 2305 mg/L·d (18.3 $g/m^2 \cdot d$) and 1202 mg/L·d (9.5 $g/m^2 \cdot d$), respectively. HRT of first stage was considered as 0.35 d for the calculation of degradation rates, as the total HRT of the system was 0.70 d.

Effect of first stage chlorophenol concentrations on the degradation rates of 4-CP and 2,4-DCP are shown in Figure 3.68 and 3.69, respectively. In the calculation of degradation rates, HRT of first stage was taken as 0.35 d as the total HRT of the system was 0.70 d and the reactor had two stages with the same volume. Removal rates increases with increasing chlorophenols concentrations in the reactor.

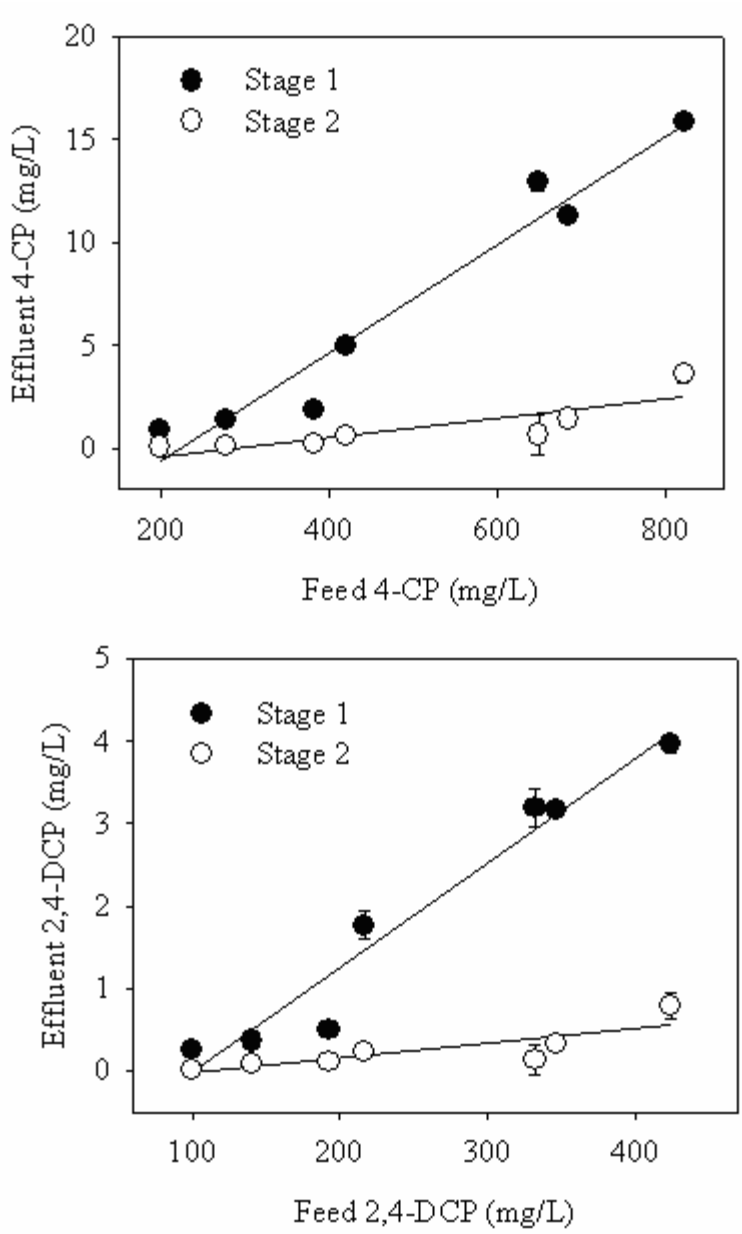

Figure 3.67. 4-CP and 2,4-DCP concentrations at the RBC effluent during shock loading experiments

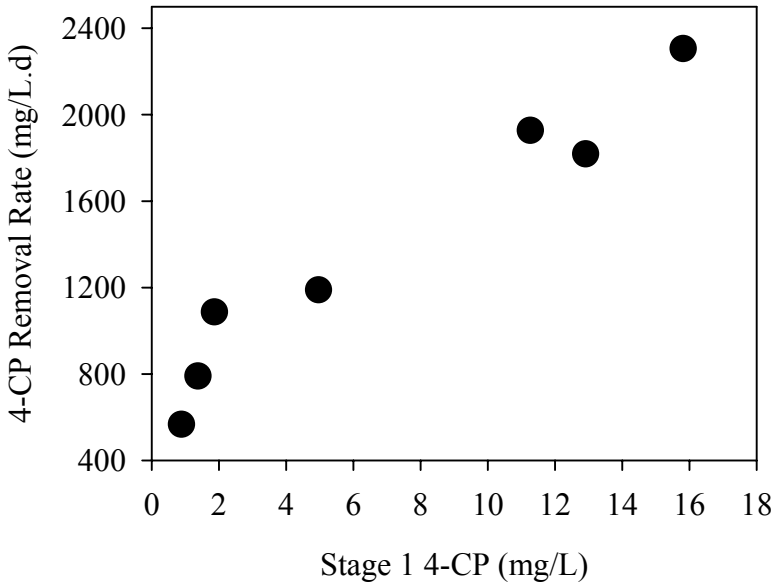

Figure 3.68. Effect of 4-CP concentration on the removal rate in the first stage

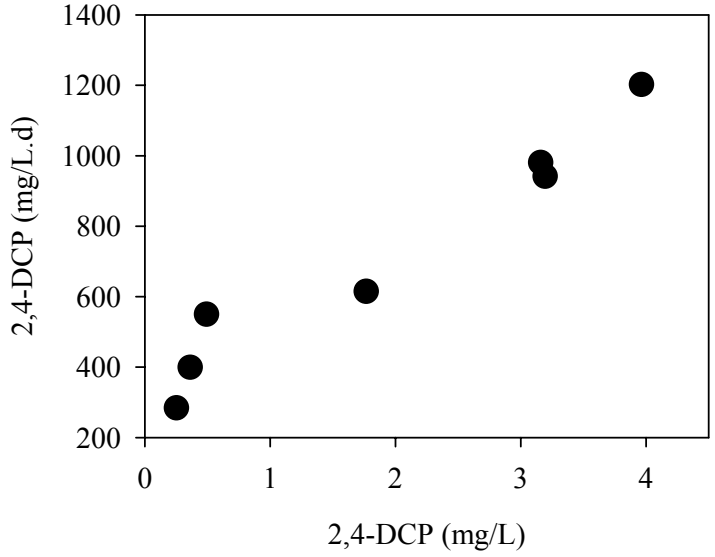

Figure 3.69. Effect of 2,4-DCP concentration on the removal rate in the first stage

Another significant point is that as the chlorophenols concentrations were increased the color of effluent turned to straw yellow due to the presence of CHMS at the effluent. The production of the intermediate was noteworthy especially on day 4

and 5 when feed 4-CP and 2,4-DCP concentrations were around 650 and 333 mg/L, respectively. In Figure 3.70, effluents from second stage of the RBC on day 2 and 4 are illustrated. The maximum reached ABS_{380} were slightly lower than 1.0 and using the extinction coefficient given by Farrell and Quilty (1999), the maximum CHMS at the effluent can be calculated as around 3.8 mg/L. When feed 4-CP concentration was 650 mg/L, the theoretically produced amount of CHMS concentration was calculated as 813 mg/L, therefore the effluent CHMS concentration is only 0.47% of its theoretical amount.

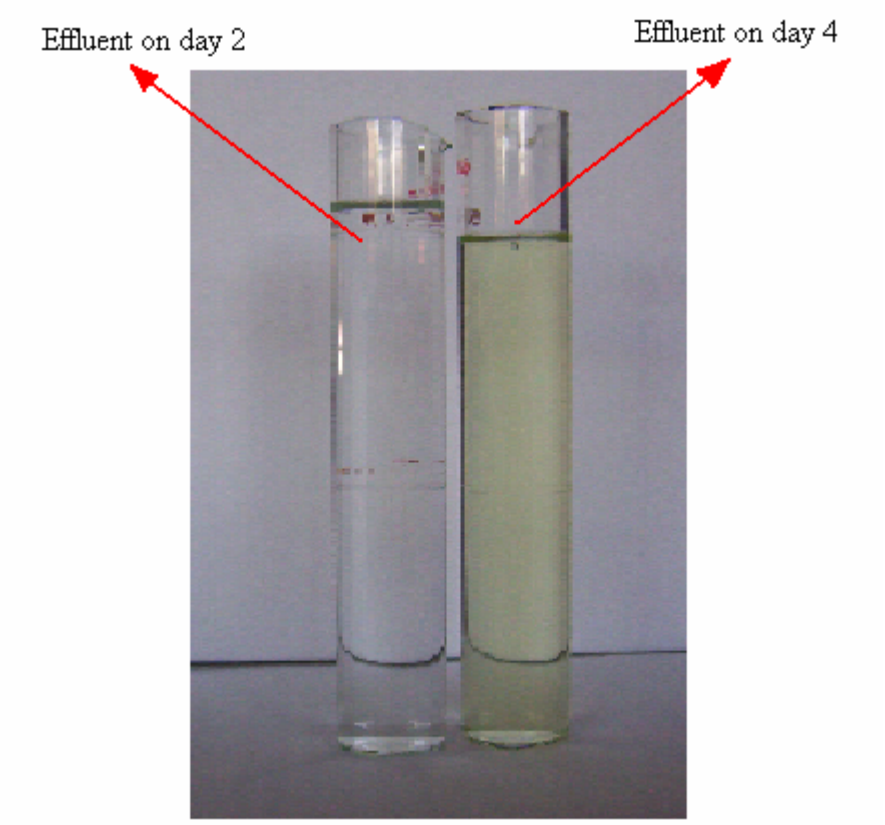

Figure 3.70. Effluent from second stage of RBC on day 2 and 4

Chlorophenols removal and loading rates are shown in Figure 3.71 and 3.72 for the first and second stage, respectively. Loading rates were almost equal to removal rates when 4-CP and 2,4-DCP loading rates in the first stage were 2300 and 1200 mg/L.d (total CP loading rate = 3500 mg/l.d), respectively. When the 4-CP and 2,4-DCP loading rate increased to 2847 and 1441 mg/L.d (total CP loading = 4288

mg/L.d), the removal rate decreased sharply and no removal of chlorophenols were further observed. Hence, it can be concluded that almost complete removal of 4-CP and 2,4-DCP can be observed up to loading rates of 2300 and 1200 mg/L.d, respectively. When both stages were considered, loading and the removal rates were around one half of those calculated for the first stage (Figure 3.71) as the most of the chlorophenols were removed in the first stage and both stages were equal in volume.

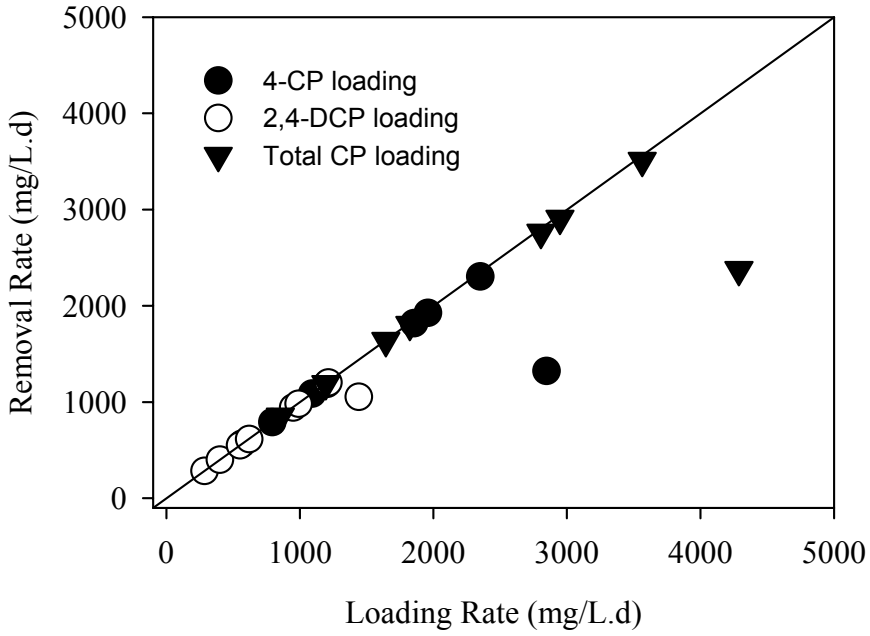

Figure 3.71. Removal rates vs. loading rates of chlorophenols in the first stage

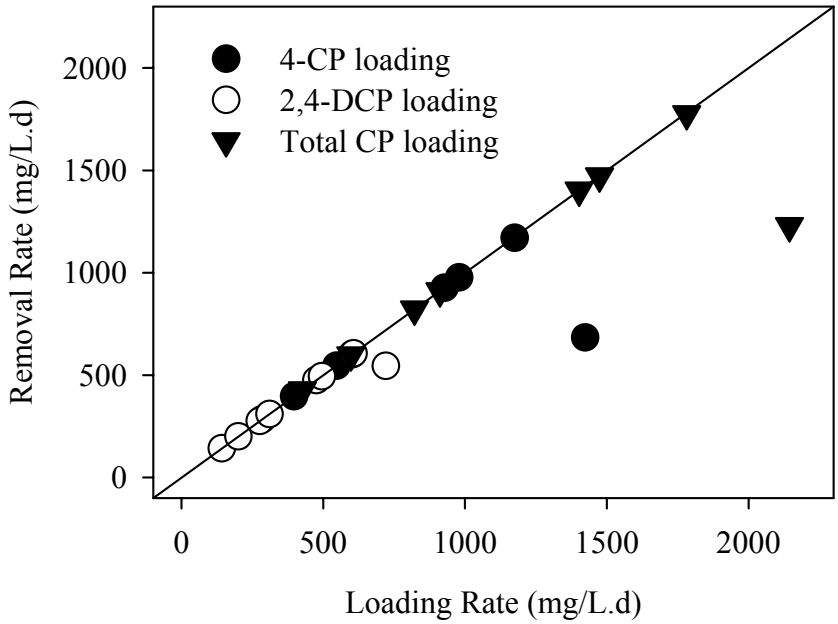

Figure 3.72. Removal rates vs. loading rates of chlorophenols in the second stage

CHAPTER 4

CONCLUSIONS

The main conclusions that can be drawn from this study can be summarized as follows:

Batch experiments

(1). Both 4-CP and 2,4-DCP started to inhibit their own degradation at 53 and 25 mg/L, respectively and the Andrews equation well described the inhibition kinetics.

(2). Culture acclimated to mixture of 4-CP and 2,4DCP (Culture M) had higher 4-CP and 2,4-DCP biodegradation rates than culture acclimated to 4-CP only (Culture 4). Both of the mixed cultures settled well. Although the pure strains isolated from mixed cultures were able to degrade chlorophenols, they did not settle well under quiescent conditions.

(3). 2,4-DCP competitively inhibits 4-CP biodegradation. The competitive inhibition was more remarkable in the case of culture acclimated to 4-CP only compared to culture acclimated to mixture of 4-CP and 2,4-DCP.

Sequencing Batch Reactor (SBR) Experiments

(4). The complete degradation of chlorophenols up to 200 mg/L 4-CP and 100

mg/L 2,4 DCP was observed in SBRs. Although CHMS accumulated during the degradation of 4-CP, the peak CHMS concentrations were only within 3-10% of its stoichiometrically calculated value, which indicated simultaneous removal of 4-CP and CHMS.

(5). Only specialist biomass was assumed to be responsible for chlorophenols degradation and the fraction of specialist biomass was estimated equal to the feed contribution of chlorophenols on COD basis. The developed models based on this assumption well fitted to the experimental data.

(6). The specific degradation rates of chlorophenols increased with increasing feed chlorophenols concentrations due to increase in competent biomass fraction. Specific degradation rates calculated on the basis of competent biomass concentrations (SDR_c) were relatively constant and did not show any correlation with feed 4-CP concentrations.

(7). High COD removal efficiencies and complete chlorophenol removals were observed even in the absence of peptone. Decreasing peptone (biogenic substrate) concentration did not cause significant change in 4-CP and 2,4-DCP biodegradation patterns and developed models well fitted to the experimental data.

(8). With long filling time, the accumulation of chlorophenols and the intermediate can be avoided in SBRs. Therefore, filling and reaction times should be decided based on the degradation kinetics and substrate interactions in multi-substrate systems. In the SBR operated at 8 h filling time, chlorophenols accumulated with decreasing peptone in the feed, whereas, specific degradation rates increased. Chlorophenols accumulation was due to the lag period after settling and strong competitive inhibition of 2,4-DCP on 4-CP degradation.

Rotating Biological Contactor (RBC) Experiments

(9). High chlorophenols (>98%) and COD (>94%) removals were achieved throughout the reactor operation in the first stage and the second stage

behaved only as a polishing step.

(10). Gradual exclusion of peptone did not adversely affect the chlorophenols removal performance of the RBC.

(11). Shock loading of 4-CP and 2,4-DCP up to 820 and 420 mg/L, respectively, did not interfere remarkably with the system performance.

(12). Attached growth reactors can tolerate higher chlorophenols loads compared to suspended growth reactors.

(13). Although the change of operational conditions may cause change of dominant specie, the community was quite robust as *Pseudomonas* species were generally dominant.

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SELECTED PUBLICATIONS

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