Reductive dechlorination under acidic condition

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**REDUCTIVE DECHLORINATION UNDER ACIDIC
CONDITION**

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SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING
2013**

REDUCTIVE DECHLORINATION UNDER ACIDIC CONDITION

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ABSTRACT

Chlorophenols with their toxicity, persistence and detrimental effects on the environment raise great concerns. Biological treatment seems to be the most economical way when dealing with wastewater containing these chlorinated compounds. Anaerobic dechlorination of chlorophenols has been extensively investigated. Despite it being effective in degrading chlorophenols, the process suffers from instabilities during operation. The imbalanced growth kinetics between acidogens and methanogens, accumulation of fermentative products leading to acidification of the system and methanogens' sensitivity to toxic compounds, like chlorophenols, make the whole process less stable and difficult to maintain. To solve the problem of process instability, acidogenic dechlorination of chlorophenols as pretreatment becomes a possible alternative. However, there are concerns still unaddressed such as:

- 1) To date, very few findings have been reported on acidogenic dechlorination process. Contradictory conclusions were drawn among different researchers;
- 2) Acidic condition is not the appropriate environment for the growth of chlororespiring bacteria;
- 3) The role of methanogens in dechlorination is arguable as different researchers reported contradictory findings. Excluding methanogens in the acidogenic environment for dechlorination is not conclusive in this case.

In this project, the anaerobic sequencing batch reactor (anSBR) was acclimated and operated at pH 5.5. The reduction of pH followed the step-wise reduction manner at the rate of 0.5 per week. The acclimated acidic sludge demonstrated capability to dechlorinate 2-MCP, 2,4,6-TCP and PCP but this was limited to attacking *ortho*-chlorines only. Besides the long acclimation period (80 days) initially, the acclimated sludge was dechlorinated TCP and PCP without lag phase.

When inhibited, caused by PCP overloading and extreme low pH (2.0), dechlorination was severely curtailed. It was found the inhibition was associated with methanogen's activity curtailment, whereas acidogenesis still continued. The qPCR analysis of microbial communities revealed the *Methanosarcinaceae* family

was the only group, which dropped sharply in terms of copy number. These results led to the hypothesis that methanogens participated in the dechlorination of chlorophenols under acidic condition.

Specific inhibitors to methanogens and gram-positive bacteria were applied to investigate the roles of different groups of anaerobes in dechlorination. Both inhibitors affected dechlorination, with the methanogenic specific inhibitor (BES) inhibiting dechlorination of 2-MCP no matter which electrons were applied. The antibiotic of gram-positive bacteria (vancomycin) did inhibit dechlorination as well. In addition, more methane was produced in batches with dosage of vancomycin suggested that acetate was directed to methanogenesis from other pathways during the inhibition. The findings in this study implied a syntrophism was involved in dechlorination. Both methanogens and the acetate-oxidizing bacteria should be properly maintained in order to perform dechlorination of 2-MCP under acidic condition.

The results found in this project implied it was not possible to exclude methanogens from the dechlorination process hence the instability issues associated with the conventional anaerobic dechlorination may not be solved by phase separation in the traditional sense. Community analysis of the sludge did suggest there were in fact limited phenotypes of methanogens, which could survive and participate in the dechlorination process. The system preferentially selected certain groups of methanogens, which demonstrated the ability to survive in the low pH environment.

This project confirmed the notion the acidic reactor could dechlorinate chlorophenols but with the participation of methanogens and acetate-oxidizing bacteria. The process was therefore not strictly phase separated but was shown to be a viable treatment process for chlorophenols.

LIST OF SMBOLS

anSBR	Anaerobic Sequencing Batch Reactor
BES	2- bromoethanesulfonic acid
C _e	Residual concentration of adsorbate at equilibrium
CF	Chloroform
CH ₄	Methane
CO ₂	Carbon Dioxide
DCA	Dichloroethane
DCP	Dichlorophenol
H ₂	Hydrogen
HAc	Acetic Acid
HBu	Butyric Acid
HPr	Propionic Acid
HRT	Hydraulic Retention Time
HVc	Valeric Acid
K	Adsorption capacity
K _s	Half Saturation Constant
MCP	Monochlorophenol
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
P/S ratio	Primary to Secondary Substrate Ratio
PCB	Polychlorobiphenyl
PCE	Tetrachloroethylene
PCP	Pentachlorophenol
q _e	Amount of adsorbate by adsorbent at equilibrium
qPCR	Quantitative Polymerase Chain Reaction
SRT	Solid Retention Time
TBP	Tribromophenol
TCE	Trichloroethylene
TCP	Trichlorophenol
TeCP	Tetrachlorophenol
U.S. EPA	United States Environmental Protection Agency

VFA

Volatile Fatty Acid

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CHAPTER 1 Introduction

1.1 Background

Chlorinated organic compounds are widely spread in the environment and are among the largest and most toxic groups of hazardous chemicals. Because they are generally resistant to both chemical and biological degradation processes, they can be persistent in all kinds of environments ranging from natural water bodies, groundwater, soil, and sediments (Stringer and Johnston, 2001). Due to the chronic and acute toxic effects on human health as well as the detrimental effects on biological treatment systems, government environmental agencies have implemented very stringent regulations on the discharge limits on these pollutants. Sixty-one chlorinated compounds are listed on the U.S. Environmental Protection Agency (U.S. EPA) priority pollutants list and chlorinated compounds make up about half of the total of 126 hazardous compounds on the list (EPA, 1989). Various industrial processes generate chlorinated compounds. Pre-treatment of the polluted effluent stream seems the best way to reduce discharge into the natural environment.

Chlorophenols are an important class of chlorinated pollutants (Ozkaya, 2005) with toxicity increasing with the number of substitute chlorines. Highly chlorinated phenols, like pentachlorophenol (PCP), are generally more resistant to biological treatment processes and more toxic than lower chlorinated compounds. The increased number of substitute chlorines increases the oxidative nature of the compounds and make the compounds less soluble in water. Biological treatment is almost always the most economical measure to deal with wastewater containing chlorophenols.

Due to the highly oxidative nature of chlorophenols, aerobic degradation is typically not appropriate and anaerobic treatment for degradation of chlorophenols is the better alternative because of the consequent reductive environment. Today, extensive researches and investigations are conducted in anaerobic reductive dechlorination of chlorophenols (Basu *et al.*, 1996; Chang *et al.*, 1999; Collins *et al.*, 2005; Katayama *et al.*, 2009). Anaerobic treatment of industrial wastewaters

containing both primary organics and chlorophenols is preferable because the process offers a few advantages beside reductive dechlorination of the chlorophenols. The anaerobic process produces less sludge, requires no aeration and produces methane (CH_4) as the end product for energy recovery. These features make the anaerobic process more cost effective.

However, there are limitations in the anaerobic process despite all of the above-mentioned attractive attributes. The successful operation of anaerobic systems requires close monitoring and maintenance due to the complexity inherent in the anaerobic process. The stability of the overall performance depends on the cooperation among different microbial species involved in the system. Any disturbance such as pH fluctuations, and sharp surges in loadings could easily cause process failure. The imbalanced growth kinetics between acidogens and methanogens make it difficult to maintain overall optimal performance. Accumulation of fermentative products, like VFAs, could easily acidify the system, which inhibits the proper function of methanogens. This problem can become worse if treating wastewaters containing chlorophenols. Methanogens are very sensitive to such toxic compounds and methanogenic activity can decrease very quickly even at a low concentration (Speece, 1996).

In order to tackle this problem, two-phase anaerobic process for treatment of inhibitory compounds has been advocated by several researchers (Ng *et al.*, 1999; Demirel and Yenigun, 2002; Yu and Fang, 2003). The phase separated anaerobic process can supposedly establish desired optimal environments for both acidogens and methanogens, and thereby limiting exposure of methanogens to inhibitory compounds. The acidogenic phase leads the methanogenic phase as a pre-treatment process to partially convert the inhibitory compounds to less toxic forms for the downstream anaerobic process.

However, dechlorination of chlorophenols acidogenically has not been studied extensively so far. Limited information is available. Two different research groups investigated the feasibility and fate of dechlorination of PCP (Piringer and

Bhattacharya, 1999; Mun *et al.*, 2008b), but these groups drew different conclusions regarding the acidogenic dechlorination of the compound because of the different acidification techniques and acclimation processes. Mun *et al.* (2008b) successfully proved the feasibility of dechlorination by the acidogenic bacteria with application of appropriate inducing strategies, however the other group (Piringer and Bhattacharya, 1999) found that PCP was not dechlorinated when the system was operated at pH 5.5 (See Chapter 2). Despite this conclusion, that the chlorophenols could be degraded under acidic condition (Mun *et al.*, 2008a; Mun *et al.*, 2008b), contradictory information about dechlorination under acidic condition has been reported. Firstly, the pH range for the identified dechlorinating bacteria falls mainly between 6.0 to 9.0 and the optimum pH is believed to be slightly above 7.0 (Mohn and Kennedy, 1992b; Cole *et al.*, 1994; Sanford *et al.*, 1996; Sun *et al.*, 2000). Villemur *et al.* (2006) found that *Desulfitobacterium* spp. could be inhibited at pH 6.0. The literatures then implied that the acidic condition was not the appropriate environment for cultivating dechlorinating bacteria. Secondly, some researchers reported the involvement of methanogens in dechlorination of aliphatic compounds such as chloroforms (Yu and Smith, 1997), dichloroethane (Holliger *et al.*, 1990) and tetrachloroethylene (Fathepure and Boyd, 1988) as well as the aromatic polychlorobiphenyl (Ye *et al.*, 1995). Whereas, others found the dechlorination of chloroform and 2,4,6-trichlorophenol (TCP) continued when methanogenesis was inhibited by the inhibitor (Perkins *et al.*, 1994; de Best *et al.*, 1999). The involvement of methanogens in the dechlorinating process suggested the importance of maintaining the methanogenic population, whereas the acidic environment was inhibitory to methanogens. The conflicting information requires more investigations in detail about the dechlorination under acidic condition.

1.2 Problem statement

The possibility of dechlorination of chlorophenols under acidic condition has been studied but so far only limited information is available. Firstly, the literature has reported conflicting findings regarding the dechlorination of PCP at pH 5.5. The process of such acidic dechlorination needs to be verified. Secondly, because of the inconsistent conclusions on the role of methanogens in the dechlorinating process,

investigations are required for proper determination and hence operation of the dechlorination process. Lastly, the dechlorinator was not identified even in the case where dechlorination of chlorophenols was successful under acidic condition. Such information will be important for the design and operation of anaerobic dechlorinating facilities. This project seeks to investigate acidic dechlorination to address these issues.

1.3 Objectives

The overall objectives of this project are to verify the feasibility of dechlorination under acidic condition and to investigate the possible microbial candidates involved in the process. The following areas are investigated:

- 1) To establish and verify the process of dechlorinating chlorophenols under an acidic environment;
- 2) To examine the inhibitory factors on and the responses of the dechlorination process and lastly;
- 3) To investigate and identify the possible microorganisms participating in dechlorination under acidic condition.

1.4 Organization of the thesis

This thesis consists of the following chapters. Each chapter elaborates a specific area of the project, which would contribute to meeting the overall objectives.

Chapter 2 – Literature Review

The chapter presented information on chlorinated compounds, treatment process, dechlorinating mechanisms, and the problems associated with the current anaerobic dechlorinating techniques. The conflicting findings regarding dechlorination under acidic condition are discussed.

Chapter 3 – Materials and Methods

The description of the anaerobic sequencing batch reactor (anSBR), the feed media, and the analytical methods are presented in this chapter. In each of the following

chapters there is also a specific section describing the experiment designs for that particular chapter.

Chapter 4 – Dechlorination of Chlorophenols under Acidic Condition

This chapter described establishment of the acidic reactor (pH 5.5) for dechlorinating various chlorophenols following the start-up methods described by Mun *et al.* (2008a). 2-monochlorophenol (MCP) was dechlorinated after long acclimation period and the acclimated sludge could be utilized for dechlorinating 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP). Dechlorination under acidic condition was found to be limited to *ortho*-dechlorination.

Chapter 5 – Inhibition on Dechlorination under Acidic Condition

The inhibitory effects of overloading with PCP, extreme low pH (pH 2) and the metabolite (phenol) were discussed in this chapter. The quantitative polymerase reaction (qPCR) technique was applied to track the microbial community changes during inhibition. It was found that dechlorination was inhibited when methanogenesis was severely affected. Hence, it led to suspicion on the role methanogens may have in dechlorination.

Chapter 6 – Syntrophism in Dechlorination under Acidic Condition

In Chapter 6, the role of different groups of microorganisms was investigated in the dechlorination of 2-MCP by applying specific inhibitors. The results indicated complete dechlorination required proper maintenance of acetate-oxidizing bacteria and methanogens. It was proposed there is syntrophic relationship between the two microorganisms.

Chapter 7 – 16S rDNA Clone Library of The Acidic Dechlorinating Community

The 16S rDNA clone libraries were constructed for bacteria and archaea communities to analyze the microbial community that dechlorinated chlorophenols under acidic condition. It was found that specific methanogens (probably

conducting dechlorinations) were present in the reactor sludge even after it was operated at pH 5.5 for over 2 years.

Chapter 8 – Conclusions and Recommendations

This last chapter listed the major findings and drew conclusions from the results discussed in the previous chapters. Dechlorination under acidic condition was feasible with appropriate acclimation and operations. The microbial community should be maintained properly to ensure performance of the system. A possible dechlorinating mechanism under acidic condition was proposed. Finally, recommendations on additional investigations on dechlorination under acidic condition were suggested in order to cover the remaining questions, which were not addressed in this project.

CHAPTER 2 Literature Review

2.1 Chlorinated compounds and chlorophenols

Various industrial processes generate chlorinated organic wastes. In general there are two major types of chlorinated compounds, chlorinated aliphatic organics (e.g. carbon tetrachloride, trichloroethylene and tetrachloroethylene) and chlorinated aromatic compounds (e.g. chlorophenols and chlorobenzenes). Both of these have high production volumes annually. The presence of chlorinated organics in wastewater raises environmental concerns due to their toxicity, carcinogenicity, and poor biodegradability (Fetzner and Lingens, 1994). In the list of priority pollutants compiled by U.S. EPA, sixty-one of the 126 priority pollutants are chlorinated organics (EPA, 1989). They are widely used as insecticides, solvents, cleaning agents, gasoline additives, herbicides and so on. Due to improper storage, handling and disposal, their residuals are widely spread over groundwater, soil, and surface water bodies (Bhatt *et al.*, 2007). These chlorinated wastes impose detrimental effects on biological treatment systems due to their inhibitory nature to microorganisms.

Chlorophenols constitute an important class of chlorinated pollutants (Ozkaya, 2005; Savant *et al.*, 2006; Bajaj *et al.*, 2008). As with other chlorinated organics, chlorophenols are persistent and accumulative in the environment. The recalcitrance of chlorophenols results from two factors: 1) stability of the aromatic structure and 2) the carbon-hydrogen bond, which is difficult to cleave (Farrell and Quilty, 2002). With additional chlorine atoms attached to the benzene ring, the higher chlorinated phenols become more hydrophobic. Although they transport less in the aquatic media, microorganisms have less access to these highly chloro-substituted compounds. They usually persist longer in the environment, such as the most chlorinated phenol, pentachlorophenol (PCP). PCP is widely used as a bactericide, antifungal agent, herbicide, pesticide and wood preservative (Kao *et al.*, 2004). PCP has a wide spectrum of toxic effects and it is known as an endocrine disrupter (Beard and Rawlings, 1999). In cells, the proton gradient across membranes is disrupted by PCP. It has been identified as a probable human carcinogen (Keith and Telliard, 1979). As the toxicity and bioaccumulation potential increase with the

degree of chlorination (Loehr and Krishnamoorthy, 1988), researchers paid more attention to the dechlorination process of highly chlorinated phenols (Alleman, 1995; Chang *et al.*, 1998; Dercova *et al.*, 2003; Kao *et al.*, 2004; Ye *et al.*, 2004; Machado, 2005; Chen *et al.*, 2006; Freire *et al.*, 2008; Shen *et al.*, 2008; Katayama *et al.*, 2009; Suyin *et al.*, 2009).

Because of their environmental concerns, the international community has placed stringent limits on PCP present in drinking water. World Health Organization imposed a standard for drinking water quality of 9 µg/L (World Health Organization, 2006). European Union (EU) set a maximum concentration of 0.5 µg/L for total chlorophenols and individual concentration below 0.1 µg/L (Quintana and Ramos, 2008).

2.2 Biodegradation and dechlorination of chlorinated compounds

Chlorinated compounds can be degraded, both aerobically and anaerobically. Biological treatment is currently considered the most economical technology. The biological process allows simultaneous removal of primary organics and chlorinated compounds hence simplifying the process of treating wastewater containing chlorinated organics (Mun *et al.*, 2008a).

2.2.1 Aerobic dechlorination

During aerobic dechlorination, mono- or dioxygenase enzymes function via metabolic or cometabolic reactions and molecular oxygen serves as the electron acceptor (Bhatt *et al.*, 2007). The oxidative (aerobic) degradation of chlorinated compounds is mainly due to co-metabolism by several oxygenases (Fetzner, 1998). The oxygenases either epoxidate the carbon-carbon double bond or cleave the benzene ring for dechlorination. This process is quite successful when dealing with aliphatic compounds. A few studies have shown successful degradation of chlorinated compounds, including PCP, by *Phanerochaete* spp. (Lamar *et al.*, 1990). Carucci *et al.* (2008) applied aerobic granules and successfully removed 4-chlorophenol. It is well documented that chlorobenzenes with up to four chlorine substituents could be aerobically mineralized (de Bont *et al.*, 1986; Spain and

Nishino, 1987; Haigler *et al.*, 1988; Sander *et al.*, 1991). However, under aerobic conditions, the dechlorination rate decreases with increase in chlorine atoms in the compound. As shown in Figure 2.1, the aerobic process is effective for chlorinated compounds with a low degree of chlorine-functional group substitution. Since the chlorine functional group is at a high oxidative state, further oxidation of highly chlorinated organics is less likely to occur.

Figure 2.1 Relative trends of oxidative and reductive dechlorination as a function of chlorination from Bossert *et al.* (2003)

2.2.2 Anaerobic dechlorination

The reductive environment in the anaerobic process makes it possible to conduct reductive dechlorination of chlorinated organics by replacing the chlorine atom with hydrogen. Anaerobic dechlorination of chlorinated compounds has been reported extensively, including both aliphatic and aromatic (Dolfing and Tiedje, 1991; Holliger *et al.*, 1998; Boopathy, 2002; Chin *et al.*, 2005; Chen *et al.*, 2006). Even with the more toxic chlorophenols, anaerobic dechlorination showed a high efficiency. In fact anaerobic dechlorination could be more suitable due to the high oxidative status of highly chlorinated phenol. Ye and Li (2007) stated PCP, over a range of 170mg/L to 182 mg/L, was degraded to 3-chlorophenol (CP) and the PCP removal rate was 99.5%. Greer *et al.* (2002) also achieved a PCP removal rate of 99%. Hence the anaerobic process is considered to be an effective process to treat wastewater containing chlorinated pollutants. Besides removing the chlorinated compounds, the anaerobic process for dechlorination and simultaneous primary

organics degradation enjoys significant advantages like lower sludge production, lower energy requirement and production of useful methane (CH₄) gas (Demirel and Yenigun, 2002).

2.2.3 Respiratory dechlorination and dechlorinating bacteria for chlorophenols

Anaerobic dechlorination could be a respiratory process if the dechlorinating bacteria exist in the system. The bacteria use the chlorinated compounds as the energy source from the dechlorinating process.

Desulfomonile tiedjei strain DCB-1 was first discovered to dehalogenate 3-chlorobenzoate (DeWeerd *et al.*, 1990). The same strain was found to dechlorinate PCP to 2,4,6-TCP by Mohn and Kennedy (1992b). Since then, a few isolates or pure culture of dechlorinating bacteria have been reported. Most of them belong to the genera, *Desulfomonile*, *Desulfovibrio* and *Anaeromyxobacter* in the phylum *Proteobacter* (Class *Deltaproteobacteria*) and the genus *Desulfitobacterium* in the phylum *Firmicutes* (Itoh *et al.*, 2011). *Anaeromixobacter dehglogans* strain 2CP-1 was isolated and it was capable of *ortho*-dechlorinating 2-MCP, 2,5-DCP and 2,6-DCP with acetate as the electron donor (Cole *et al.*, 1994). Another strain, *Desulfovibrio dechloracetivorans* Strain SF3, was isolated by Sun *et al.* (2000). This isolate could use acetate, lactate, propionate, pyruvate, alanine and ethanol as the electron donor coupled with dechlorination of 2-MCP but was limited to *ortho*-dechlorination of 2-MCP and 2,6-DCP.

Deulfitobacterium contains a large number of strains capable of dechlorinating chlorophenols. *Desulfitobacterium dehalogans* strain JW/IU-DC1 is capable of removing the *ortho*-chlorines from DCPs, TCPs and PCP (Utkin *et al.*, 1994; Utkin *et al.*, 1995). Stanford *et al.* (1996) reported a related strain of JW/IU-DC1, *Desulfitobacterium chlororespirans* Co23, which dechlorinated DCPs and TCP at the *ortho*-position but not MCPs. *Desulfitobacterium hafniense* DCB-2 is capable of both *ortho*- and *meta*-dechlorination of DCPs, TCPs and PCP (Madsen and Licht, 1992) and the PCP-1 strain is capable of removing all substituted chlorines at

ortho-, *meta*- or *para*-positions from DCPs, TCPs, TeCPs and PCP with exceptions of 2,3-DCP, 2,5-DCP, 3,4-DCP and MCPs (Bouchard *et al.*, 1996).

The summary of isolates capable of dechlorinating chlorophenols is presented in Table 2.1. From the table it may be concluded that: 1) dechlorinating bacteria usually could utilize a wide range of electron donors from H₂, pyruvate to VFAs; 2) the pH range for the bacteria to dechlorinate falls between 6.0 and 9.0 but normally the optimum pH is above 7.0 to a slightly alkaline environment; 3) the dechlorinating bacteria works better with poly-chlorinated phenols, only a few strains could dechlorinate MCP, and 4) besides the *Desulfitobacterium frappieri* PCP-1 strain, which is capable of removing chlorines from all three positions, the dechlorinating bacteria target chlorines at specific substituted positions in the chlorophenols. This may become a problem in treatment of chlorophenols with poly-substituted chlorines. In order to remove the chlorines completely, different dechlorinating bacteria might be required to be present in the system.

Table 2.1 Isolates or pure culture to dechlorinate chlorophenols

Species	Electron acceptor	Electron donor	Position of dechlorination	pH range	References
<i>Deltaproteobacteria</i>					
<i>Desulfomonile tiedjei</i> DCB-1	PCP	H ₂ , Formate, Pyruvate,	<i>meta</i>	6.5- 7.8	(DeWeerd <i>et al.</i> , 1990; Mohn and Tiedje, 1991; Mohn and Kennedy, 1992b)
<i>Anaeromixobacter</i> <i>dehalogens</i> strain 2CP-1	2-MCP, 2,5-DCP, 2,6-DCP	Acetate,	<i>ortho</i>	7.5	(Cole <i>et al.</i> , 1994)
<i>Desulfovibrio</i> <i>dechloracetivorans</i>	2-CP, 2,6-DCP	Acetate, Lactate, Propionate, Pyruvate, Ethanol	<i>ortho</i>	7.3- 7.5	(Sun <i>et al.</i> , 2000)
<i>Firmicutes</i>					
<i>Desulfitobacterium</i> <i>dehalogenans</i> JW/IU-DC1	2,4-DCP, 2,3-DCP, 2,6-DCP, 2,4,6-TCP	Formate, H ₂ , lactate, pyruvate	<i>ortho</i>	6.0- 9.0	(Utikin <i>et al.</i> , 1994; Utkin <i>et al.</i> , 1995)
<i>Desulfitobacterium</i> <i>chlororespirans</i> Co23	2,3-DCP, 2,6-DCP, 2,4,6-TCP	Formate, Butyrate, H ₂ , Crotonate	<i>ortho</i>	7.2- 7.5	(Sanford <i>et al.</i> , 1996)
<i>Desulfitobacterium</i> <i>hafniense</i> strain DCB-2	2,4-DCP, 3,5-DCP, 2,4,6-TCP, 2,4,5-TCP, PCP	Pyruvate	<i>ortho, meta</i>	7.0	(Madsen and Licht, 1992)
<i>Desulfitobacterium</i> <i>hafniense</i> PCP-1	PCP, 2,3,4,5-TeCP, 2,3,5,6-TeCP, 2,3,4-TCP, 2,3,5-TCP, 2,3,6-TCP, 2,4,5-TCP, 2,4,6-TCP, 3,4,5-TCP, 2,6-DCP, 2,4-DCP 3,5-DCP	Pyruvate, Formate	<i>ortho, meta,</i> <i>para</i>	6.0- 9.0	(Bouchard <i>et al.</i> , 1996)

2.2.4 Anaerobic dechlorination as a cometabolic process

Anaerobic dechlorination could be carried out by anaerobic microorganisms cometabolically. The anaerobes utilize external carbon sources as the primary substrate to gain energy while degrading the secondary substrate using the enzymes produced. Cometabolism can be an important mechanism for dechlorination anaerobically. Several anaerobic strains had been shown to be related to the dechlorinating process. Fathepure and Boyd (1988) demonstrated a strong interdependence between the dechlorination of tetrachloroethylene (PCE) and substrate consumption by *Methanosarcina* strain DCM. Their results clearly indicated the positive correlation between consumption of carbon substrate (methanol) and accumulation of the dechlorinating metabolite, trichloroethylene (TCE), and CH₄. The same strain was unable to dechlorinate PCE if no primary substrate was added. Hence they proposed the electrons transferred during methanogenesis were transferred to PCE by an unknown electron transporter involved in CH₄ production, which suggested dechlorination of PCE was a cometabolic process. Holliger *et al.* (1990) reported the dependence of dechlorination of 1,2-dichloroethane on the methanogenic activities of *Methanosarcina barkeri* cells. The same authors found involvement of the corrinoid and the protein-bound factor F₄₃₀ was the catalyst involved to transform 1,2-dichloroethane to ethylene and chloroethane (Holliger *et al.*, 1992a; Holliger *et al.*, 1992b). The F₄₃₀ is only found in methanogenic archaea (Thauer, 1998). Other anaerobes including homoacetogens containing a high level of corrinoid have shown capability to dechlorinate PCE by *Sporomusa ovate*, and trichloroethane and chloroform by *Clostridium* sp. strain TCAIIB respectively (Gälli and McCarty, 1989; Terzenbach and Blaut, 1994). These results suggested it was possible to dechlorinate chlorinated compounds when there was lack of dechlorinating bacteria. The process involved the participation of homoacetogens or methanogens as described above.

2.3 Acclimation of biomass for reductive dechlorination

Dechlorination of poly-substituted chlorophenols may require a proper acclimation process. An example is dechlorination of PCP. Reductive dechlorination of PCP

experiences diverse pathways by exposing anaerobic sludge to different chlorophenols (Mikesell and Boyd, 1986; Nicholson *et al.*, 1992; Wang *et al.*, 1998; Takeuchi *et al.*, 2000). Details of the acclimation process and dechlorinating intermediates are summarized in Table 2.2. The results showed the dechlorinating sludge with various acclimation procedures were qualitatively different. As indicated by Mikesell and Boyd (1986), sludge acclimated with 2-CP was able to remove the *ortho*-chlorine (major) and *para*-chlorine (minor) from PCP. In 3-CP acclimated sludge, only *meta*-chlorine was removed and *para*-chlorine of PCP was removed by sludge exposed to 4-CP. Sludge acclimated with PCP directly gained the ability to remove chlorines from all different positions initially but dechlorination ceased as various TCPs accumulated. Unacclimated sludge had demonstrated very limited dechlorination capabilities. These results suggested the specificities between acclimating compounds and the sites of dechlorination of PCP. It was apparent that acclimations with different chlorophenols accumulated specific microbial species or enzymes targeting chlorines at different positions in PCP. The complete reductive dechlorination of PCP was achieved through the combined activities of different dechlorinating populations or enzymes.

Under acidic condition, no dechlorination activities were detected in sludge acclimated with PCP, and unacclimated sludge. The *ortho*-dechlorination could be achieved by exposing sludge to 2,4,6-TCP prior to PCP. As stated above, incomplete dechlorination of PCP in this case may be due to lack of *meta*- and *para*-dechlorinating microbes or enzymes since the sludge was acclimated with 2,4,6-TCP only.

In summary, a less chlorinated phenol, which is generally less toxic, is required for acclimation. The purpose of applying the acclimating compound is to ensure the sludge adapted to the toxic compounds and was induced to produce the enzymes targeting chlorines at different positions.

Table 2.2 Diverse pathways of PCP dechlorination with different inducers

References	Acclimating CP	Media	Intermediates
Mikesell and Boyd (1986)	2-CP	Anaerobic sludge	3,4,5-TCP and 3,5-DCP
	3-CP	Anaerobic sludge	2,3,4,6-TeCP and 2,4,6-TCP
	4-CP	Anaerobic sludge	2,3,5,6-TeCP and 2,3,5-TCP
	2-CP, 3-CP and 4-CP	Anaerobic sludge	3,4,5-TCP, 3,5-DCP and 3-CP
Nicholson <i>et al.</i> (1992)	N.A.*	Methanogenic sludge	2,3,4,5-TeCP and 3,4,5-TCP
	PCP	Methanogenic sludge	2,3,4,5-TeCP, 2,3,5,6-TeCP, 2,3,4,6-TeCP, 3,4,5-TCP, 2,4,5-TCP and 2,3,5-TCP
Wang <i>et al.</i> (1998)	N.A.*	Methanogenic sludge	N.D. [#]
	Phenol	Methanogenic sludge	2,3,4,6-TeCP, 2,4,6-TCP, 2,4-DCP and 4-CP
Takeuchi <i>et al.</i> (2000)	N.A.*	Methanogenic sludge	2,3,4,5-TeCP and 3,4,5-TCP
Piringer and Bhattacharya (1999)	N.A.*	Acidogenic sludge	N.D. [#]
	PCP	Acidogenic sludge	N.D. [#]
Mun <i>et al.</i> (2008b)	N.A.*	Acidogenic sludge	N.D. [#]
	2,4,6-TCP	Acidogenic sludge	3,4,5-TCP

NOTE: *Unacclimated biomass. [#] No dechlorination of PCP

2.4 Anaerobic process and its instabilities

2.4.1 Overview of anaerobic process

The conventional anaerobic process experiences four major phases. Firstly, the hydrolysis degrades complex organics to simpler organics or monomers like amino acids and sugars. This is followed by acidogenesis where fermenting (acidogenic) bacteria convert these organics into H_2 , volatile fatty acids (VFA), and ethanol etc. Thirdly, the acetogenic bacteria transform these to acetate and lastly methanogens utilize the acetate, H_2 and CO_2 to produce methane gas (shown in Figure 2.2).

Figure 2.2 Conventional anaerobic metabolism (McCarty and Smith, 1986)

2.4.2 Instabilities of anaerobic process

A properly functioning anaerobic system requires balance between the various microorganisms involved in the processes (Cohen *et al.*, 1982). In other words, intermediates should not accumulate. The produced fermentative metabolites, VFAs, should be removed immediately by methanogens. However, biokinetics of the various groups of microorganisms involved differ widely (IWA, 2002). From Table 2.3, it is noted the average specific growth rate of acidogens is almost 2 times of that of

methanogens (H_2 -utilizing) and about 20 times faster than acetoclastic methanogens. In the anaerobic reactor about 2/3 of the methane gas produced is derived from acetate by acetoclastic methanogens as shown in Figure 2.2 (McCarty and Smith, 1986). This probably indicates that intermediates like VFAs can be produced much faster than the utilization rate by methanogens, which could lead to accumulation of organic acids and a subsequent pH drop. The low pH could inhibit methanogenesis more profoundly because of the energy-generating mechanism of the methanogens. At low pH, VFAs in the molecular form could diffuse freely into the cell and disrupt the establishment of proton motive force (Henderson, 1971; Madigan *et al.*, 2006). The acidogens generate energy through substrate phosphorylation depending on ATP production, in which case the proton concentration does not have direct effect (Madigan *et al.*, 2006).

Furthermore, the average half saturation constant (K_s) of acidogenic bacteria is 456 mg COD/L whereas the K_s of methanogens is only 0.21 mg COD/L. The large difference between the two groups indicates that with high organic loading to the system, methanogens will likely be inhibited. Again, an imbalance between the acid producers and methane formers leads the system to become acidic and fail.

Table 2.3 Kinetic constants for the various groups of microorganisms in the anaerobic process*

Microorganisms	$\mu_{\max}$ (d ⁻¹)			Yield (COD/COD)			K _s (mg COD/L)			Sample Size
	Ave	Lower	Upper	Ave	Lower	Upper	Ave	Lower	Upper	
Acidogens	8.96	0.41	21.25	0.11	0.01	0.17	456	23	1280	7
Acetogens (Valerate)	0.96	0.69	1.20	0.053	0.05	0.06	210	62	357	2
Acetogens (Butyrate)	1.37	0.23	2.70	0.057	0.026	0.079	185	12	318	7
Acetogens (Propionate)	0.97	0.02	2.68	0.045	0.019	0.089	334	30	1146	9
Methanogens (Aceticlastic)	0.49	0.047	1.406	0.048	0.025	0.076	229	11	930	15
Methanogens (H ₂ -utilizing)	5.2	0.02	12	0.069	0.014	0.18	0.21 [#]	0.001 [#]	0.6 [#]	6

Note: *Data summarized by Mun (2008b) from IWA (2002). [#] H₂ concentrations was represented by the concentration of formate by the following equation: HCOOCH → H₂ + CO₂

2.4.3 Inhibition of methanogens by chlorophenols

In terms of treating chlorophenols, methanogens are the consortium members most sensitive to chlorophenols (Wu *et al.*, 1993; Speece, 1996; Stuart and Woods, 1998; Demirel and Yenigun, 2002). Golden *et al.* (1994) studied the toxicity assay of chlorophenols to anaerobes. The reported IC₅₀ values of various chlorophenols on methanogens (Table 2.4) revealed that the toxicity effects increased dramatically with the increasing number of chlorines attached to the aromatic ring. The IC₅₀ of MCP concentrations against acetoclastic methanogens range from 200 to 320 mg/L, whereas the value decreases to 87 mg/L for 2,4,6-TCP and to as low as 9 mg/L for PCP. They also found that the acetoclastic methanogens were more susceptible to inhibitory effects from chlorophenols than the hydrogenophilic methanogens (with exception of 2-MCP). As discussed above, among the various methanogens, it is believed that about 2/3 of the methane produced comes from acetoclastic methanogens (McCarty and Smith, 1986) and about 90% of the methanogenic population belongs to the acetoclastic family (Karakashev *et al.*, 2005). The system could be inhibited more easily when dealing with chlorophenols as the acetoclastic methanogens are more sensitive to the toxic compounds. The inhibition of the overall methanogenic process hence leads to accumulation of intermediates. The accumulation of fermentative intermediates will cease the activities of acidogens in turn. The whole system would then stop functioning.

Table 2.4 Toxicity of chlorophenols against the methanogens

Inhibitor	IC ₅₀ Concentration (mg/L)	
	Acetoclastic	Hydrogenophilic
2-MCP	320	110
3-MCP	200	510
4-MCP	230	480
2,4,6-TCP	87	250
PCP	9	43

In conclusion, although anaerobic dechlorination of chlorophenols enjoys operating advantages, the instabilities and the inhibitory effects of chlorinated compounds make the conventional anaerobic process unstable. A new solution is needed in order to have a better performing anaerobic dechlorination process.

2.5 Phase separation and acidogenic pretreatment

2.5.1 Phase separation of the anaerobic process

Due to the instabilities of the conventional anaerobic process, researchers have advocated consideration of the two-phase anaerobic process. Pohland and Ghosh (1971) first developed the idea of such a phase separated anaerobic process for primary organic treatment. By phase separation, optimum environments could then be provided for both acidogens and methanogens. The physical separation of the two processes could allow maintenance of dominant cultures of acidogens and methanogens in different reactors. The inhibitory effects caused by accumulation of VFAs and pH fluctuation will be circumvented. Phase separation usually can be operated in two serial reactors. The first reactor is under acidogenic condition with an optimal pH range of 4.0-6.5 (depending on the influent substrate) and the second reactor is operating with a pH around neutral to maintain the methanogenic condition (Speece, 1996). The effluent of the first reactor is then the feed of the second reactor. Various techniques have been developed by researchers to achieve this - such as biokinetic selection because of the different specific growth rates between acid and methane formers (Pohland and Ghosh, 1971; Ghosh *et al.*, 1975), dialysis separation of acidogens and methanogens, and selective inhibition in the acidogenic reactor (Jeyaseelan and Matsuo, 1995).

The two-phase anaerobic process provides a more stable and efficient solution for anaerobic treatment of wastewater, and sludge and solid waste treatment. Cohen *et al.* (1980, 1982) reported a 3-fold increase in maximum specific COD turnover rate during continuous feeding and an average 6-8-fold increase for shock loading in the phase separated process compared to the conventional single stage system. In the phase separated system, rapid conversion of all VFAs also took place during recovery stage after overloading. The authors observed that overloading of the two-phase system did not cause adaptational changes in the microbial population whereas the single-stage system was inhibited. Other researchers also found that the two-phase system was more stable to changes in pH and had higher methane production (Ghosh *et al.*, 2000).

In general, firstly, the two-phase system allows the optimum conditions for different bacteria in each phase. Secondly, it increases the stability of the reactors compared to the single-stage anaerobic system as it prevents the accumulation of organic acids, which may suppress methanogenic activities. Consequently then, the two-phase system has a better performance in terms of COD removal and CH₄ production.

2.5.2 Acidogenic pretreatment of potentially inhibitory compounds

The first phase (acidogenic) of the anaerobic process has also been extended to applications such as pretreatment of wastewater containing inhibitory compounds, which are usually persistent in the environment. Researchers have demonstrated an improved overall performance when treating wastewater containing acrylic acid, nitroaromatic compounds, biphenyl, polynuclear aromatic hydrocarbons (PAHs), and phenol (Aziz *et al.*, 1994; Qu and Bhattacharya, 1996; Ng *et al.*, 1999; Jinadasa *et al.*, 2004).

The acidogenic phase is able to convert partially the persistent compounds to more readily degradable forms for the subsequent downstream aerobic or methanogenic reactors. The less inhibitory metabolites in the effluent from acidogenic reactors stabilized the whole system and furthermore the low HRT requirement of the acidogenic process provided a possibly cost-effective solution for treatment of persistent compounds.

2.5.3 Dechlorination under acidogenic condition

Although researchers noted the better and more stable performance of acidogenic pretreatment of various potential inhibitory compounds, few investigations have been conducted on the feasibility of dechlorination under acidogenic condition to date. A review of previous researches provided evidence of dechlorination under acidogenic condition. de Best (1999) reported that CCl₄ transformation still proceeded with addition of 2-bromoethanesulfonic acid (BES) (inhibitor of methanogens). Mun *et al.* (2008a) successfully degraded CCl₄ in the acidic environment (pH 5.6 to 6.0) with negligible methane gas produced (5% of total gas produced). Perkins *et al.* (1994) also noted addition of BES inhibited

methanogenesis but dechlorination of 2,4,6-trichlorophenol continued. Their results indicated coupling of dechlorination and acidogenesis and suggested methanogens were not involved in dechlorination. In 2002, Boopathy also indicated that the maximum rate of dechlorination of carbon tetrachloride (CCl_4) occurred under fermenting condition compared to the methanogenic, iron-reducing and nitrate-reducing conditions. The conclusion drawn from their results suggest the possibility of reductive dechlorination, which applies the acidogenic condition for dechlorination instead of the complete anaerobic process.

Two studies have been conducted to dechlorinate pentachlorophenol (PCP) under acidogenic condition so far. Mun *et al.* (2008b) demonstrated the *ortho*-dechlorination of PCP to 3,4,5- TeCP together with the bio-adsorption of PCP, but the other group of researchers showed there was no PCP intermediates observed, by which they claimed there was lack of biodegradability of PCP under acidic condition (Piringer and Bhattacharya, 1999).

The different results shown by these two groups of researchers may have been caused by following reasons:

- 1) Different operating conditions - the acidogenic conditions were created through different approaches in the two studies. Mun *et al.* adjusted pH from 7.6 to 5.6 through a stepwise manner at a rate of 0.5 units per week during acclimation. This was identified as one of the key factors which ensured success in inducing dechlorination (Mun *et al.*, 2008c). Pringer and Bhattacharya operated their reactors at pH 6.0 from the very beginning of the experiments.

- 2) Prior acclimation - Mun *et al.* (2008b) concluded that PCP could only be degraded by an acclimated acidogenic sludge if it had prior exposure to a less toxic chlorophenol, like 2,4,6-TCP otherwise dechlorination of PCP failed. Pringer and Bhattacharya applied either unacclimated sludge or PCP-exposed sludge. In both cases, no dechlorination was observed because of the lack of a proper inducer. Furthermore, in their study, the PCP to MLVSS ratio was from 78 to 266 $\mu\text{moles/g}$,

which might have been too high compared to to Mun *et al.*'s (2008b) 3.5-4.2 $\mu\text{moles/g}$.

2.5.4 Advantages of acidogenic process for treatment of chlorophenols

The acidogenic dechlorinating process was proposed in order to eliminate or reduce the instability issues associated with conventional anaerobic dechlorination. The process was thought to possess the following advantages.

Firstly, system failure due to accumulation of VFAs and lowering pH leading to inhibition of methanogenesis could be prevented since the methanogenic process is removed. Although accumulation of VFAs will eventually affect further acidogenesis, the whole system is considered more robust compared with the conventional anaerobic process. Previous studies described above (Cohen *et al.*, 1980, 1982; Ghosh *et al.*, 2000) had shown that the two-phase process had better performance in terms of total organic loading, less impact from pH fluctuations and recovered faster after overloading than the single stage system.

Secondly, researchers have shown chlorinated phenols could serve as favorable electron acceptors while utilizing H_2 , VFAs or simple organics as electron donors by dechlorinators (Perkins *et al.*, 1994; Holliger *et al.*, 1998; Mazur *et al.*, 2003; Villemur *et al.*, 2006). This helps to establish a symbiotic relationship between acidogens and dechlorinators. Hence, the dechlorinating process is probably enhanced.

Thirdly, dechlorinating bacteria do not have to compete for electron donors with methanogens while this would have occurred commonly in the conventional anaerobic system (Yang and McCarty, 1998). Methanogenesis is severely inhibited in the acidogenic condition, which reduces the risk of incomplete dechlorination due to methanogens outcompeting dechlorinators.

Fourthly, the methanogenesis is often considered to be most sensitive to toxicity of chlorinated compounds (Yang and Speece, 1986). The kinetic data shown in Table

2.3 also show that the acidogens may be more robust to the toxicity of the compounds. The treatment process would be more stable under acidogenic condition with less risk of inhibition of methanogens.

Lastly, due to the instabilities of the conventional anaerobic process, close monitoring of the system is unavoidable. Specific loadings of organic wastes and pH should be carefully controlled. It may not be feasible to adjust the pH of incoming wastewater when dealing with acidic industrial wastewater containing chlorophenols. It is believed the acidogenic process for dechlorination could be monitored and operated more easily and hence more cost effectively.

2.6 Knowledge gap and concerns about dechlorination under acidic condition

There is the potential of dechlorinating chlorophenols under acidogenic condition and the above-mentioned advantages make the process more attractive over the conventional anaerobic dechlorination, but a few concerns associated with the idea remain.

Firstly, there have been no extensive investigations of the biodegradation of chlorinated compounds under acidogenic conditions so far. Two groups of researchers made efforts to evaluate the possibility of acidogenic dechlorination of PCP. However, the results drawn from them were opposite. In Piringer and Bhattacharya's study (1999), they did not find any metabolites of PCP even though there was 18 to 31% of PCP reduction. With extracts recovered from the biomass, it was confirmed that all PCP removed was due to bio-adsorption. This agreed with Mun *et al.*'s study (2008b) when PCP loading was high (9.3 $\mu\text{moles/g MLVSS/d}$). Mun *et al.* (2008c) developed the start-up procedure for acidogenic dechlorination of chlorophenols by reducing pH in a step-wise manner and applying dechlorination inducer during acclimation. They have been the only group, which had demonstrated dechlorination of chlorophenols under acidic condition. The existence of such contradictory conclusions raises doubt on process feasibility.

Secondly, as presented earlier in this chapter, dechlorinating bacteria typically thrive at neutral pH. An acidic environment could prevent their growth and hence cause process failure. It has been reported *Desulfitobacterium* spp. was inhibited at pH 6.0 (Villemur *et al.*, 2006).

Thirdly, in the case of cometabolism, numerous evidences have been found that cometabolic dechlorination involved the methanogens and their specific enzyme F₄₃₀ (Section 2.2.4). The acidic environment is detrimental to methanogens. Although avoidance of methanogens to establish the two-phase anaerobic process could increase the stability of the system, lack of methanogens may suggest inability to conduct cometabolic dechlorination if dechlorinating bacteria could not be cultivated. Meanwhile the roles of different groups of anaerobes in dechlorination under acidic condition have not been studied in detail yet. Due to the conflicting information regarding whether methanogens are involved in the dechlorination process, it is necessary to determine whether dechlorination under acidic condition is a cometabolic process conducted by methanogens. In this sense, the acidic dechlorinating reactor is not a phase-separated process, but it is an acidic single-stage reactor, which included both acidogenesis and methanogenesis.

Lastly, Mun *et al.* (2008b; 2008c) had only demonstrated dechlorination under acidogenic condition of 2,4,6-TCP and PCP at the *ortho*- position (PCP to 3,4,5-TCP). No *meta*- or *para*- dechlorination was observed. This had seemed to be a major drawback for dechlorination under acidogenic condition. Studies on PCP degradation revealed the optimum pH for dechlorination was in fact slightly alkaline (Bouchard *et al.*, 1996). Other reasons why *meta*- or *para*- dechlorination did not occur may be lack of proper inducers in the acclimation stage and insufficient acclimation period. Complete dechlorination could possibly be achieved if the acclimating compounds could induce the accumulation of different species or enzymes targeting chlorines at all three positions (Mikesell and Boyd, 1986).

CHAPTER 3 Materials and Methods

3.1 Sequencing Batch Reactor

Two anaerobic sequencing batch reactors (anSBR 1 & 2) were constructed with a working volume of 4 L each (total volume of 5 L). Both reactors were operated at $35\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$. Temperature was maintained with heating tapes wrapped outside the reactor. Biogas was collected using gasbags (Tedlar, U.S.) and the collected biogas was re-circulated to the bottom of the reactors for the purpose of mixing. The schematic diagram of the reactor setup is shown in Figure 3.1. The SBR operational mode created a concentration gradient after feeding during the cultivation period. The concentration gradient was a selective force for the enrichment of specific dechlorination microbes.

Figure 3.1 Schematic diagram of anSBR setup: (a) Alkaline tank; (b) Acid tank; (c) Feed tank; (d) Discharge tank; (e) Gas collector; (f) pH probe; (g) ORP probe; (h) Water level sensor; (P1) Feeding pump; (P2) Gas recycle pump; (P3) Acid Dosing Pump; (P4) Alkaline Dosing Pump

The basic feed comprised (units in mg/L): Sucrose (9000), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (110), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (125), NH_4Cl (430), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (118), KH_2PO_4 (30), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$

(15), Na₂SO₄ (66), and a trace elements supplement (1ml/L). The trace elements supplement stock solution comprised (units in g/L): CoCl₂·6H₂O (1.25), H₃BO₃ (1.25), MnCl₂·4H₂O (3.06), NaMoO₄·2H₂O (0.1), NiCl₂·6H₂O (1.25), ZnCl₂ (1.25) and thiamine hydrochloride (1.95). The feed medium was diluted to the appropriate concentrations of sucrose required in the batch studies in the following chapters.

3.2 Cycle Study

Besides regular evaluations of target (COD, chlorophenols and VFAs) of influent and effluent parameters, cycle studies were conducted to investigate changes in chlorophenol content within an operating cycle (12 h or 6 h). The sampling frequencies are described in the table below (Table 3.1).

Table 3.1 Cycle study sampling frequencies

12-Hour Cycle			6-Hour Cycle		
Sampling Frequency	Sampling Time (mins)	Remaks	Sampling Frequency	Sampling Time (mins)	Remaks
	10	End of FEED		10	End of FEED
Every 30 mins	40	REACTION	Every 30 mins	20	REACTION
	70			50	
	100			80	
	130		Every 60 mins	140	
Every 60 mins	190			200	
	250			260	
	310			340	End of REACTION
Every 120 mins	430			355	End of Settling
	550				
	655	End of REACTION			
	715	End of Settling			

3.3 Chlorophenols extraction and acetylation

The liquid samples were centrifuged at 12000 rpm for 10 minutes. Two milliliters of clear supernatant was collected for extraction and acetylation following the protocol modified from previous studies (Rodríguez *et al.*, 1996; Bagheri and Saraji, 2001). The collected supernatant was spiked with an internal standard (10 µl/ml), mixed with 1 ml of 5% K₂CO₃, 200 µl of acetic anhydride and 1 ml of n-hexane. The mixture was shaken for 1 minute and phase separation was then allowed. The aqueous phase was further extracted with 1 ml of n-hexane by shaking for another minute. The two n-hexane portions were mixed, dried over anhydrous sodium sulfate (granular) and injected into a GC-µECD for analysis. The calibration standards were prepared in the same way as samples and 2,4,6-tribromophenol (TBP) was used as internal standard (50 mg/L).

3.4 Analytical procedures

3.4.1 Quantification of chlorophenols

The chlorophenols were analyzed by an Agilent (U.S.) gas chromatograph model 7890N equipped with a micro electron capture detector (µECD) and a split/splitless injector. Separations of chlorophenols were carried out using a capillary column DB-5ms (Agilent, 30 m × 0.25 mm x 0.25 µm). The column was initially held at 50 °C for 2 minutes, increased to 80 °C at 100 °C/min, increased to 100 °C for 1 minute at 5 °C/min and increased to 250 °C minutes at 100 °C/min. It was held at the final temperature for 5 minutes. The injector was set at 200 °C, splitless mode. The detector temperature was 300 °C with a constant makeup gas (nitrogen) flow of 30 ml/min. Helium was used as the carrier gas with a constant flow velocity of 20 cm/s.

3.4.2 Quantification of phenol

Quantification of phenol was performed via high performance liquid chromatography (HPLC) (1260 Infinity, Agilent). The HPLC was equipped with a reverse C-18 column (Eclipse Plus, Agilent) and UV-Visible detector. Phenol was detected at 254 nm wavelength. The mobile phase was a mixture of acetonitrile and 0.01 M H₃PO₄ in the proportion of 1:4. The flow rate was controlled at 1.5 ml/min. The column was kept at 35 °C. Samples were centrifuged at 12000 rpm for 10

minutes, 2 ml of supernatant was passed through a 0.2 μm syringe filter and 100 μl was injected.

3.4.3 Analysis of volatile fatty acids

VFAs were measured using an Agilent gas chromatograph system (7890N) equipped with flame ionization detector (FID) according to Zhou *et al.* (2009). Different VFAs were separated with a 30 m $\times$ 0.32 mm $\times$ 0.50 μm DB-FFAP fused silica capillary column. The injector and detector temperatures were set at 260 $^{\circ}\text{C}$ and 300 $^{\circ}\text{C}$ respectively. Oven temperature was initially 60 $^{\circ}\text{C}$, then ramped up to 120 $^{\circ}\text{C}$ at a rate of 20 $^{\circ}\text{C}/\text{min}$, held for 1 min, and further increased to 240 $^{\circ}\text{C}$ at 20 $^{\circ}\text{C}/\text{min}$ and held for 3 min. Helium was the carrier gas with a constant pressure of 103.0 kPa. The injector was in split mode with split ratio of 50:1. Prior to analysis, 0.1 ml of 10% formic acid was added to each 0.9 ml of samples and standards.

3.4.4 Analysis of COD, TSS/VSS

COD and MLSS/MLVSS was determined in accordance with *Standard Methods* (APHA, 2005).

CHAPTER 4 Dechlorination of Chlorophenols under Acidic Condition

4.1 Introduction

Anaerobically reductive dechlorination is an efficient process. However, information regarding dechlorination under acidic or acidogenic environment is limited and contradictory. Chin *et al.* (2005) reported that 2,4-dichlorophenoxyacetic acid with a concentration of 20 – 200 mg/L can be degraded in an acidogenic anaerobic sequencing batch reactor (anSBR). Mun *et al.* (2008b; 2008c) found that 2,4,6-TCP and PCP could be dechlorinated using acidogenic sludge but only *ortho*-dechlorination occurred. However, other researchers stated that removal of PCP at pH 6.0 was due to adsorption only (Piringer and Bhattacharya, 1999). The different results in dechlorination of chlorophenols under acidogenic and acidic condition from these two research groups might be attributed to the different acclimation strategies. Mun *et al.* (2008c) used a less toxic compound, 2,4,6-TCP, to cultivate the acidogenic sludge and applied a step-wise reduction of pH to reach the value of pH 5.5 whereas Pringer and Bhattacharya (1999) reduced the pH to 6.0 at the initial stage to eliminate the methanogens, which usually experienced the optimum growth at neutral pH. The pH-shocked sludge was used directly to evaluate PCP removal efficiency. Therefore, the sudden reduction of pH and dosage of toxic PCP in the latter case might have resulted in a different microbial consortium in which the dechlorinating microbes were inhibited or removed.

The different conclusions drawn from the literatures led to this study in order to investigate the feasibility of acidic dechlorination. In this study, various chlorophenols, such as monochlorophenols (MCP), trichlorophenol (TCP) and pentachlorophenol (PCP) were tested. The possibility of *meta*- or *para*-dechlorination under acidic condition was also explored.

4.2 Materials and methods

4.2.1 Chemicals and materials

Chlorophenols (>99%) including monochlorophenols (2-MCP, 3-MCP and 4-MCP), dichlorophenols (2,4-DCP, 2,6-DCP and 3,5-DCP), trichlorophenols (2,4,6-TCP and 3,4,5-TCP), tetrachlorophenols (2,3,4,5-TeCP, 2,3,5,6-TeCP and 2,3,4,6-TeCP) and pentachlorophenol (PCP) were purchased from Sigma Aldrich (Singapore). All chlorophenol stock solutions (1 M) were prepared in 0.05 M of sodium hydroxide solution.

The inoculating sludge was collected from two different local wastewater treatment plants (Singapore), Ulu Pandan Water Reclamation Plant (UPWRP) and Jurong Industrial Water Works (JIWW). The former plant was treating domestic wastewater while the other treated municipal wastewater with a substantial pretreated industrial wastewater component.

4.2.2 Sequencing batch reactors

Two anaerobic sequencing batch reactors (anSBR) were set up according to the description in Chapter 3. The anSBRs were initially operated with a 12-hour cycle with the following sequences: 1) Feeding: 10 minutes, 2) Reaction: 10 hours 45 minutes, 3) Settling: 1 hour, and 4) Decanting: 5 minutes. Hydraulic retention time (HRT) was 2 days. The settling time was reduced to 30 minutes and further to 15 minutes when the settleability of the sludge improved. Subsequently the reaction time was increased to 11 hours 15 minutes and further to 11 hours 45 minutes. After about 1 year, the HRT was reduced to 1 day by operating the anSBRs with a 6-hour cycle with 10 minutes of feeding, 5 hours 30 minutes of reaction, 15 minutes of settling and 5 minutes of decanting.

4.2.3 Dechlorinating anSBR start-up procedures

The seed sludge was collected from two different local anaerobic digesters (Singapore) where one treated excess sludge generated from domestic wastewater treatment (UPWRP) while the other dealt with sludge from municipal (with industrial component) wastewater treatment (JIWW).

The seed sludge was filtered through a 600 μm sieve after collection to remove large particles and stored at 4 $^{\circ}\text{C}$ before use. N_2 was applied to keep anaerobic conditions during seeding. The initial pH of the reactors was 7.30 – 7.55. A step-wise reduction of pH was implemented at a rate of 0.5 units per week in order to reach the acidic condition and the final pH was 5.50. The pH was controlled with an automated programmable logic control (PLC) system with 0.5 M sodium hydroxide solution and 0.2 M hydrochloric acid. Initial mixed liquor volatile suspended solids (MLVSS) in the reactor were around 5685 mg/L with a MLVSS/MLSS ratio of 0.74 in anSBR 1 and 6083 mg/L with a MLVSS/MLSS ratio of 0.71 in anSBR2 respectively.

4.2.4 Synthetic wastewater and anSBR operating phases

The synthetic feed was prepared by diluting the basic feed (9000 mg/L of sucrose) described in Chapter 3 to contain 1600 mg/L of sucrose and then dosed with chlorophenols to the target concentrations (Table 4.1). The operation/acclimation period of two anSBRs can be divided into 4 phases.

1) anSBR 1 (UPWRP sludge)

In order to acclimate the sludge to have the capabilities to degrade the *ortho*-, *meta*- and *para*-chlorines, 3 monochlorophenols, namely 2-MCP, 3-MCP and 4-MCP were applied to anSBR 1 simultaneously during SBR1/Phase I (100 μM each). In the same phase, pH was reduced stepwise to 5.5 in the first 4 weeks. When the sludge showed stable dechlorinating activity of 2-MCP only, 3-MCP and 4-MCP was removed from the feed and concentration of 2-MCP was doubled to 200 μM in SBR1/Phase II, and each cycle was reduced to 6 hours from 12 hour in SBR1/Phase III. The HRT was 1 day then. The acclimated sludge with dechlorinating ability was used to deal with the synthetic feed containing higher-chlorinated compounds, 2,4,6-TCP and PCP (SBR1/Phase IV).

2) anSBR 2 (JIWW sludge)

Initially, both reactors were fed and operated under the same conditions with seed sludge from UPWRP. After about 200 days, degradation of 3-MCP and 4-MCP was

still not observed. SBR 2 was re-seeded with anaerobic sludge from JIWW where there was an industrial wastewater component in order to evaluate the *meta*- and *para*-dechlorinating capabilities with a different sludge. In the new feed, only 3-MCP and 4-MCP (50 μM each) were used to acclimate the sludge after re-seeding in SBR2/Phase I. Concentrations of the MCPs were increased to 100 μM each during SBR2/Phase II until Day 140. No dechlorination of 3-MCP and 4-MCP was observed in SBR2/Phase I and II. Following that, the SBR 2 was challenged with 2-MCP (100 μM) during SBR2/Phase III and PCP (5 μM) in Phase IV. Details of anSBR operation with various chlorophenols are described in Table 4.1.

Table 4.1 Reactor operation phases and chlorophenol loadings

Reactor/Phase	Sludge	pH	HRT	Chlorophenols fed (Concentration)	Chlorophenols loadings*
SBR1/I	UPWRP-Unacclimated	7.5 initially and reduced to 5.5	2 days	2-MCP/3-MCP/4-MCP (100 μM each)	50 $\mu\text{moles/day}\cdot\text{L}^{-1}$ each MCP
SBR1/II	UPWRP-Capable to dechlorinate 2-MCP	5.5	2 days	2-MCP (200 μM)	100 $\mu\text{moles/day}\cdot\text{L}^{-1}$
SBR1/III	UPWRP-Capable to dechlorinate 2-MCP	5.5	1 day	2-MCP (200 μM)	200 $\mu\text{moles/day}\cdot\text{L}^{-1}$
SBR1/IV	UPWRP-Capable to dechlorinate 2-MCP	5.5	1 day	PCP (5 μM)	5 $\mu\text{moles/day}\cdot\text{L}^{-1}$
SBR 2/I	JIWW-Unacclimated	7.5 initially and reduced to 5.5	2 days	3-MCP/4-MCP (50 μM each)	25 $\mu\text{moles/day}\cdot\text{L}^{-1}$ each MCP
SBR 2/II	JIWW-Exposed to 3-MCP and 4-MCP	5.5	2 days	3-MCP/4-MCP (100 μM each)	50 $\mu\text{moles/day}\cdot\text{L}^{-1}$ each MCP
SBR 2/III	JIWW-Exposed to 3-MCP and 4-MCP	5.5	1 day	2-MCP (100 μM)	100 $\mu\text{moles/day}\cdot\text{L}^{-1}$
SBR 2/IV	JIWW-Capable to dechlorinate 2-MCP	5.5	1 day	PCP (5 μM)	5 $\mu\text{moles/day}\cdot\text{L}^{-1}$

Note:* Chlorophenol loadings is measured in μmoles of chlorophenols per day per liter of reactor volume

4.2.5 Batch test for TCP dechlorination

After acclimation with MCPs, anSBR 1 sludge was withdrawn to be challenged with the higher chlorinated compounds. The sludge had displayed excellent removal of 2-MCP by removing the *ortho*-chlorine. The test was conducted with 120 ml serum bottles (with 100 ml liquid volume). 300 ml of sludge was collected from anSBR 1 and spun down for 5 minutes at 3000 rpm. The sludge pellet was rinsed with PBS to remove residual organics. Rinsing was repeated thrice. The sludge was then re-suspended with synthetic wastewater containing 5mM sucrose, 100 μ M 2,4,6-TCP and mineral/trace element solutions as described in Chapter 3. All the bottles were flushed with N₂ for 5 minutes before capping and were incubated on the shaker (Sartorius Stedim) at 35 °C. The serum bottles were sealed with Teflon-lined butyl rubber stoppers and capped with aluminum crimp-top caps. During the first 5 days, 25 ml of the supernatant after settling was withdrawn and replaced with fresh media. From Day 6 onwards, 50% of the supernatant (50 ml) was exchanged with the fresh media. The test was performed in triplicates.

4.2.6 Dechlorination of 3-MCP and 4-MCP at pH 7.0

Sludge (600 ml) from anSBR 2 (seeded with JIWW anaerobic sludge) was collected on Day 140 in order to test whether the sludge could remove 3-MCP or 4-MCP at neutral pH. Two sets of batch tests were prepared in this experiment. The MCPs were used individually in tests. The collected sludge was subjected to the same cleaning process as described above to remove adsorbed organics. The sludge was then re-suspended with the synthetic media with 1600 mg/L of sucrose and 100 μ M of each MCP. 100 ml of the mixed liquor was transferred to the 120 ml serum bottles sealed with Teflon-lined butyl rubber stopper and capped with aluminum crimp-top caps. The pH was maintained at around 7.0 by using phosphate buffer. On each day, 50 ml of the supernatant after settling was replaced with fresh media. Each set of the tests was conducted in triplicates.

4.2.7 Effects of external carbon source

The effects of external carbon source were investigated by comparing the dechlorinating efficiency of 2-MCP in the presence of sucrose and without sucrose

(control). Sludge was collected from anSBR 1 in Phase II when it showed stable dechlorinating activity of 2-MCP. The sludge was inoculated in the 120 ml serum bottles and mixed with the same media fed to the reactor (200 μ M of 2-MCP and 1600 mg/L of sucrose). 50% of the supernatant was exchanged on daily basis. The control batches were prepared in the same manner except for the addition of sucrose. Both sets were prepared in triplicates.

4.2.8 Analytical Methods

Analysis of chlorophenols, volatile fatty acids and chemical oxygen demand were conducted according to the protocols described in Chapter 3.

4.3 Results

4.3.1 Acclimation and dechlorination of monochlorophenols

The UPWRP sludge was acclimated with 2-MCP, 3-MCP and 4-MCP simultaneously in both reactors. One effluent sample was collected on the scheduled collection date and the collected sample was only measured once for MCPs and VFAs. The measured value did not show significant variations after the system had stabilized. The GC quality control results confirmed that the measured values were reliable. Hence, there was no duplicate measurement for the analysis of monochlorophenols and VFAs in the effluent.

The profile of MCPs in the effluent were similar in both reactors as shown in Figure 4.1, hence only the results from anSBR 1 are presented here.

Figure 4.1A showed 2-MCP concentration in the anSBR 1 effluent in Phases I, II and III. In the start-up stage, 2-MCP accumulated in the reactors during the step-wise reduction of pH and was in the effluent until after 80 days. Thereafter, 2-MCP declined with phenol as the end product. From Day 100 onwards, the removal rate of 2-MCP was over 99% when influent concentration was 100 μM (Phase I). 2-MCP loading was doubled on Day 330, which resulted in an increase of 2-MCP concentration in the effluent. The 100% removal performance was quickly resumed after 3 to 4 days. In Phase III, when HRT was reduced from 2 days to 1 day, there was no accumulation of 2-MCP observed in the system.

In contrast, removal of 3-MCP or 4-MCP was not observed even after a long acclimation period (330 days) (Figure 4.1B). According to the mass balance, the variation of the 3-MCP and 4-MCP concentrations in the effluent was mainly due to adsorption and desorption of 3-MCP and 4-MCP from the sludge. The presence of these two monochlorophenols did not inhibit growth of the biomass and dechlorination of 2-MCP at the influent concentration of 100 μM . Since the sludge did not degrade the two monochlorophenols, 3-MCP and 4-MCP were not fed into the reactor in Phases II and III.

Figure 4.1 MCP concentrations in effluent of anSBR 1 (UPWRP sludge): A) 2-MCP; B) 3-MCP and 4-MCP

Note: Each point represents a single measured value for each effluent sample collected.

Compared with *ortho*-dechlorination, *meta*- and *para*-dechlorination are more difficult (Mikesell and Boyd, 1986; Wang *et al.*, 1998). This study confirmed the preceding report. Two possibilities are raised as follows: 1) the lack of *meta*- or *para*-dechlorinating microorganism in the seed and acclimated sludge; or 2) the presence of a more readily dechlorinated compound (2-MCP) inhibiting dechlorination of 3-MCP or 4-MCP.

In order to evaluate the above hypothesis of 3-MCP/4-MCP dechlorination, anSBR 2 was re-inoculated with sludge collected from JIWW and acclimated with a mixture of 3-MCP and 4-MCP. JIWW sludge might have been exposed to more inhibitory wastewater and so might have the potential to deal with *meta*- or *para*-chlorines. Dechlorination of 3-MCP or 4-MCP would also not be disadvantaged given the absence of 2-MCP.

The performance of the newly seeded anSBR 2 is shown in Figure 4.2. Initially anSBR 2 was fed with 50 μM (6.4 mg/L) of 3-MCP and 4-MCP for around 100 days. Against expectation, neither MCP was dechlorinated nor phenol production was detected. It was thought concentration of the MCPs might have been too low and so below the threshold for utilization of the MCPs. The concentrations of both MCPs were increased to 100 μM (12.6 mg/L) and the reactor was operated for another 40 days (Phase II). Unfortunately, MCPs in the effluent increased immediately after the loading was doubled and finally reached the same level as in the incoming feed. Without the presence of 2-MCP and with a different source of seed sludge, *meta*- or *para*-dechlorination was still not observed.

Figure 4.2 MCP concentrations in the effluent from anSBR 2 (JIWW Sludge)

Note: Each point represents a single measured value for each effluent sample collected.

4.3.2 Dechlorination of 3-MCP and 4-MCP at neutral pH

Efforts were further made to dechlorinate 3-MCP or 4-MCP in a neutral pH environment. Sludge from anSBR 2 (Phase II) fed with these two MCPs were withdrawn and inoculated into serum bottles to conduct the test at around pH 7.0. After incubation for about 2 weeks, there was still no removal of 3-MCP or 4-MCP as indicated in Figure 4.3. The variations were caused by adsorption/desorption as no metabolites, such as phenol, was found in the supernatant.

Figure 4.3 Results of batch tests to dechlorinate 3_MCP and 4-MCP under neutral pH: (A) 3-MCP in supernatant; (B) 4-MCP in supernatant
 Note: Data presented are the average of triplicates and error bar is the standard deviation.

4.3.3 Dechlorination of 2-MCP in anSBR 2 acclimated with 3-MCP/4-MCP

Although the efforts including different acclimating strategies and application of pH around 7.0 were made, negative results of dechlorination of 3-MCP and 4-MCP were observed. AnSBR 2 was then challenged with other chlorophenol by starting with 2-MCP (SBR2/Phase III). It was found that 2-MCP was removed without any lag period. The concentrations of 2-MCP in the effluent during the Phase III are illustrated in Figure 4.4. 2-MCP in the influent was 100 μ M (12.856 mg/L). The removal efficiency was 50%, 90%, 90% in the first three days and 100% was achieved from Day 5. The fast response to 2-MCP in fact was not expected since it took more than 80 days for the fresh sludge to degrade 2-MCP initially. Although the biomass in anSBR 2 was never exposed to 2-MCP, it had been cultivated in the acidic environment for over half a year. The sludge was adapted to the acidic condition and readily performed reductive dechlorination.

Figure 4.4 Dechlorination of 2-MCP with the sludge acclimated with 3-MCP and 4-MCP only

Note: Each point represents a single measured value for each effluent sample collected.

4.3.4 Effects of external carbon source

The effect of the primary substrate (sucrose) on dechlorination of 2-MCP was studied in 100 ml serum bottles. The acidic environment sludge from anSBR 1/Phase II was used in this batch experiments. The results are presented in Figure 4.5. In the batches where external carbon source was supplied, the 2-MCP was completely degraded within 6 days. The degradation rates of 2-MCP in the batches without sucrose were slower with dechlorination activity very low from Day 2 onwards. It is proposed that when there was no external carbon source available, the sludge may conduct endogenous decay to utilize biomass as the carbon source for dechlorination. This may explain why fast removal of 2-MCP occurred only in the initial 2 days in the batches without sucrose. This finding was in agreement with other researchers (Vallecillo *et al.*, 1999), who had observed external carbon sources enhanced or stimulated dechlorination.

Figure 4.5 Dechlorination of 2-MCP with and without sucrose addition

Note: Data presented are the average of triplicates and error bar is the standard deviation.

4.3.5 Dechlorination of 2,4,6-TCP

In Phase IV, after biomass from anSBR 1 had been acclimated with monochlorophenols and had shown capability to *ortho*-dechlorinate, higher chlorinated compounds like TCP and PCP were used to challenge the biomass. Since PCP is the most inhibitory chlorophenol, the sludge was firstly challenged with 2,4,6-TCP in serum bottles before PCP was fed to the reactor.

In Figure 4.6, the dechlorination metabolites of 2,4,6-TCP are presented. It was found that *ortho*-chlorines of 2,4,6-TCP were removed immediately from Day 1. The dechlorinating intermediate, 2,4-DCP was found in the supernatant and disappeared quickly from Day 2. 4-MCP as the final product of this experiment continuously accumulated while both 2,4,6-TCP and 2,4-DCP were completely removed from the mixed liquor. The loading of 2,4,6-TCP was doubled from Day 6, which resulted in the reduction of 4-MCP concentration on Day 6 due to the dilution with fresh media. With the doubled loading, the system was still able to achieve 100% of 2,4,6-TCP removal (Figure 4.6). Extraction of chlorophenols from the sludge was also performed to determine the amount of chlorophenols removed by biosorption. The results showed that only 4-MCP was recovered from the sludge and no 2,4,6-TCP or 2,4-DCP was extracted and detected. This supported the conclusion that disappearance of TCP and DCP in the tests was due to biological dechlorination.

Figure 4.6 *Ortho*-dechlorination of 2,4,6-TCP by acclimated sludge

Note: Data presented are the average of triplicates and error bar is the standard deviation.

The mass balance of the chlorophenols in the above tests was determined and is shown in Table 4.2. Total recovery of the chlorophenols was above 94%. It was found that the total amount of 4-MCP generated was 48 μ moles, which was about 91% of the total 2,4,6-TCP added to the process. Hence, biological removal of 2,4,6-TCP in the acidic environment had occurred with the less toxic 4-MCP as end product.

Table 4.2 Mass balance analysis of chlorophenols

Compounds	Discarded ^a	Aqueous ^b	Adsorbed ^c
2,4,6-TCP (μmoles)	0.74	0	0.18
2,4-DCP (μmoles)	0.91	0.20	0
4-MCP (μmoles)	27.68	17.47	2.73
Total (μmoles)	29.37	17.67	2.91
Recovered (a+b+c) (μmoles)	49.95		
2,4,6-TCP dosed (μmoles)	52.99		
Recovery (%)	94		

Note: ^a“Discarded” means the amount of chlorophenols in the supernatant withdrawn from the test bottles; ^b“Aqueous” means the amount of chlorophenols remaining in solution in the test bottles; ^c“Adsorbed” means the amount of chlorophenols extracted from the biomass

4.3.6 Dechlorination of PCP

With the batch test results of successful dechlorination of 2,4,6-TCP under acidic condition, anSBR 1 and 2 were challenged with the most chlorinated compound, PCP, in Phase IV. Due to PCP's toxicity, only 5 μM was dosed in the feed. Figure 4.7 shows the residual concentrations of PCP and the degradation metabolites in the effluent for both reactors. It was found PCP was quickly degraded through the *ortho*-dechlorination pathway and 2 chlorines at position 2 and 6 were replaced with hydrogen atoms from the first day of feeding. The intermediate 2,3,4,5-TeCP appeared transiently (< 0.5 μM) in the first 5 days and transformed to 3,4,5-TCP as the end product. The results indicated the transformation was fast with removal efficiency of 96 %. In anSBR 1, where the sludge was initially acclimated with 2-MCP, removal of PCP did not encounter any problem during the one-month of operation but removal efficiency was reduced in anSBR 2 after Day 25. The reasons for the decline are not clear at this stage. The reduction in dechlorination efficiency of PCP might, however, have been caused by accumulation of the metabolite, 3,4,5-TCP.

Figure 4.7 PCP concentration in anSBRs effluent: A) anSBR 1; B) anSBR 2

Note: Data presented are the average of three measurements of a single effluent sample collected and error bar is the standard deviation of the three measurements.

In summary, both the acclimated biomasses, from different sources, dechlorinated 2,4,6-TCP and PCP with removal of *ortho*-chlorine immediately without lag phase.

The intermediates of dechlorination, such as 2,4-DCP or 2,3,4,5-TeCP were efficiently removed as well without showing inhibitory effect to the process.

4.4 Discussions

As shown in previous studies by Mun *et al.* (2008c), a step-wise reduction of pH was a key factor in inducing dechlorination under acidic condition. In this study, the same operating strategy was applied to investigate the feasibility of acidic anaerobic dechlorination of different chlorinated phenolic compounds. The less toxic compounds e.g. 2-MCP, 3-MCP and 4-MCP were used to acclimate the sludge for different dechlorination pathways.

This study found that dechlorination was feasible under acidic condition. *Ortho*-dechlorination could be successfully conducted at pH 5.5. The removal efficiency of 2-MCP was more than 99% when 2-MCP loading was 200 $\mu\text{moles/d}\cdot\text{L}^{-1}$. The effluent concentration of 2-MCP was always below 0.5 μM . However, the acidic environment also had negative impact on process development. Firstly, the acclimation period was longer (80 days) than previously reported, where the reductive dechlorination was carried out under neutral pH conditions. Comparative data of acclimation periods between that noted in this study and other dechlorination processes under conventional anaerobic condition is illustrated in Table 4.3. The acclimation period for mono-chlorophenol was usually less than one week (Hrudey *et al.*, 1987; Ye and Shen, 2004). In some cases, the dechlorination activity took place immediately after the sludge received 2-MCP (Boyd and Shelton, 1984). In the start-up period, MCPs were hardly degraded in this study. However the low concentrations of MCPs used did not inhibit the growth of anaerobes as increase in TSS and VSS was observed. On the other hand, the growth of particular microbes that are responsible for the dechlorination process could be inhibited by the acidic conditions, which resulted in the longer acclimation needed for the microbes to grow or proliferate until there were sufficient numbers in the system. It seemed that the culture would need to adapt to acidic condition before dechlorination could occur. This was shown to be so in the re-inoculated anSBR 2 where the reactor had not been exposed to 2-MCP during the acclimation period but

could remove 2-MCP immediately when the feed was changed after the system had been operated in the acidic environment for more than 140 days.

Table 4.3 Comparisons of acclimation period

Reference	Acclimating Compound	Media	Acclimation Period
Boyd and Shelton (1984)	2-MCP	Anaerobic	Immediate
	3-MCP		3 Days
	4-MCP		4 Days
Hrudey <i>et al.</i> (1987)	2-MCP	Anaerobic	< 1 Week
Ye and Shen (2004)	2-MCP	Anaerobic	< 7 Days
	3-MCP		
	4-MCP		
This Study	2-MCP	Acidic	80 Days

Secondly, dechlorination under acidic condition was limited to *ortho*-dechlorination of 2-MCP, 2,4,6-TCP to 4-MCP and PCP to 3,4,5-TCP. During the acclimation period, it was originally thought the presence of 2-MCP could have prevented the degradation of 3-MCP and 4-MCP because 2-MCP is the easiest congener to be degraded. Microbes might utilize it first before attacking the more difficult MCPs. It was also believed the seed sludge from a domestic wastewater treatment plant was lacking in dechlorinating species which could remove 3-MCP and 4-MCP. Attempts were made with a different acclimating strategy using a different seed sludge in the hope this could address the different positions of chlorines and so induce removal of *meta*- or *para*-chlorines. Unfortunately, the above attempts were not successful. 3-MCP and 4-MCP were also not degraded at neutral pH (Section 4.3.2). Other researchers have suggested a much longer acclimation period (14 months) was required for *meta*-dechlorination to take place (Mun *et al.*, 2008a). AnSBR 1, however, had already been operated for more than 300 days with 3-MCP and 4-MCP and there was no removal of either MCPs. Similar results were obtained with 3,4,6-TCP or PCP degradation in which case only *ortho*-dechlorination was possible. The results may suggest no *meta*- or *para*-dechlorination capable species functioned under the acidic conditions in the system.

As 3-MCP and 4-MCP are less chlorinated compounds, they can be easily metabolized by aerobic microorganisms (Schollhorn *et al.*, 1994; Fava *et al.*, 1995). Since *meta*- and *para*-dechlorination could not be achieved in the acidic dechlorination system in this study, the acidic dechlorination process could be utilized as the pre-treatment process to detoxify the more highly chlorinated phenols to a less toxic compound. The non-degradable 4-MCP could then be aerobically mineralized by an aerobic process downstream. Both Armenante *et al.* (1999) and Atuanya *et al.* (2000) had demonstrated efficient sequential anaerobic-aerobic biodegradation of 2,4,6-TCP and 2,4-DCP respectively. The more chlorinated TCP and DCP were firstly attacked in the anaerobic system for reductive dechlorination and the resulting 4-MCP was then mineralized in the aerobic process.

Lastly, the end product of 2-MCP dechlorination in this study was phenol and it was accumulated in the effluent without being further mineralized, whereas, complete mineralization of chlorophenols to carbon dioxide and methane was observed in the conventional anaerobic dechlorination process (Boyd and Shelton, 1984; Zhang and Wiegel, 1990). The presence of primary substrate (sucrose in this study) may discourage microbes from using the chlorophenols as carbon source to support their growth. This study did, however, find an external carbon source could enhance the dechlorination process and with its effectiveness much reduced without addition of the co-substrate. It implied the dechlorination process in acidic environment could be a cometabolism process. As suggested in the literature (Chapter 2), cometabolism could be carried out by some anaerobes like methanogens by transferring the electrons in the methanogenesis to the chlorinated compounds. Without the external carbon source, methanogenesis could not take place and hence enzymes production and electron transfer would not occur as well.

The biomass acclimated under acidic condition in this study was able to dechlorinate higher chlorinated phenols without lag phase. As presented in Table 4.3, there were a short lag period (1 day) to treat 2,4,6-TCP using acclimated sludge (Chang *et al.*, 1999) and 14 days to treat PCP with unacclimated sludge (Ye and Shen, 2004) under neutral pH. Under acidic condition, there was either no PCP

degradation observed (Piringer and Bhattacharya, 1999) or a lag period of 14 days (Mun *et al.*, 2008b). Comparing with other studies, the lag period in this study was similar with results obtained from the conventional anaerobic dechlorination process but shorter than those in other acidic environment reports. Although the biomass in this study required a longer acclimation period, the biomass was ready to treat chlorophenols without any lag period after acclimation. The faster response time respect to 2,4,6-TCP and PCP suggested it was a stable process after acclimation.

Table 4.4 Comparisons of incubation and lag periods

Reference	Reactor	Acclimating Compound	Acclimation Period	Compound	pH	Lag Phase
Chang (1999)	Batch	2,4-DCP 3,4-DCP	6 months	2,4,6-TCP	6.8-7.0	1 day
Ye & Shen (2004)	Batch	N.A.	N.A.	PCP	Neutral	14 days
Piringer & Bhattachary (1999)	Chemostat	N.A.	N.A.	PCP	6.0	No degradation
Mun (2008b)	SBR	2,4,6-TCP	30 days	PCP	5.6-6.1	14 days
This Study	SBR	2-MCP	80 days	PCP 2,4,6-TCP	5.5	0 days

Capability of acclimated sludge for removing chlorines at *ortho*-positions from 2-MCP, 2,4,6-TCP and PCP indicated that all three chlorophenols may be degraded by the same group of microorganism or the same dechlorinating enzyme was functioning in the system. Various researchers had reported the *ortho*-chlorine atoms were the most preferred sites to be removed from CPs whereas the *para*-chlorines were the most toxic agents (Droste, 1998) and thereby dechlorination rate of *para*-dechlorination was the lowest (Takeuchi *et al.*, 2000). Although other acclimating protocol may induce *meta*- or *para*-dechlorination (Mohn and Kennedy, 1992a; Magar *et al.*, 1999), it was not successful in either Mun *et al.*'s (2008b) or this study where various *meta* position chlorophenols (3-MCP, 2,3,6-TCP, 2,3,4-TCP, 3,4,5-TCP and 3,5-DCP) were used as incubating substrate in acidic environment. Both results suggested failure to acclimate the *meta*-dechlorination population in the microbial community.

Two different sources of sludge were acclimated and used to conduct dechlorination in this study. Although two different types of sludge were acclimated with different protocols, both acclimated sludge showed very close *ortho*-dechlorination efficiency of 2-MCP as well as PCP. It suggested similar dechlorinating microbial communities were generated in both acidic systems regardless of the seed sludge source. This implied the different sources of sludge did not make significant difference in dechlorination activities under acidic condition. This might be because dechlorination under acidic environment was a co-metabolic process, in which case no specific chloro-respiration species were cultivated or required.

4.5 Conclusions

This study investigated the feasibility of degrading various chlorophenols under acidic condition. The following conclusions can be drawn from the study: (1) the acidic system required longer acclimation period compared with the conventional anaerobic dechlorination process. The long acclimation period allows accumulation of the dechlorinating microorganisms to grow and adapt to the condition; (2) the acclimated sludge with *ortho*-dechlorinating capability did not have a lag phase when challenged with more toxic chlorinated compounds such as 2,4,6-TCP and PCP; (3) the acidic environment may have negative impact on cultivating *meta*- or *para*-dechlorinating population. Attempts were made in this study but these failed to induce dechlorination at these two positions. Hence the acidic dechlorination process should be applied as a pre-treatment system to make toxic chlorophenols less inhibitory before the wastewater is treated by a downstream process; and (4) the possibility of acidic dechlorination implied that it was not necessary to adjust the pH of real industrial wastewaters (if it is acidic) for the anaerobic reductive dechlorination process. With proper operating strategies, acidic dechlorination could be an alternative option to the conventional anaerobic dechlorination process.

CHAPTER 5 Inhibition on Dechlorination under Acidic Condition

5.1 Introduction

From the previous chapter, it had been shown feasible to dechlorinate various chlorophenols by acclimated culture under acidic condition. Acclimation of biomass with proper pH control (i.e. a step-wise reduction of pH value) and exposure to a less toxic chlorinated compound (MCP), the acclimated sludge could *ortho*-dechlorinate 2-MCP, 2,4,6-TCP and PCP at pH as low as 5.5. It was previously believed the optimum condition for dechlorination was slightly alkaline (Armenante *et al.*, 1993). Thus, resistance of the sludge to pH variation would be investigated in this study.

As described in Chapter 4, the reactors were not inhibited by the various loadings of chlorophenols used. It would be interesting to understand the various inhibitory effects generated from the high loadings and metabolites of higher chlorinated compounds under acidic condition.

Although the reactors in this study were operated at low pH of 5.5, at which it was believed methanogens would be inhibited, it was found there was production of methane (CH₄). VFAs were not observed in the effluent of the two reactors. The quantitative polymerase chain reaction (qPCR) detected existence of different types of methanogens. The presence of methanogens made it difficult to understand the role of acidogens and methanogens in dechlorination. Various researchers have claimed that methanogens did not participate in dechlorination of chlorinated compounds since the dechlorination still proceeded after methanogenesis was inhibited in their studies (Perkins *et al.*, 1994; de Best *et al.*, 1999; Mun *et al.*, 2008b). In this chapter, the response and functionality of methanogens in dechlorination were investigated.

5.2 Materials and methods

5.2.1 Acidic anaerobic sequencing batch reactor and feed media

AnSBR 1 was still operated following the operating parameters described in Chapter 4. The anSBR showed a stable removal efficiency of PCP (Phase IV) when the following four studies were carried out.

The feed was diluted from the basic feed described in Chapter 3 to the sucrose concentration of 1600 mg/L.

5.2.2 Inhibition by PCP overloading

In Chapter 4, it was reported the two anSBR systems removed PCP without obvious inhibition at PCP loading of 5 $\mu\text{moles/L}\cdot\text{day}$. In this study, the concentration of PCP was increased to 10 $\mu\text{moles/L}\cdot\text{day}$. During the PCP overloading test, the basic feed was mixed with PCP (with a final concentration of 10 μM). The synthetic feed was then fed to the reactor (1 L/cycle). The reactors were operated for about 30 days before cycle study was conducted (Chapter 3)

After the systems were completely inhibited by PCP (details can be found in the results section), PCP in the feed was replaced with 100 μM of 2-MCP during the recovery period. Parameters such as 2-MCP, phenol, VFAs and biogas composition were monitored during the recovery period.

5.2.3 PCP biosorption test

The biosorption capacity of the sludge cultivated in this study was investigated after PCP dechlorination was completely inhibited. The sludge showing no sign of dechlorination was withdrawn from the reactor. The harvested sludge was centrifuged at 10,000 rpm for 5 minutes and resuspended by 1x PBS solution. The process was repeated 3 times to remove any organic matters on the surface of the sludge. The biosorption test was conducted in 100 ml serum bottle with a final biomass concentration of 1.3 ± 0.1 g/l. The initial concentrations of PCP were prepared at 3.8, 18.8, 37.6, 56.4 and 75.2 μM . Higher concentration of PCP was also tried, but at the testing pH of 5.7, PCP exceeding 75 μM became insoluble. The

test bottles were placed in the incubator shaker (Sartorius Stedim) and temperature was kept at 35 °C. As suggested by Bell and Tsezos (1987) and Kennedy *et al.* (1992), biosorption of PCP was usually a rapid process which could be accomplished within 12 hours. Thus samples were taken for analysis at 0, 0.5, 2, 6 and 12 hours to determine the time required to reach equilibrium. Two types of controls were prepared. One was dosed with PCP at each concentration tested without sludge to determine the amount of PCP adsorbed by the serum bottles. The other was prepared with sludge only to investigate the amount of PCP desorbed. Results showed the amounts of PCP adsorbed by the serum bottles and desorbed from sludge were negligible.

The amount of PCP adsorbed by the sludge was determined by subtracting residual PCP in the aqueous phase from the initial determined value. The Freundlich equation was used to model the adsorption phenomenon.

$$q_e = KC_e^n \quad (5-1)$$

Where q_e represents the amount of adsorbate adsorbed by adsorbent at equilibrium ($\mu\text{moles/g}$), K is the adsorption capacity, C_e is the residual concentration in the aqueous at equilibrium (μM) and n represents the adsorption intensity. The constants in the equation were obtained by non-linear regression using the data from the adsorption experiment.

5.2.4 Inhibition by pH shock

This study was carried out after systems were fully recovered from PCP inhibition, and both systems were able to remove 100% of 2-MCP when pH shock study was initiated. The purpose of the pH shock study was to examine resistance of the system to extreme low pH since it was proposed that long-term operation at low pH might make the system more robust to pH fluctuation although extreme low pH (2.0) could inhibit methanogenesis completely. In that case, the role of methanogens in dechlorination could be evaluated. The pH in the reactor was reduced to around pH 2 by dosing 1 M HCl and incubated for two days. 2-MCP and dechlorinating metabolite concentrations and VFAs in the effluent were measured to investigate the effects of pH shock on the dechlorination process.

5.2.5 qPCR analysis

The copy number of different microorganisms was determined by quantitative polymerase reaction (qPCR). Five groups of microbes were tracked - bacteria, *Metanobacteriale* (Mbt), *Methanomicrobiale* (Mmb), *Methanosarcinaceae* (Msc) and *Methanosaetaceae* (Mst). Mbt and Mmb were the hydrogen utilizing methanogens, and Msc and Mst were classified as acetoclastic methanogens in the order of *methanosarcinale*. The DNA was extracted using MagNA Pure Compact Nucleic Acids Isolation Kit (Roche, Germany). In brief, sludge withdrawn from the reactor was diluted to contain less than 2 g/L of TSS and washed with 1x PBS three times. Following that, 100 µl of diluted sludge was extracted with a fully automated nucleic acid extractor (MagNA Pure Compact, Roche). The extracted DNA was stored at -20 °C until analysis. qPCR was conducted using the TaqMan Probe technique. The primers and probe sequences were obtained from Yu *et al.*'s study and summarized in Table 5.1.

The qPCR reactions were performed in a 96-well white plate with LightCycler 480II (Roche, Germany). Each well was separately loaded with 2 µl of DNA sample, 1 µl of forward and reverse primers (500 nM), 2 µl of the TaqMan probe (200 nM), 4 µl of distilled water, and 10 µl of LightCycler 480 probe master (Roche) to a final reaction volume of 20 µl. A negative control was included in every assay performed. All experiments were performed in duplicate.

A two-step program was applied to amplify the target DNA as follows: an initial temperature at 95 °C for 10 minutes; denaturing temperature at 95 °C for 10 seconds; combined annealing and extension temperature at 60 °C for 30 seconds and the process was repeated for 55 cycles. Finally the cooling temperature was set at 40 °C for 10 seconds. The fluorescent signal data were processed by LightCycler 480 software (version 1.5.0 SP4).

Table 5.1 Primers and Probes sequences for real-time qPCR reactions

Name	Target group	Sequence (5'-->3')	Tm (°C)	Size (bp)
BAC338F	<i>Bacteria</i>	ACTCC TACGG GAGGC AG	63.4	468
BAC516F*		TGCCA GCAGC CGCGG TAATA C	70.8	
BAC805R		GACTA CCAGG GTATC TAATC C	60.7	
MBT857F	<i>Methanobacteriales</i>	CGWAG GGAAG CTGTT AAGT	60.7	343
MBT929F*		AGCAC CACAA CGCGT GGA	67.2	
MBT1196R		TACCG TCGTC CACTC CTT	63.2	
MMB282F	<i>Methanomicrobiales</i>	ATCGR TACGG GTTGT GGG	63.8	506
MMB749F*		TYCGA CAGTG AGGRA CGAAA GCTG	70.2	
MMB832R		CACCT AACGC RCATH GTTTA C	61.5	
Msc380F	<i>Methanosarcinaceae</i>	GAAAC CGYGA TAAGG GGA	61.2	408
Msc492F*		TTAGC AAGGG CCGGG CAA	66.7	
Msc828R		TAGCG ARCAT CGTTT ACG	59.9	
Mst702F	<i>Methanosaetaceae</i>	TAATC CTYGA RGGAC CACCA	61.0	164
Mst753F*		ACGGC AAGGG ACGAA AGCTA GG	70.0	
Mst862R		CCTAC GGCAC CRACM AC	62.1	

Note: * denoted the sequences of TaqMan Probes

5.2.6 Inhibition by phenol

The inhibitory effect of phenol was investigated in 150 ml serum bottles. Sludge withdrawn from the reactor was centrifuged down and washed with 1x PBS and the process was repeated three times to remove residual compounds on the surface of the sludge. The washed sludge was then resuspended in the prepared solution, which contained 200 μM of 2-MCP and various initial levels of phenol at 0, 180, 450, 600, 1300, 1800, 2300 and 2800 μM respectively. One-hundred milliliter of the mixed liquor was then transferred to the serum bottles and sealed with Teflon-lined butyl rubber stopper and aluminum crimp cap. The process above was performed under a nitrogen stream to ensure anaerobic condition. In the test, 10 mM of sodium acetate was used as external carbon source for each batch test. The serum bottles were placed in the shaker incubator at 35 °C for 24 hours. Samples were taken for analysis of 2-MCP, phenol and acetic acids after incubation. Three replicates were prepared at each concentration of phenol tested. The average VSS in the serum bottle was 1.7 ± 0.1 g/L.

5.2.7 Analytical Methods

Analysis of chlorophenols, volatile fatty acids and chemical oxygen demand were conducted according to the protocols described in Chapter 3.

5.3 Results

5.3.1 Inhibition by overloading with PCP

In the previous chapter, the acclimated sludge showed capability of *ortho*-dechlorination of PCP at loading of 5 $\mu\text{moles/day}\cdot\text{L}^{-1}$. PCP was quickly degraded through an *ortho*-dechlorinating pathway. After SBR1/Phase IV, the loading of PCP was increased to 10 $\mu\text{moles/day}\cdot\text{L}^{-1}$ in order to determine the maximum treatment capacity of the acclimated sludge. Figure 5.1A shows the profiles of chlorophenols in the effluent before and after increasing PCP loading. The PCP concentration in the effluent increased rapidly after loading was increased. Meanwhile the dechlorination product 3,4,5-TCP reduced. The intermediate 2,3,4,5-TeCP increased slightly 5 days after the loading doubled, which suggested a portion of the PCP was still degraded to TeCP. Dechlorination did not, however, proceed further to 3,4,5-TCP. Dechlorination of PCP ceased after 5 days with PCP accumulating in the system with no intermediates or end products detected.

Although the reactor was operated under acidic conditions, methanogens were not completely inhibited in this study. The step-wise reduction of pH during acclimation, recycling of biogas generated for mixing, and application of long SRT helped the system to maintain the population of methanogens. Meanwhile, the formation of granules and large flocs would provide a layered structure for methanogens to be protected within. Thus COD mineralization in the system was above 80-90% when dechlorination was not inhibited (Phase II – Phase IV). As shown in Figure 5.1A, the remaining COD was always below 100 mg/L before inhibition occurred but increased thereafter as inhibition of PCP dechlorination set in. COD removal was then reduced to less than 25% and CH_4 production was reduced to less than 10% of pre-inhibition values.

Figure 5.1 Chlorophenols, VFAs and COD concentration before and after inhibition of dechlorination of PCP: A) Chlorophenols and COD; B) VFA profiles

Note: Figure A), data presented are the averages of three measurements of a single effluent sample collected and error bar is the standard deviation of the three measurements. Figure B), each point represents single measured value for each effluent sample collected.

VFAs, fermentation products of sucrose, mainly consisted of acetic acid (HAc) and propionic acid (HPr) before inhibition. Concentrations of both acids were below 1 mM. After the inhibition, conversion of HPr to HAc and HAc to CH₄ were affected with accumulation of both acids in the system as shown in Figure 5.1B. A comparison of VFA compositions before and after inhibition is listed in Table 5.2. Besides the accumulation of HAc and HPr, longer chain VFAs, such as butyric acids (HBu) and valeric acids (HVa), were present as well. The results suggested the acetogenesis was affected.

Table 5.2 VFAs concentrations before and after inhibition

	Average Volatile Fatty Acids Concentration (mM)			
	HAc	HPr	HBu	HVa
Before inhibition	0.2	0.4	N.D. ^a	N.D. ^a
After inhibition	7.6	7.4	0.5	1.2

Note: N.D. means non-detectable

The theoretical COD values of VFAs (COD_{VFA}), listed in Table 5.3, were calculated and compared with the measured soluble COD (SCOD) in the effluent. As illustrated in Figure 5.1A, the COD of remaining VFAs in the effluent was very close to the measured SCOD, which indicated 1) the fermentation of sucrose was completed during the inhibition period as the VFAs contributed to nearly all remaining SCOD in the effluent; and 2) the acidogenesis process was not inhibited.

Table 5.3 Theoretical COD of VFAs

	Volatile Fatty Acids COD (g/mole)			
	HAc	HPr	HBu	HVa
Theoretical COD ^a	64	112	160	208

NOTE: ^a The theoretical COD was calculated based on the acid form of VFAs and complete mineralization to CO₂ and H₂O.

5.3.2 Adsorption of PCP

When the system was inhibited by PCP, it was found that biosorption of PCP by the sludge became significant (Piringer and Bhattacharya, 1999; Mun *et al.*, 2008b). A cycle study was conducted to investigate the fate of PCP. As shown in Figure 5.2,

the concentration of PCP in the aqueous phase remained constant after the initial dilution and adsorption.

Figure 5.2 PCP in the aqueous phase during inhibition within a 6-hour cycle

The amount of PCP remaining in the aqueous phase and adsorbed by the biomass before and after the cycle is shown in Figure 5.3. More than 85% of PCP in the reactor can be recovered from the sludge. Comparing the amount of PCP in different phases before and after the cycle, it clearly showed the sludge was saturated with the adsorbed PCP at this stage. The amount of PCP present in the influent was directly discharged in the effluent without any bio-transformation. The recovery of PCP from both aqueous phase and sludge at the end of the cycle was 96.5%. The results of mass balance of PCP showed that about 88% of PCP in the system was adsorbed to sludge (Table 5.4).

Figure 5.3 Amount of PCP in different phases before and after a 6-hour cycle

Table 5.4 Mass balance calculation of PCP

	PCP amount (μmoles)			
	Influent	Adsorbed	Aqueous	Total
Begin of the cycle	4.9	68.6	6.6	80.1
End of the cycle	N.A.	68.3	9.1	77.4
Recovery				96.5%

In order to understand the biosorption behavior of PCP under acidic conditions, a series of adsorption tests was conducted at pH 5.7 at 35 °C. Figure 5.4 shows the residual PCP concentrations in the aqueous phase following different contact times. The results were similar with either 6 hour or 12 hour contact time regardless of different initial concentrations of PCP which suggested adsorption of PCP by sludge reached equilibrium within 6 hours in this study. Other researchers have found that adsorption of PCP was complete within 2 hours at neutral pH or less than 24 hours under anaerobic conditions (Tsezos and Bell, 1989; Kennedy *et al.*, 1992; Antizar-Ladislao and Galil, 2004).

Figure 5.4 Effects of contact time on biosorption of PCP at pH 5.7, 35°C

The Freundlich's adsorption constants were then derived and summarized in Table 5.5 together with information from the literature.

Table 5.5 Comparisons of Freundlich's constants for biomass adsorption of PCP

	pH	Temperature	Sorption capacity, K	Sorption intensity, n
This study	5.7	35 °C	2.01	0.80
Kennedy <i>et al.</i> (1992)	7.2	35 °C	3.78*	0.68
Mun (2008a)	5.8	26 °C	1.42	0.90

Note:* The value was recalculated by converting the units to μmoles instead of μg in Kennedy's experiment

Based on the information gathered, comparisons of adsorption capacity of sludge operating under different conditions are presented in Figure 5.5. The adsorption capacity of the sludge in this study was close to that obtained by Mun *et al.*'s (2008a) work, which also used sludge incubated under acidic conditions. Compared with anaerobic dechlorinating sludge obtained at pH 7.2, adsorption capacity of the acidic sludge was about 30% less.

Figure 5.5 Comparison of the biosorption of PCP.

Note: The testing pH and temperature were: 1) pH 5.7 and 35°C in this study; 2) pH 5.8 and 26°C in Mun's study and 3) pH 7.2 and 35°C in Kennedy's study

5.3.3 Recovery from inhibition by PCP overloading

Recovery was initiated, after the reactor completely lost dechlorination capability, by feeding with sucrose only with intention to remove residual chlorophenols in the reactor (5 days). 2-MCP (100 μM) was fed into the system when PCP could no longer be detected. As shown in Figure 5.6, a 25-days lag period occurred before the dechlorinating function resumed. The total recovery period was about 1 month including the time when only sucrose was fed. A loading of 100 μmoles/L·day 2-MCP was completely removed from day 40 onwards. On Day 110, the 2-MCP loading was further doubled to 200 μmoles/L·day. Similar performance of 99% of 2-MCP removal was obtained. Phenol was found in the effluent. Based on the mass balance, the 2-MCP removed was fully converted to phenol which not further degraded.

Figure 5.6 Concentrations 2-MCP and phenols in the effluent during recovery period

Note: Each point represents a single measured value for each effluent sample collected.

VFAs production during the recovery period is presented in Figure 5.7. Initially the longer VFAs (e.g. Hbu and HVa acids), which were the main products during the inhibitory period, were converted to HPr and HAc. Then consumption of short chain VFAs (HAc and HPr) and production of biogas was observed with resumption of dechlorinating capability. The CH₄ content in the biogas produced was about 60%. Residual acetic acid and propionic acid were further reduced and could not be detected after the 2-MCP loading increased. This suggested that the larger amount of 2-MCP dechlorinated required more consumption of acetic acid and propionic acid and recovery of methanogenesis. From the accumulation and consumption of VFAs, it would suggest that dechlorination activity was related to the removal of the fermentative products. Although it was difficult to conclude which group of microorganisms was responsible for the dechlorination under acidic environment at this moment, methanogenesis may have a role.

Figure 5.7 VFAs profile in the effluent before and after recovery from PCP overloading inhibition

Note: Each point represents a single measured value for each effluent sample collected.

5.3.4 Dechlorinating inhibition by and recovery from pH shock

Since the reactor was running in the acidic environment at pH 5.5 for more than 2 years, it was interesting to test the system's robustness against pH variations. It was suggested that the optimum pH range for dechlorination of 2,4,6-TCP was from 6.0 to 6.3 (Mun *et al.*, 2008c). Dechlorination ceased when pH was below 5.0 and required about 30 days to recover from the pH inhibition (pH 5.3). In this study, the pH of anSBR 1 was reduced to 2.0 for two days to investigate performance of the dechlorinating process. The test was conducted from 210 days after the systems were fully recovered from the inhibition by PCP overloading. Figure 5.8 shows the profiles of phenolic compounds and VFAs in the effluent during pH shock and recovery periods.

The sharp drop of pH took the immediate effect on the dechlorination of 2-MCP. Dechlorination of 2-MCP stopped completely after one day under the extremely low pH (Figure 5.8A). The pH inhibition caused VFAs' accumulation, which

suggested interruption of acetogenesis and methanogenesis (Figure 5.8B). Further, CH₄ content in the biogas fell to 6-8% from more than 60%. The dechlorination process was gradually resumed in around 30 days with the accumulation of phenol. The period of 30 days for recovery also implied the dechlorinating activity only resumed when methanogens accumulated as the growth rate of methanogenic archaea was much slower than the acidogenic bacteria. As described in the next section, it could be found that the dechlorination activity resumed along with the increment of copy numbers of one the methanogens. (Section 5.3.5)

The duration required to recover the dechlorinating capability was the same as those needed for recovery from inhibition by PCP overloading and in Mun *et al.*'s (2008c) study. Both their and this study conducted the dechlorinating process under acidic condition. Although different pH values were tested to investigate pH shock on dechlorinating activity, dechlorination could be resumed in 30 days' time no matter whether the pH was 5.3 or as low as pH 2.0. Armenante *et al.* (1993) suggested the dechlorination of 2,4,6-TCP was only possible within the pH range of 8.0-8.8. Apparently, given a proper acclimation period, acclimated biomass could perform dechlorination under acidic conditions. The activity of dechlorination was only inhibited at extremely low pH.

Figure 5.8 2-MCP, phenol and VFAs profile during pH shock: A) 2-MCP and phenol; B) VFAs

Note: Each point represents a single measured value for each effluent sample collected.

5.3.5 Microbial community change during inhibition

In the previous two inhibition studies, it had seemed possible that the dechlorinating activities were related to the functions of methanogens. In other words, dechlorination required methanogens to perform properly. In order to better understand the involvement of methanogens, if any, quantifications of different methanogens by qPCR were performed. In this study, two order levels of H₂-utilizing methanogens, *Metanobacterial* (Mbt), *Methanomicrobials* (Mmb) and two family levels of aceticlastic methanogens, *Methanosarcinaceae* (Msc) and *Methanosaetaceae* (Mst), were quantified. The copy numbers of the different groups of methanogens and bacteria are presented in Figure 5.9.

Among the methanogens, as Mst was present in the seed sludge but was absent in the system thereafter, it was not shown in Figure 5.9. As shown in the highlighted area, which showed the changes during the two inhibition periods described in the previous section, it was found that Msc was the most affected group of methanogens. The absolute number reduced over 1000 times during inhibition and the relative percentage among the total methanogens became only 0.01% from the original above 2% when dechlorination had proceeded. This was so in both cases of inhibition. The results supported the observation on accumulation of HAc and HPr during the inhibition, as Msc was the only acetate-utilizer in the reactor in this study. The total bacteria was, however, relatively stable throughout the period when it was monitored. Although the Mmb was also found affected, it did not recover immediately when the system regained dechlorinating function (on 09 Sep, 2012). It did not seem as directly related to dechlorination as the Msc.

Figure 5.9 qPCR quantification of bacteria and methanogens

Note: Data presented are the average of triplicates and error bar is the standard deviation.

5.3.6 Effects of phenol on dechlorination

The influence of a dechlorination metabolite (phenol) should be investigated in order to understand and efficiently control dechlorination under acidic condition.

Figure 5.10 presents the amount of 2-MCP consumed per gram of biomass at various initial phenol concentrations. Compared with the phenol-free control, the degradation of 2-MCP declined as of phenol increased. Degradation of 2-MCP was reduced to 60% of the control at the highest phenol concentration determined in this study (2.8 mM), whereas the activity was about 90% and 80% of the control test when phenol concentrations were below 0.6 mM and 2.3 mM respectively. From the results, it can be concluded that the inhibitory effect of phenol on dechlorination of 2-MCP was not progressive but had thresholds at each inhibitory level. In this study the inhibitory thresholds observed were 0.2, 1.3 and 2.8 mM respectively, above which, the dechlorinating activity could be reduced by about 10-20% from previous concentration level.

Figure 5.10 Influence of initial phenol concentrations on dechlorination of 2-MCP

Note: Data presented are the average of triplicates and error bar is the standard deviation.

5.4 Discussions

In this chapter, different factors, which were believed to influence the dechlorination of PCP and 2-MCP were investigated.

First, dechlorination of PCP was completely inhibited by a PCP loading of 10 μM/d. Boyd (1984) using typical anaerobic sludge for dechlorination reported dechlorination ceased when PCP loading was over 6 μM/d. Compared with Boyd's findings, the results from this study suggested the dechlorinating ability of PCP by anaerobic sludge and acclimated sludge under acidic condition were similar. In other words, the functioning dechlorinating microorganisms although cultivated under different conditions might still be the same. After the system was inhibited by PCP overloading, the immediate effect observed was the accumulation of VFAs. The appearance of longer chain VFAs other than acetic acid e.g. propionic acid, butyric acid and valeric acid suggested acetogenesis was interrupted. Biogas production was severely reduced and methane in the biogas was less than 10%,

which indicated methanogenesis was inhibited by the PCP overloading. On the other hand, fermentation of sucrose continued. These results are contradictory with (Mun *et al.*, 2008c) who reported dechlorination of 2,4,6-TCP by an acidogenic sludge was not inhibited when methanogenesis was stopped by inhibitors. Although the dechlorinating patterns were similar in both studies including only *ortho*-dechlorination was observed, the argument regarding the involvement of methanogens in the dechlorination was not consistent. In this study, it was clear that dechlorination was completely stopped when methanogenesis was inhibited. This phenomenon indicated dechlorination was coupled with methanogenesis. While a similar step-wise pH reduction strategy was applied in both studies, MCPs were used as the inducer during the start-up period in this study whereas Mun *et al.* (2008b) had applied 2,4,6-TCP directly. The recirculation of produced biogas to enhance mixing in this study could also have encouraged further utilization of CO₂ and H₂ by methanogens and hence their growth. The long SRT and formation of big biomass flocs could also have protected the methanogens from the acidic environment. These operating differences might have resulted in the different dechlorinating microorganisms, methanogens in particular, being accumulated in the reactors leading to the contradictory results.

Secondly, dechlorination of 2-MCP was severely inhibited by pH shock when the sludge was incubated at pH 2.0 for 2 days. Similar to what was found during the PCP overloading inhibition, the immediate effect of the pH shock was inhibition of methanogenesis and accumulation of VFAs in the system. As described above, the acetic acid and propionic acid were accumulated to about 7.0-7.5 mM, followed by the accumulation of butyric and valeric acids and the reduction of methane production during the inhibition period. In both inhibitions, it was found dechlorination of chlorophenols under acidic condition coupled with methanogenesis or dechlorination required the methanogenesis to proceed properly. As long as the methanogens were inhibited by either overloading of PCP or the low pH, dechlorination ceased. Although the dechlorinating role of methanogens could not be conclusively determined at this stage, acidogenesis alone was not sufficient to complete the dechlorination. In both inhibitory studies, it was found that sucrose

was completely fermented into VFAs based on the COD balance calculation, which suggested the acidogenic bacteria was not inhibited.

Further evidence was provided by the qPCR analysis of the microbial community. The qPCR results revealed that the *Methanosarcinaceae* family was the most affected group during both inhibition studies. The copy number of this family was reduced sharply by more than 1000 times during inhibition, whereas the total bacteria number remained relatively consistent. After the inhibition episode, the system required about 30 days to recover the dechlorinating capability. The duration required for recovery agreed with the results obtained from qPCR. During this period, *Methanosarcinaceae* recovered to the same level as it was before inhibition. This might also explain the long acclimation period (80 days) required at the start-up phase, during which, this particular group of methanogens would need to accumulate to a sufficient level for dechlorination to proceed. The results further indicated a copy number of *Methanosarcinaceae* should not be below the order of 10^6 copies/ml in the reactor in order to support dechlorination. It had been demonstrated the isolates or pure culture of methanogens from *Methanosarcinaceae* family could conduct dechlorination of various chlorinated compounds including tetrachloroethylene, chloroform and vinyl chloride (Fathepure and Boyd, 1988; Mikesell and Boyd, 1990; Heimann *et al.*, 2006). It was believed that members from *Methanosarcinaceae* family were also involved in the chlorophenols dechlorination in this study although there had been no reports in the literature of involvement of this group in dechlorinating aromatic chlorinated organics.

In the phenol inhibition test, it was found the dechlorinating rate and efficiency decreased as the concentration of phenol increased. However the inhibitory effect was not progressive. The activity of dechlorination was reduced only when phenol concentration exceeded a certain threshold. This indicated failure to further degrade phenol could limit the treatment capacity of wastewater containing 2-MCP. The results suggested the level of phenol should be closely monitored to avoid inhibition as it was not further degraded in acidic environment. The inhibitory pattern of phenol on dechlorination is consistent with reported inhibition of phenol

on methanogenesis. Fang and Chan (1997) investigated the toxicity effects of phenol on acetate-, propionate- and benzoate-degrading biogranules. There was also a threshold above which phenol toxicity took effect on substrate degradation and methanogenesis. The above add support to the argument methanogens participated in the dechlorination of 2-MCP even under acidic condition.

Even when the inhibition occurred, more than 88% of PCP was found to be adsorbed by the biomass. PCP is a hydrophobic compound with limited solubility in water. The biomass's biosorption capacity of PCP in this study was very close to that obtained by Mun *et al* (2008a). With the low pH, a larger fraction of PCP would be in molecular form, (Figure 5.11) and hence more PCP would be attracted to the biomass. However, compared with conventional anaerobic sludge, the acidic sludge had lower biosorption capacity even when the experiment was conducted at low pH. It has been reported that acidogens have a more hydrophilic surface than methanogens (Daffonchio *et al.*, 1995). The sludge generated in this study was cultivated at low pH for more than 1 year. The sludge would consist of a higher proportion of acidogens even though methanogenesis still occurred. As shown in Figure 5.9, the bacteria copies in the system ranged in the order of 10^{10} and 10^{11} , whereas the methanogens only counted in the order of 10^6 to 10^9 . The total methanogens (sum of the four groups of methanogens) were always below 10% of the copies of bacteria. Hence it suggested the acid sludge was more hydrophilic as compared to the anaerobic sludge in nature, which led to the lower adsorption capacity for chlorophenols.

Figure 5.11 Fraction of molecular form of PCP as a function of pH.
Note: $pK_a = 4.81$

5.5 Conclusions

Different factors that were inhibitory to the dechlorination process were investigated in this chapter. It was determined PCP loading on the acidic sludge at $5 \mu\text{moles/day} \cdot \text{L}^{-1}$ already had adverse impact. A sharp decrease in pH also adversely impacted dechlorination of 2-MCP. The response of the various microbial communities in the sludge during and after inhibition suggested methanogenesis should not be inhibited in order to maintain dechlorination under acidic environment.

CHAPTER 6 Syntrophism in Dechlorination under Acidic Condition

6.1 Introduction

In the previous chapter, it was noted dechlorination and methanogenesis were inhibited simultaneously. Dechlorination of PCP was inhibited when there was overload of PCP. During the inhibition episode, methane production ceased although fermentation of sucrose still proceeded. In the pH shock test, dechlorination of 2-MCP could not continue when methanogenesis was inhibited by the low pH. In both inhibition cases, the qPCR results revealed the *methanosarcinaceae* family had declined significantly. This led to the argument methanogens could have been involved in the dechlorination of chlorophenols under acidic condition. More detailed studies on the role of methanogens were conducted by applying the methanogenesis inhibitor, 2-bromoethanesulfonate (BES). BES is a structural analog of coenzyme M, which is involved in the last step of methanogenesis (Ellermann *et al.*, 1988) and is found in methanogens only but not bacteria or other archaea (Balch and Wolfe, 1979). BES works as the competitor of coenzyme M to prevent the reduction of methyl-coenzyme M to methane (Perkins *et al.*, 1994). Thus, BES was selected in this study as it specifically inhibits methane formation. Against this, the bacterial antibiotic vancomycin that inhibits formation of cell walls of gram-positive bacteria (Godbole *et al.*, 1991; Madigan *et al.*, 2006) was used to investigate the role of bacteria in dechlorination.

In this study, the objective was to investigate the relation between dechlorination and methanogenesis when different electron donors (including sucrose, acetate and hydrogen) were used.

6.2 Materials and methods

6.2.1 Cycle study before each batch test

In this study, the focus was to investigate the participation of methanogens in the dechlorination process. After the system recovered from the PCP overload discussed in the previous chapter, anSBR 1 was fed with 2-MCP and sucrose as the carbon source to work as the mother reactor. The 2-MCP concentration in the feed media was 100 μM during recovery and was increased to 200 μM after anSBR 1 had recovered. All the tests were carried out in serum bottles with the sludge collected from anSBR 1. Before each test, one cycle study was conducted to confirm the dechlorinating capability of the biomass. In the cycle study, 10 ml of mixed liquor was collected as described in Chapter 3. The collected samples were then centrifuged at 10,000 rpm for 5 minutes. Two ml of clear supernatant was used for analysis of chlorophenol. The rest of the sample was filtered through a 0.45 μm syringe filter and the filtrate used for COD and VFAs analysis while filtrate from a 0.2 μ syringe filter was used for phenol quantification.

6.2.2 Biogas quantification

Biogas generated in the batch tests was quantified according to the ideal gas law in the following form:

$$PV = nRT \quad (6-1)$$

where P is the pressure, V represents the total volume of the gas, n is the amount of substance of the gas (moles), R is the gas constant ($8.3144621 \times 10^{-5} \text{ m}^3 \text{ bar K}^{-1} \text{ mol}^{-1}$) and T is the absolute temperature measured in kelvin (K). In this study, the temperature was maintained at 35 °C (308.15 K). The value of R is as recommended by the Committee on Data for Science and Technology (CODATA) (Mohr *et al.*, 2008).

In each batch test, the pressure was first determined by the Dwyer Mark II manometer (Series 475, U.S.) before any liquid or gas sample was taken. The determined pressure was used to calculate the total moles of the gas mixture (n). The amount of individual gas was then calculated by multiplying this value with the percentage of each gas determined by GC-TCD/TCD.

6.2.3 Preliminary test with BES

In the test, 120 ml serum bottles were used with liquid volume of 100 ml. The serum bottles were operated in the manner of the sequencing batch reactor. Fifty percent of the supernatant (50 ml) after settling was replaced with fresh incubation media daily. The media contained 170 μM of 2-MCP and 1600 mg/L of sucrose as the primary substrate. The inorganic macro-nutrients supplements and trace elements were the same as described in Chapter 3. On Day 0, 50 ml of mixed liquor collected from the sludge was mixed with 50 ml of media and transferred into the serum bottles, which were closed with Teflon-lined butyl rubber stoppers and aluminum crimp caps. Each serum bottle was flushed with nitrogen gas for 5 minutes before it was sealed to ensure anaerobic condition. BES was dosed into the serum bottles after the supernatant was replaced on Day 6. The final concentration of BES in each bottle was 10 mM. The same amount of BES was dosed daily afterwards until the test was completed. A negative control was prepared in the same way as above with autoclaved biomass. All tests were performed in triplicates.

6.2.4 Batch Tests with inhibitors

Sucrose and acetate were selected as the carbon sources in this experiment and the tests were conducted in the 120 ml serum bottles with 100 ml of liquid volume. The serum bottles were sealed with Teflon-lined butyl rubber stoppers and capped with aluminum crimp-top caps. In each test, three parallel batches were prepared. The control batch contained the culture, media and no inhibitors. The BES and the vancomycin batch included the methanogenic specific inhibitor BES (50 mM) and vancomycin (200 mg/l) respectively besides having the same culture and media as in the control batch. The sucrose medium contained 1600 mg/L of sucrose, 200 μM of 2-MCP with the inorganic macronutrients and trace elements solutions (1 ml/L) described in Chapter 3. The acetate medium was prepared in the same way except for replacing sucrose with acetate (5mM). The culture (600 ml) was collected from the anSBR 1 after the cycle study and dechlorination had been observed. The biomass was then equally transferred to the centrifuge tubes under the nitrogen stream and spun for 5 minutes at 3000 rpm. The pellet was rinsed with 1x PBS and the process was repeated three times. The final centrifugant was then resuspended

in 100 ml of the media and transferred to the serum bottles. All the bottles were flushed with N₂ for 5 minutes before capping and were incubated in the shaker (Sartorius Stedim) at 35 °C. On every second day, 50% (50 ml) of the supernatant after settling was replaced with fresh media. The inhibitors were dosed to maintain the above-mentioned concentrations when applying fresh media. From Day 10 onwards, 5 ml of pure (100%) H₂ produced by a hydrogen-generator (LNI Schmidlin SA, Switzerland) was introduced to the vancomycin and BES batches after the media was exchanged. The H₂ was collected in the gasbag (Tedlar, U.S.) and then transferred to the serum bottles using a gas tight syringe (Agilent, Singapore). All tests were conducted in triplicates.

6.2.5 Dechlorination using H₂ as the electron donor

Two sets of serum bottles were prepared in this experiment. One set was cultivated with H₂ and the other with H₂ and BES (50 mM). The biomass and inoculating process were prepared in the same way as described above. The media in this experiment contained 1 mM of 2-MCP without any external carbon sources. Only 5 ml of the supernatant was exchanged with the fresh media daily and 10 ml of H₂ was injected in the bottles. All tests were prepared in triplicates.

6.2.6 Analytical methods

The analysis for 2-MCP, phenol and VFAs were performed according to the procedures in Chapter 3.

6.3 Results

6.3.1 Cycle study before batch test

The remaining concentrations of 2-MCP and phenol in the reactor within a 6-hour cycle are shown in Figure 6.1. Figure 6.1A showed the phenol profile when 100 μM of 2-MCP was dosed in the feed while Figure 6.1B represented the 200 μM 2-MCP case. In both cases, the 2-MCP was largely removed within 80 minutes with a linear removal rate during this period. The removal rates were 56.8 $\mu\text{moles/h}$ and 94.8 $\mu\text{moles/h}$ when the 2-MCP concentration was 100 and 200 μM respectively. The residual 2-MCP profiles and removal rates determined in the cycle studies would be used to confirm dechlorination by the biomass before each batch test. As shown in the figures, the dechlorination rate had remained relatively constant until dechlorination stopped within a cycle, although the substrate concentration had progressively reduced. Similarly, linear removal rates of 2-MCP were observed in each cycle study throughout the project when the system was in steady state. The batch tests were only conducted when the system had stabilized.

Figure 6.1 2-MCP and phenol concentrations within one operating cycle: A) 100 μM of 2-MCP in the feed; B) 200 μM in the feed

Note: Each point represents a single measured value for each effluent sample collected.

6.3.2 Preliminary test with BES

In this test, 10 mM of BES was applied in the batches to investigate whether the dechlorination of 2-MCP could continue when methanogenesis was affected. Before the dosage of BES, there was a rapid reduction of 2-MCP in the bottles with live biomass. On Day 6, no residual 2-MCP could be detected in the effluent withdrawn (Figure 6.2). Dechlorination continued for one more day after the dosage

of BES, then accumulation of 2-MCP occurred. In the control batches, the concentration of 2-MCP increased to the level as it was in the feed. This indicated that there was no dechlorination in the negative controls. The rate of accumulation of 2-MCP during the first 6 days then became an indicator to examine whether dechlorination occurred. As shown in the figure, the rate was around 12.3 $\mu\text{M}/\text{d}$ calculated based on the linear portion of the curve.

Figure 6.2 Degradation of 2-MCP before and after addition of BES

Note: Data presented are the average of triplicates and error bar is the standard deviation.

Although the residual 2-MCP increased after the inhibitor was applied, dechlorination did not cease completely as accumulation rate of 2-MCP was 7.4 $\mu\text{M}/\text{d}$. It was about 60% of the accumulation rate where there was no dechlorination in the controls. This suggested dechlorination could have continued at a reduced pace after BES was dosed. This preliminary result indicated dechlorination efficiency decreased when methanogenesis was affected and supported the findings in the previous chapter. The reason why dechlorination was not inhibited completely might be due to the concentration of BES applied was not sufficient to inhibit methanogenesis completely. Mun and his coworkers suggested methanogenesis could be inhibited severely (96%) at the same level of BES used in

this study while degradation of 2,4,6-TCP was unaffected (Mun *et al.*, 2008a). However, the required concentration of BES to cease methanogenesis varied in different conditions. The suggested values were from 2mM to 50mM from the literatures (Zinder *et al.*, 1984; Siriwongrungson *et al.*, 2007; Wüst *et al.*, 2009).

6.3.3 Dechlorination with sucrose and inhibitors

In order to inhibit methanogenesis completely, 50 mM BES was applied. Vancomycin was also used in parallel batches to determine its effect on dechlorination activity by inhibiting gram-positive bacteria. Cumulative 2-MCP removed from the bulk liquid in both batches and in the control which did not contain any of the inhibitors is presented in Figure 6.3A. The detailed calculations are presented in Table 6.1. The total amounts of 2-MCP degraded in the batches were 32.7 ± 0.5 (control), and 14.8 ± 0.7 (BES) and 22.2 ± 0.7 μ moles (vancomycin) during the 8-day test. Dechlorination was affected in the batches dosed with BES. The total amount of 2-MCP removed was only 45% of the amount degraded in the control. The 2-MCP removed was largely degraded within the first 2 days and small amount of methane could still be detected during these first 2 days. Following that, dechlorination was not active in the BES batches when no methane production could be detected (Figure 6.3B). On Day 8, dechlorination was severely inhibited in the BES batches (87%). On the other hand, in the vancomycin batches, the dechlorination was only slightly reduced in first 4 days. The amount of 2-MCP removed was about 83% and 95% of the amount removed in the control batches on Day 2 and Day 4 respectively. However, the inhibition in vancomycin batches became more severe after forth day. On Day 6 and 8, the inhibition levels were 61% and 79% respectively, which were close to the values in BES batches (64 and 87%). The vancomycin prevents eubacteria cell wall synthesis, hence it might take some time to prevent the formation of new cells and this may explain why dechlorination activity decreased after 4 days. As shown in Table 6.1, the amount of 2-MCP dechlorinated was very similar in both BES and vancomycin batches. Both inhibitors prevented the dechlorination of 2-MCP after the inhibitor took effects.

Table 6.1 Amount of 2-MCP removed on each sampling date with inhibitors					
Time (Day)	2-MCP removed (μ moles)			Inhibition ^a (%)	
	Control	BES	Vancomycin	BES	Vancomycin
2	14.6 (0.01)	9.5 (0.24)	12.1 (0.01)	35	17
4	7.5 (0.02)	2.6 (0.29)	6.8 (0.65)	66	9
6	5.9 (0.10)	2.1 (0.82)	2.3 (0.57)	64	61
8	4.7 (0.59)	0.6 (0.52)	1.0 (0.22)	87	79
Total	32.7 (0.50)	14.8 (0.74)	22.2 (0.66)	-	-

Note: Results are the average of triplicates and values in the brackets are the standard deviations. ^a Inhibition was calculated with respect to the control without any inhibitors applied.

In addition, methane production in the vancomycin batches was 18% higher than the amount of methane generated in the control batches as shown in the Figure 6.3B. Because a complex carbon source, sucrose, was used in the test, it was difficult to determine the source for the extra methane based on a COD balance. Hence a similar test but using acetate as the carbon source was conducted and this presented in the next section. It was noted dechlorinating efficiency reduced about 79% in the vancomycin batches, whereas the efficiency reduced 87% in BES batches. The results suggested that both inhibitors affected the dechlorination efficiency. This phenomenon led to the hypothesis that dechlorination under acidic environment might require another group of bacteria besides methanogens to maintain the capability.

Figure 6.3 Effects of inhibitors on dechlorination and CH₄ production with sucrose: A) Cumulative 2-MCP removed; B) Cumulative CH₄ gas produced.
Note: * The cumulative 2-MCP removed was calculated based on the residual concentration of 2-MCP in the supernatant.
 Note: Data presented are the average of triplicates and error bar is the standard deviation.

6.3.4 Dechlorination with acetate and inhibitors

A similar experiment as that described above was conducted in serum bottles but with acetate as the carbon source. The effects of inhibitors on dechlorination of 2-MCP under the acidic environment are shown in Figure 6.4. Similar to the results in the test with sucrose as carbon source, dechlorination only occurred in the first 2 days in the BES batch and was inhibited from Day 4 onwards in the vancomycin batch. The amount of 2-MCP removed as of Day 4 was 77.6 ± 0.3 , 9.2 ± 0.1 and 23.2 ± 0.2 μ moles in the control, BES, and vancomycin batches respectively. Both the inhibitors seriously affected dechlorination, although the impact of the methanogenic inhibitor BES was more severe. Cumulative 2-MCP removed in the BES batch was only 12% of the amount in the control batch but in the vancomycin batch, cumulative 2-MCP removed was about 30% of the amount in control until Day 4. After the inhibition had occurred in the batches with inhibitors, 5 ml of H_2 was injected into the headspace of the BES and vancomycin batches to provide the electron donor for dechlorination on Day 10 but this did not help in resuming the dechlorinating capability.

Figure 6.4 Effects of inhibitors on dechlorination with acetate

Note: Data presented are the average of triplicates and error bar is the standard deviation.

In Figure 6.5, the amount of CH₄ generated (A) and acetate consumed (B) is presented. As suggested by the graph, higher acetate consumption is related to higher CH₄ production in the vancomycin batches but BES inhibited methanogenesis completely. In the batches with sucrose as the carbon source, it was also found that there was extra CH₄ produced in the vancomycin batches. In this experiment, it was confirmed the additional CH₄ was generated through acetoclastic methanogenesis since the simpler carbon source acetate was used and the correlation between production of the gas and utilization of the substrate. The COD balance of the extra acetate consumed, CH₄ produced and H₂ dosed was calculated and shown in the Figure 6.6. From the graph, it was suggested that the COD of the extra CH₄ was very close to the COD of extra acetate utilized in the vancomycin batches and H₂ injected ($\frac{COD_{CH_4}}{COD_{HAc}+COD_{H_2}}$ ranged between 70-120%). About 32-39% of the extra CH₄ originated from the H₂ supplied. The results indicated that acetate was directed to methanogenesis from other pathways like oxidation of acetate to CO₂ and H₂ by acetate oxidation bacteria (Hatamoto *et al.*, 2007). As shown in the graphs (Fig 6.4 and Fig 6.5A), the differences in CH₄ production and acetate consumption started on Day 4, which coincided with the time when dechlorination was also inhibited. The results again supported the argument dechlorination likely did not depend on methanogenesis alone but required the syntrophic bacteria of methanogens to function properly.

Figure 6.5 Effects of inhibitors on CH_4 production and acetate consumption:
A) Cumulative CH_4 production (mmoles); B) Cumulative acetate consumption (mmoles)

Note: Data presented are the average of triplicates and error bar is the standard deviation.

Figure 6.6 COD balance of CH₄, acetate and H₂

Note: The extra amount was calculated by subtracting the values of control batches from vancomycin batches. H₂, CH₄ and acetate COD equivalent was calculated based on following reaction equations: 1) $H_2 + \frac{1}{2}O_2 \rightarrow H_2O$; 2) $CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$; 3) $CH_3COOH + 2O_2 \rightarrow CO_2 + 2H_2O$

DiStefano *et al.* (1992) had suggested some growth factor produced by bacteria might be necessary for proper dechlorination of tetrachloroethene. They used the filtrate of the supernatant from batches with proper growth of bacteria to resume the dechlorinating activity in those batches supplied with H₂ where dechlorination was inhibited by vancomycin. The same procedure was repeated in this experiment by filtering supernatant from the control batches and injecting this into the vancomycin batches. Unfortunately, the dechlorination of 2-MCP did not recover (data not shown). Hence, it is possible dechlorination of 2-MCP under acidic environment not only required the “growth factor” but also the activity of live bacteria to maintain the syntrophic environment with methanogens.

6.3.5 H₂ as the electron donor

The effect of H₂ serving as the electron donor for dechlorination of 2-MCP under acidic environment is presented in Figure 6.7. The graph shows dechlorination

could be sustained when H₂ was supplied, at least for a short period. In the H₂ fed batch, the removal efficiency of 2-MCP was more than 95% initially. However the efficiency declined after 4 days. At Day 7, only about 32% of the 2-MCP could be degraded. Utilization of H₂ as shown in the Figure 6.8 had then also declined and had accumulated in the headspace. Biomass activity could have declined when supplemental carbon was not provided. BES was applied in parallel batches, where only H₂ was used as well and significant removal of 2-MCP was only observed in the first day and only 20% of 2-MCP was removed on Day 2. Less than 5% of 2-MCP was removed in the following days. Figure 6.8 indicates utilization of H₂ was blocked in the batches with BES and this supported the argument methanogens were involved in the dechlorinating process.

Figure 6.7 Dechlorination of 2-CP with H₂ as electron donor

Note: Data presented are the average of triplicates and error bar is the standard deviation.

Figure 6.8 H₂ content in the headspace in batches with only H₂ supplied

Note: Data presented are the average of triplicates and error bar is the standard deviation.

6.4 Discussions

Efforts were made to identify the group of microorganisms responsible for the dechlorination of 2-MCP under acidic environment. Various electron donors or carbon sources (sucrose, acetate and hydrogen) were used together with the two inhibitors, BES and vancomycin in these experiments.

Results from batches dosed with the methanogenic inhibitor BES were consistent regardless of the electron donor used. In all three cases, dechlorination of 2-MCP was inhibited. This suggested involvement of methanogenesis in dechlorination of 2-MCP. These results were expected since it was found that dechlorination could not proceed when methanogenesis was suppressed by overloading of PCP or extreme low pH in the previous chapter (Chapter 5). Yu and Smith (1997) reported dechlorination of chloroform was inhibited by 90% in the BES amended samples and the inhibition could be reversed significantly by addition of co-enzyme M. Their results indicated that methanogens were the main dechlorinator and the dechlorination process could proceed if the methanogenesis functioned. Freeman

and Gossett (1989) also demonstrated methanogenesis played a key role in transforming trichloroethylene (TCE) to ethylene under methanogenic conditions. Despite of these results, the role of methanogens in dechlorination remains arguable as the above-mentioned authors only proved BES could inhibit the dechlorination of aliphatic chlorinated compounds. Mun *et al.* (2008c) demonstrated that BES had no effect on dechlorination of 2,4,6-TCP while vancomycin had a more severe inhibitory effect, while results in this study only partially agreed with their findings as BES could also prevent the dechlorination of 2-MCP. The possible reason might be due to the different microbial consortia obtained because of the different acclimating compounds used during the period to cultivate the biomass. In their study (Mun *et al.*, 2008c), the more toxic 2,4,6-TCP was used and this inhibited most of the methanogens initially while in this study MCPs were applied. This hypothesis could be supported by data on methane in the biogas produced. In their study it was only around 10% while methane content could reach 50-60% in this study. Perkins *et al.* (1994) illustrated BES only reduced 2,4,6-TCP dechlorination rate when acetate was used but did not affect the dechlorinating process in the bottles with H₂ or fructose plus titanium citrate. Thus they concluded methanogens should not be the responsible dechlorinator. Unlike their results, BES stopped dechlorination of 2-MCP in all three experiments no matter the electron donors used in this study. It suggested the involvement of methanogens in dechlorinating 2-MCP. In addition, BES altering eubacteria community structure was reported previously (Chiu and Lee, 2001) although the findings suggested that the results from BES inhibition test solely could not be conclusive with respect to the role of methanogens in dechlorination. However in their research (Chiu and Lee, 2001), the anaerobic enrichment was affected after a long period following application of BES (18 months). In the short period test, the application of BES did not alter the dechlorination pathway of trichloroethene with their culture (Chiu and Lee, 2001), which suggested application of BES in short period would not alter the anaerobic bacteria community yet. In this study, BES took effect from the second day. In such a short duration, it was believed BES probably would not have affected the eubacteria yet. The other argument regarding the inhibitory effect of BES on dechlorination was that the sulfonate moiety of BES could serve as the electron

acceptor, which might compete for electrons with the dechlorinators. Such an argument was not valid in this study as BES inhibited the dechlorination of 2-MCP in the test with H_2 where sufficient free H_2 was available to work as the reducing equivalent. Furthermore, the results in Chapter 5 had already shown dechlorination was inhibited together with methanogenesis inactivity while acidogenesis was not severely affected. The qPCR results also revealed only the *Methanosarcinaceae* family declined sharply during the inhibition period. In Chapter 2, it had been reported the correlation between cometabolic dechlorination and the activity of methanogens. The results in this chapter could be due to cometabolic dechlorination of chlorophenols by methanogens. In summary, although other researchers had reported dechlorination was not related to methanogenic activities, the involvement of methanogens in dechlorination was conclusive in this study.

The earlier results had shown dechlorination of 2-MCP proceeded when methanogenesis was properly maintained. However, interestingly the reverse was not true. In the vancomycin batches, inhibition of 2-MCP dechlorination was also observed although it was slightly less significant than the BES batches where either sucrose or acetate was used as the carbon source. In this case, although methanogenesis was not affected, it would seem dechlorination could not be completed by methanogenesis alone. The additional CH_4 produced from acetoclastic methanogenesis in the bottles with vancomycin added implied involvement of other acetate utilizers besides the methanogens in dechlorination. A possible candidate for the acetate utilizers might be acetate-oxidizing bacteria. Dolfing and Teidje, and He *et al.* (1991; 2002) reported association of syntrophic acetate-oxidizing bacteria (AOB) and reductive dechlorinators in dechlorinating 2,5-dichlorobenzoate and tetrachloroethane. They suggested syntrophic AOB could oxidize acetate to produce the more preferable reducing equivalent H_2 for the dechlorinators. In this study, although it was found that the H_2 could be utilized as the electron donor for the dechlorination, the role of AOB was not limited to producing the reducing power. Dechlorination did not resume after H_2 was supplied. The addition of filtrate from the control batches, which performed dechlorination, into the vancomycin batches did not make the biomass to regain dechlorinating

ability. This suggested the acetate-utilizers inhibited by vancomycin were participating in dechlorination instead of just producing “growth factors” for methanogens to dechlorinate.

In general, based on the results from using the two inhibitors, it is proposed dechlorination of 2-MCP under acidic environment by the biomass cultivated in this study required the cooperation between acetate oxidizing bacteria and methanogens. In other words, the dechlorinating process is a result of syntrophism of the two groups of microorganisms.

6.5 Conclusions

In this chapter, the effects of two inhibitors, vancomycin and BES, were examined in order to clarify the role of bacteria and methanogens in dechlorination of 2-MCP under acidic environment. Methanogens were shown to be involved in the process as BES inhibited dechlorination irregardless of which carbon source or electron donor was used. However, the gram-positive bacteria inhibitor could also affect dechlorination although to a much lesser extent. This indicated dechlorination of 2-MCP needed both types of microorganisms to work properly. The results implied that the operation of an acidic reactor for dechlorination should be carefully monitored in order to maintain the proper microbial population diversity.

CHAPTER 7 16S rDNA Clone Library of The Acidic Dechlorination Community

7.1 Introduction

Typically methanogens prefer the neutral pH environment with the optimum pH range at 6.5-8.2 with the lower bound at pH 6.0 (Speece, 1996). In this study, it was observed there were methanogens surviving in a distinctly acidic environment (pH 5.5) and participating in dechlorination. The existence of these methanogens in the community led to interest to understand the diversity of methanogenic microbes therein. While it had earlier been argued there was syntrophic relation between acetate-oxidizing bacteria and methanogens in dechlorination of 2-MCP, it was still possible there were specific dechlorinating bacteria existing in the culture.

Clone library analysis based on the 16S rDNA of the microbes could offer a more in-depth understanding of the microbial community in terms of the detailed genera or species of microorganisms in the culture. Statistical analysis could help obtain information on abundance of the microbes and hence the likely dominating groups.

In this chapter, a clone library was established for the biomass cultivated in this study in order to gather relevant information on the dechlorinating culture under acidic environment at the microbial level.

7.2 Materials and methods

7.2.1 DNA extraction

Biomass sample was collected from the anSBR 1 when stable dechlorination was observed. The DNA was extracted using the automated nucleic acid extractor (MagNA Pure Compact, Roche) following the procedures described in Chapter 5. The extracted DNA was stored in a -20 °C freezer until used.

7.2.2 PCR amplification

Bacteria specific forward primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and universal reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3') (Eden *et al.*, 1991; Weisburg *et al.*, 1991) were used for amplifying the eubacteria (Bacteria library). Methanogens were amplified with two sets of archaeal forward/reverse primers, 21F (5'-TTCCGGTTGATCCYGCCGA-3')/1492R (5'-GGTTACCTTGTTACGACTT-3') (DeLong, 1992) (Archaea library 1) and 63F (5'-GAYTAAGCCATGCRAAGTC-3')/1392R (5'-ACGGGCGGTGTGTRC-3') (DeLong, 1992; Zheng *et al.*, 1996; Galkiewicz and Kellogg, 2008) (Archaea library 2), in order to cover a broader range of methanogens. A 50 µl PCR mixture was prepared with 0.25 µl *Ex Taq* polymerase (Hot Start, TaKaRa, Japan), 5 µl 10x *Ex Taq* buffer, 4 µl dNTP mixture, 1 µl of each forward and reverse primer, 5 µl extracted DNA template and 33.75 µl of deionized water (dH₂O). The touch-down PCR thermal profile was performed as follows: initial denaturation at 94 °C for 5 minutes followed by 30 cycles with denaturation at 94 °C for 30 seconds, annealing at 64 °C for 30 seconds with 1 °C every 2 cycles decrement (final annealing temperature was 50 °C), extension at 72 °C for 1 minute, followed by additional 5 cycles of regular PCR with annealing temperature at 50 °C and the final elongation at 72 °C for 10 minutes. A negative control replacing DNA template with dH₂O was carried out in the PCR reactions with each set of primers. The PCR product was then loaded in 1.2% agarose gel for electrophoresis to check success of the PCR reaction. The successful PCR amplicons should be around 1300-1400 base pairs (bp) in length.

7.2.3 Cloning of 16S rDNA , OTU assignment and sequencing

The PCR amplified product was first cleaned using Wizard® SV Gel and PCR Clean-up kit (Promega, Japan) following the instructional manual. The cleaned amplicons were ligated with the pTA2 vector from TArget Clone Plus kit (Toyobo, Japan) according to the manufacturer's protocol and the ligated vectors were transformed to DH5 α competent *Escherichia coli* cells (Toyobo, Japan) in accordance with the manufacturer's instruction (1 ml). The competent cells were then grown on Luria-Bertani (LB) agar plate with 100 μ g/ml of ampicillin at 37 °C overnight. Before the competent cells were spread on the plate, 20 μ l of 4% X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside and 20 μ l of 100 mM IPTG were applied and spread on the plates. Three plates containing 10 μ l, 30 μ l and 60 μ l of the transformed competent cells respectively were prepared for each tube of competent cells.

White colonies were picked from the plates and cultivated overnight in 2 ml of LB medium with ampicillin at 37 °C. The culture was then stored at 4 °C until plasmid was extracted. In total, 122, 98 and 148 colonies were picked for the bacteria (Bac), archaea 1 (Arc 1) and archaea 2 (Arc 2) libraries respectively. Plasmid DNA containing the inserted 16S rDNA fragments were extracted using Wizard® *Plus* SV Minipreps DNA purification kit (Promega, Japan) following the protocols in the product manual. The extracted plasmid DNA was digested with *EcoR I* restriction enzymes (Nippon Gene, Japan) at 37 °C for 4 hours in a 20 μ l reaction mixture containing 2 μ l plasmid DNA, 0.25 μ l EcoRI enzyme, 2 μ l 10x HB buffer (Nippon Gene, Japan) and 15.75 μ l dH₂O. Resultant fragments were separated in 1% agarose gel electrophoretically to determine if the recombinant plasmids contained the appropriate DNA fragment insert (positive clones).

Amplified Ribosomal DNA Restriction Analysis (ARDRA) was carried out to classify the extracted DNAs. The plasmid DNA from the identified positive clones was subjected to digestion with *Rsa I* restriction enzyme (Toyobo, Japan) at 37 °C for 4 hours. The reaction mixture contained 3 μ l plasmid DNA, 0.25 μ l *Rsa I* enzyme, 2 μ l 10x T buffer (Nippon Gene, Japan) 2 μ l 0.1% BSA and 12.75 μ l dH₂O.

Plasmid DNAs were screened according to their restriction fragment length polymorphism (RFLP) patterns. Operational taxonomic units (OTUs) were assigned according to the electrophoresis patterns of the digested plasmid DNA fragments on 2% agarose gel. Clones representing each OTU were selected for sequencing (1-5 representatives from each OTU) by Center for Biological Resources and Informatics (Tokyo Institute of Technology). The OTUs were re-assigned after the sequences were obtained if the sequence results appeared the same even if the RFLP patterns differed.

7.2.4 Sequence analysis and phylogenetic tree construction

The sequences were assembled by LaserGene-SeqMan software (DNASAR, USA). The assembled sequences were then blasted with the NCBI Basic Local Alignment Search Tool (BLAST) program with the 16S ribosomal RNA sequences (Bacteria and Archaea database) (Altschul *et al.*, 1990) and processed for the chimera check by using the Find Chimera tool (<http://decipher.cee.wisc.edu/FindChimeras.html>) in the Ribosomal Database Project II (RDP-II). Chimera sequences were then removed from the subsequent analysis. The phylogenetic tree was constructed with Mega 5 software after alignment by ClustalW (Tamura *et al.*, 2011). The phylogenetic tree was constructed with Neighbor-Join algorithm. The tree was tested with Bootstrap method for reliability of the tree.

7.3 Results and discussions

7.3.1 PCR amplification and selection of positive clones

Electrophoresis of the PCR amplicons on a 1.2% agarose gel is presented on Figure 7.1. In the graph, the bands in Lane 3, 5 and 7 represented the products from PCR amplification using primer sets 21F/1492R (Bacteria), 27F/1492R (Archaea I) and 63F/1392R (Archaea II). According to the positions of the primers, the resultant amplicons should be around 1300 - 1400 bps in length. As suggested by the electrophoresis results shown in the figure, the PCR successfully amplified targeted regions of the rDNA. The negative controls in Lane 4, 6 and 8 did not return any positive readings, which suggested that PCR amplification did not generate false positive results. Finally, in the lanes with PCR amplicons, there was no other significant band. This indicated that none specific binding due to the mismatching of primers and target genes during PCR was not significant. The smears in Lane 3 suggested the existence of impurities. Hence the purification of PCR amplicons was carried out before further processing. In conclusion, the selected primer sets and optimized PCR thermal program could achieve the goal to amplify the target rDNA for this study.

Figure 7.1 Electrophoresis of PCR products on a 1.2% agarose gel, 100 V and 30 minutes: Lane 2, DNA ladder (200 – 4000 bps, Toyobo, Japan); Lane 3, PCR amplicons with 21F/1492R primers; Lane 5, PCR amplicons with 27F/1492R; Lane 7, amplicons with 63F/1392R and Lane 4, 6, 8, negative controls

The plasmid DNA extracted was digested with the restriction enzyme *EcoR I* in order to verify the inserted DNA into the vectors was appropriate. The digested fragments were separated on the agarose gel as shown in the Figure 7.2 (electrophoretic images of all samples were presented in Appendix B). The criterion for identifying positive clones with correct inserts if the RFLP patterns were as follows: one fragment with around 3000 bps (pTA2 vector) and the other fragment with about 1500 bps (the insert with part of the vector) (e.g. Lane 2 etc.); or one fragment with around 3000 bps and two shorter fragments with total length around 1500 bps (e.g. Lane 3 etc.). Otherwise, it was defined as negative clones, which either had the incorrect insert (Lane 5) or no insert (Lane 18). The negative clones were removed from further analysis.

Figure 7.2 Electrophoresis of extracted plasmid DNA with *EcoR I* on 1% agarose gel: Lane 1 and 13, DNA ladders (200 – 4000 bps)

As a result, 123, 67 and 137 clones were identified as positive for Bac, Arc 1 and Arc 2 library respectively. The plasmid DNAs from these positive clones would be processed for further analysis.

7.3.2 Amplified Ribosomal DNA Restriction Analysis (ARDRA) classification and sequencing

The plasmid DNAs from the positive clones were subjected to digestion with *Rsa I* and the electrophoretical patterns of the digested product are shown in Figure 7.3 (images of all samples are in Appendix C). The electrophoretical patterns were compared and classified manually based on the length of the fragments. One operational taxonomic unit (OTU) was assigned if patterns were unique (e.g. one OTU was assigned to samples loaded in Lane 3, 4, 10 and 15).

Figure 7.3 Electrophoresis of plasmid DNA (partially from Arc I library) with *Rsa I* on 2% agarose gel: Lane 1, DNA ladder (200 – 4000 bps)

Based on information from the ARDRA results, 31, 6 and 8 OTUs were assigned in Bac, Arc 1 and Arc 2 libraries respectively. The number of OTUs assigned to the bacteria and archaea libraries indicated the relative diversity of the eubacteria and archaea communities. The two archaea libraries constructed in this study only contained 6 and 8 OTUs, which implied limited species of archaea were able to survive under the acidic condition. It could be expected that majority of the archaea in the reactor was methanogens and the acidic environment (pH 5.5) was not the optimum condition for them to grow (Speece, 1996). Hence, the convergence of archaea group should be expected. Whereas it was much diversified in the bacterial community as the OTUs assigned was about 4 to 5 times of the number in both archaea libraries.

Representatives were randomly selected from each OTU for sequencing. In order to ensure the manual assignment of the OTUs was appropriate, three samples from the same OTU were sequenced (if applicable) when the number of clones screened to the OTU was less than 10, otherwise 5 replicates were sequenced. In total, 65, 27 and 33 samples were selected in the Bac, Arc 1 and Arc 2 libraries. The successfully sequenced samples were 62, 25 and 31 respectively (Table 7.1).

Table 7.1 Summary of number of positive clones, OTUs and successful sequences

Library	Number of positive clones	Number of OTUs	Samples for sequencing	Successful sequencing	Clones with successful sequencing
Bac	123	32	65	62	107
Arc 1	67	6	27	25	66
Arc 2	137	8	33	31	134
Total	327	46	125	118	307

7.3.3 Phylogenetic analysis of Bacteria Library

The sequences obtained were aligned against each other and it was noted that there were identical sequences found among some of the OTUs assigned based on the ARDRA results. After re-examining the ARDRA results, it was found that the ARDRA patterns of the OTUs with the same sequence were in fact quite similar. A few bands in one of them had migrated further against the DNA ladders, which caused the conclusion of different lengths of the DNA fragments and hence assignment as a new unique OTU during the analysis. The OTUs were re-assigned based on the information from sequence alignment. The sequences were then blasted with the NCBI BLAST program. The closest matches in the database were selected and the results are presented in Table 7.2 (Bac Library), Table 7.3 (Arc 1 Library) and Table 7.4 (Arc 2 Library).

According to RDP classification, 58 of the total 107 clones were from Phylum *Firmicutes* (Bac-101, 102, 103, 104, 105, 107,108 and 110). Thirty-nine clones were from TM7, and other minority groups were from Phylum *Bacteroidetes* (Bac-112, 5 clones), *Chloroflexi* (Bac-113, 3 clones), *Proteobacteria* (Bac-109, 1 clone), and *Armatimonadetes* (Bac-111, 1 clone).

As shown in Table 7.2 and Figure 7.4, three major groups of bacteria were identified. Bac-106 related with Phylum TM7 sp. (36.5%), Bac-105 was close to *Olsenella* sp (28%). and Bac-101 (14%) was found to be related to an unknown bacterium found in trichlorobenzene-transforming microbial consortium (von Wintzingerode *et al.*, 1999).

The largest group of bacteria was related to the Phylum TM7. Members of TM7 have been reported as widely distributed in the environment from anaerobic digester (Godon *et al.*, 1997), forest soil (Borneman and Triplett, 1997) activated sludge process (Bond *et al.*, 1995) to oral cavities (Bik *et al.*, 2010). However the group lacks cultivatable representatives so far and the phylum is exclusively characterized by culture-independent techniques from environmental sequence data (Hugenholtz *et al.*, 1998). TM7 bacterium was found to be involved in the degradation of glucose and other hydrocarbons (Ariesyady *et al.*, 2007; Luo *et al.*, 2009). More interestingly, members from TM7 Phylum have been detected at chlorinated solvent contaminated locations such as TCE and *cis*-DCE-contaminated sites (Lowe *et al.*, 2002). Cannon *et al.* (2005) reported TM7 bacterium might use propane or metabolites of propane oxidation. They found the sharp drop of TCE and *cis*-DCE correlated with propane sparging and suggested TM7 members might be co-oxidizing TCE and *cis*-DCE when utilizing propane. However they did not rule out other propane utilizers' role in removing the contaminants. In this study, Phylum TM7 sp. might have become one of the dominant bacteria groups because of the primary substrate used, sucrose. Based on information from the literature, the species could have actively consumed sucrose and other hydrocarbons. Unfortunately, due to the lack of information about the isolates or cultivated pure culture of members from this phylum, it was difficult to identify their role in dechlorination of chlorophenols in this study.

Clones in OTU Bac-105 belonged to Genus *Olsenella*, which was reported as a glucose-degrader in the anaerobic digester (Dewhirst *et al.*, 2001; Nakamura *et al.*, 2003; Ito *et al.*, 2012). Fifteen clones (Bac-101) were close to the uncultured bacterium found in chlorobenzene degrading consortium. However, the author had

identified another dechlorinator, *Dehalobacter* species, and it was believed that this species were not involved in the dechlorination of trichlorobenzene (von Wintzingerode *et al.*, 1999). Clones in Bac-104 were close to a carbon monoxide-utilizing acetogen from the *Eubacterium* genus. The strain could produce acetate, butyrate and ethanol using CO as the sole carbon source (Roh *et al.*, 2011). The OTU Bac-112 (5 clones, 4.8%) affiliated with the an uncultured bacterium from the Phylum *Bacteroidetes* was reported to play an important role in long chain fatty acids degradation in the mesophilic methanogenic reactor (Shigematsu *et al.*, 2006). Other minor sequences found in this Bacteria Library could not match any defined species from the NCBI database, but they matched uncultured bacteria found in environmental samples or from anaerobic systems.

Most importantly, from the sequences obtained from this Bacteria Library and compared with database available, although some of the clones were close to some uncultured bacteria in the environment contaminated with chlorinated compounds, it was believed that these clones was not degrading any chlorinated compounds based on the information available. Hence there were no well-defined dechlorinators of chlorophenols or other chlorinated compounds detected in this study. As discussed in the literature review in Chapter 2, those dechlorinating bacteria belonged to very limited genera, *Desulfitobacterium*, *Anaeromyxobacter*, *Desulfomonile* and *Deesulfobibrio* (Itoh *et al.*, 2011). Meanwhile the pH range for them to perform the dechlorinating activities was normally above 6.0. In this project, the anSBR was running at pH 5.5 for more than 2 years, the low pH value might be the reason that the dechlorinating bacteria were not growing in the system. On the other hand, the absence of dechlorinating bacteria could be evidence supporting the argument that dechlorination of chlorophenols in this study was through a co-metabolism mechanism.

Table 7.2 Operational taxonomic units of Bacteria Library and their closest matches

OTU	Closest Match in BLAST	Accession Number	Similarity (%)	No. of Clones	Abundance (%)
Bac-101	Uncultured bacterium	AJ009486	96	15	14.0
Bac-102	<i>Propionispora hippie</i>	AJ508928	100	2	1.9
Bac-103	Uncultured <i>Veillonellaceae</i> bacterium	JX505385	94	1	0.9
Bac-104	<i>Eubacterium</i> sp.	DQ337524	93	7	6.5
Bac-105	Uncultured <i>Olsenella</i> sp.	DQ168843	99	30	28.0
Bac-106	TM7 phylum sp.	AY349415	97	39	36.5
Bac-107	<i>Pectinatus portalensis</i> strain B6	NR_042900	99	1	0.9
Bac-108	<i>Catabacter hongkongensis</i>	AB671763	100	1	0.9
Bac-109	Uncultured bacterium	AB364740	97	1	0.9
Bac-110	Uncultured bacterium	HQ716176	92	1	0.9
Bac-111	Uncultured bacterium	AJ009501	95	1	0.9
Bac-112	Uncultured bacterium	AB244311	96	5	4.8
Bac-113	<i>Bacterium</i> K-4b6	AF524858	97	3	2.8

Figure 7.4 Phylogenetic tree of Bacteria Library

7.3.4 Phylogenetic analysis of Archaea 1 and 2 Library

In the Archaea 1 Library, 41 clones in a total of 66 (Arc-101, 102, 103 and 104) were identified as methanogenic archaea, which consisted about 54.5% of the total clones. The rest (Arc-105) were close to uncultured archaeon other than methanogens (Table 7.3 and Figure 7.5). The uncultured archaeon was affiliated with the genus *Thermogymnomonas*. This genus is usually found in spring or river sediments as well as anaerobic sludge (Yashiro *et al.*, 2011; Bandara *et al.*, 2012). The results revealed that there was significant amount of archaea, which were not methanogens in the system.

Among the methanogens, 33 out of 41 clones belonged to the genus *Methanobacterium* (Arc-101 and 102), a genus of the order *Metahnobacteriales* (Mbt), which are the H₂-utilizing methanogens. The results suggested Mbt was the dominant methanogens in the sludge while only about 20% of the methanogens were from *Methanosarcina* genus (Arc-103 and 104). The abundance calculated based on the number of clones here indeed agreed with the qPCR results discussed in Chapter 5. Both results proved that Mbt was the major group of methanogens in the sludge. The recycling of the produced biogas might be an important factor to promote the growth of Mbt since the operation offered more chances for this hydrogenotrophic methanogens to utilize the H₂ and CO₂. Another reason might be due to the low pH at which the reactor was operated. Shimada *et al.* (2011) reported that acetoclastic methanogens (order *Methanosarcinales*) were not maintained at the low pH condition (5.2 – 5.5) while methane was produced by the hydrogen-utilizers in the order *Methanobacteriales*.

Arc-103 and Arc-104 were related to *Methanosarcina* sp. strains. Several authors had reported the involvement of *Methanosarcina* sp. species in dechlorinating tetrachloroethylene (PEC) (Fathepure and Boyd, 1988), transforming of chloroform (Mikesell and Boyd, 1990; Yu and Smith, 1997) and degrading chloroethane to ethane (Holliger *et al.*, 1990). The examples demonstrated the capabilities of *Methanosarcina* strains to conduct co-metabolic dechlorination. Although the process was mainly associated with the alkyl halide, it may be also offering the

measures for dechlorinating aryl chlorinated compounds as co-metabolic activities are non-specific reactions (Holliger and Schraa, 1994). During the inhibition period described in Chapter 5, it was found that the *Methanosarcinaceae* family was the member in the system most affected as copy number of the family reduced by over 1000 times.

In addition, it was noticed that the diversity of methanogens was quite limited. Only 4 OTUs were identified in total. The low pH environment limited growth of the methanogens. As a result, only a few phenotypes of the methanogens could be found in the culture. Information on the types of methanogens, which could survive under the acidic condition and dechlorinate is important. The information could serve as the indicator in operating reactors to dechlorinate chlorophenols at low pH. If the dechlorinating methanogens could be identified, the existence of the particular methanogens may suggest the potential of the anaerobic sludge to conduct dechlorination. Meanwhile, the efforts could be made to isolate the particular methanogens for further investigation of the role of methanogens in the dechlorinating process. One more advantage of the convergent methanogens community was to prevent the competition for electron donors between non-dechlorinating methanogens and the dechlorinators. As suggested in the literature review, the existence of methanogens could compete for H_2 with the dechlorinators, which cause insufficient electron donors for dechlorination. In the situation, like this study, the limited diversity of methanogenic community prevented the growth of methanogens not participating in the dechlorinating process. In this case, H_2 would not be withdrawn from the dechlorinating process.

Table 7.3 Operational taxonomic units of Archaea 1 Library and their closest matches

OTU	Closest Match in BLAST	Accession Number	Similarity (%)	No. of Clones	Abundance (%)
Arc-101	<i>Methanobacterium formicicum</i>	AY196659	98	31	47.0
Arc-102	<i>Methanobacterium beijingense</i>	AY552778	98	2	4.5
Arc-103	<i>Methanosarcina barkeri</i> strain DSM 800	NR_025303	99	1	1.5
Arc-104	<i>Methanosarcina barkeri</i> strain CM1	AJ002476	99	7	1.5
Arc-105	Uncultured archaeon	JN398040	98	30	45.5

Figure 7.5 Phylogenetic tree of Archaea 1 Library

Similar to the findings in Archaea Library 1, only 4 OTUs were defined in Archaea Library 2 as illustrated in Table 7.4. The dominant group was close to the *Methanobacterium* genus (Arc-201), which consisted of 97.8% of the total clones (131 out 134 clones). One OTU was related to the uncultured archaeon from environment sample (Arc-202), one OTU was the *Methanoregula boonei* (Arc-203) and the other OTU was found to be a bacterium (Arc-204). Since majority of the clones shared the same OTU, it implied that the primer set selected was in fact biased towards certain species during PCR amplification. The presence of the *Bacteroides* sp. meanwhile suggested that unspecific bindings occurred in the PCR reaction, which meant the primers could amplify certain bacteria groups as well. Hence construction of the Archaea 2 library was considered as failed. Nevertheless, a novel acidiphilic methanogen, *Methanoregula boonei*, was identified in the library

(Bräuer *et al.*, 2006). This suggested long term operation of the reactor at low pH selected a methanogenic population that adapted to the acidic environment.

Table 7.4 Operational taxonomic units of Archaea 2 Library and their closest matches

OTU	Closest Match in BLAST	Accession Number	Similarity (%)	No. of Clones	Abundance (%)
Arc-201	<i>Methanobacterium formicicum</i>	AY196659	98	131	97.8
Arc-202	Uncultured archaeon	AY350742	99	1	0.8
Arc-203	<i>Methanoregula boonei</i> (T) 6A8	NR_025303	99	1	0.8
Arc-204	Uncultured Bacteroides sp	AJ002476	99	1	0.8

7.4 Conclusions

The clone libraries based on the 16S rDNA of bacteria and archaea was constructed in this chapter and used to perform phylogenetic analysis.

In the Bacteria Library, sequences of the clones were generally anaerobic bacteria including glucose-degrading, hydrocarbon-degrading, long chain fatty acids degrading species and acetogens. Dechlorinating bacteria were not found in the library. The low pH environment of the reactor might not be suitable for enriching these dechlorinating bacteria, which normally work around the neutral pH. The results suggested that dechlorination of chlorophenols under acidic condition was more likely conducted through the co-metabolism process.

In both Archaea Libraries, it was found very limited phenotypes of methanogens existing in the sludge although primers used in Archaea 2 Library was not suitable due to the bias and unspecific binding issues.

In Archaea 1 Library, the majority of the methanogens belonged to *Methanobacterium* genus of the *Methanobacteriales* order, which agreed with the qPCR results. Other methanogens were related to the *Methanosarcina barkeri* strains. The *Methanosarcina* sp. or strains have been reported to be involved in dechlorinating chlorinated compounds including TCE, chloroform and chloroethane

through the co-metabolism process. Considering the findings in the previous chapter (Chapter 6) that suggested the involvement of methanogens in dechlorinating 2-MCP in this study, the presence of *Methanosarcina barkeri* provided the evidence for the hypothesis.

CHAPTER 8 Conclusions and Recommendations

8.1 Major findings

8.1.1 Limited reductive dechlorination of chlorophenols under acidic condition

In this study, it was shown dechlorination of chlorophenols was feasible under the acidic condition following a proper inducing process, which included step-wise reduction of pH during the start-up period. The process required a long acclimation period (around 80 days after pH was reduced to 5.5) to accumulate sufficient dechlorinating organisms. Acclimated sludge showing capabilities to dechlorinate 2-MCP did not experience any lag phase when challenged with higher chlorinated phenols. In this study, 2,4,6-TCP and PCP were tested. Both of the compounds followed the *ortho*-dechlorinating pathway with 4-MCP and 3,4,5-TCP as the end product respectively.

Only *ortho*-dechlorination was observed in this project. Efforts were made to induce *meta*- or *para*-dechlorination, including using seed sludge from a different source, pH adjustment and different inducers while excluding the readily-degradable 2-MCP. However, all the efforts failed. From the literature, it was found the optimum pH for dechlorinating bacteria capable of *meta*- or *para*-dechlorination was around neutral or slightly higher. The low pH environment in this study could have prevented cultivation of *meta*- or *para*-dechlorinating populations.

Interestingly, sludge could readily dechlorinate 2-MCP after exposure to 3-MCP and 4-MCP simultaneously for about 140 days even if it did not degrade 3-MCP or 4-MCP.

Summaries of the chlorophenols that were tested in this project are presented in Table 8.1 with their respective dechlorinating pathway.

Table 8.1 Dechlorination of chlorophenols under acidic condition (pH 5.5)

Compounds	Acclimating process	Lag phase (days)	Pathway	Removal due to adsorption	Maximum loading attempted (μmoles/day·L)
2-MCP	Stepwise reduction of pH	80	2-MCP → phenol	Negligible	200
2,4,6-TCP	Acclimated to low pH and showing dechlorination of 2-MCP	0	2,4,6-TCP → 2,4-DCP → 4-MCP	Negligible	Not determined
PCP	Acclimated to low pH and showing dechlorination of 2-MCP	0	PCP → 2,3,4,5-TeCP → 3,4,5-TCP	88% if dechlorination inhibited	10
3-MCP	Stepwise reduction of pH Acclimation with 3-/4-MCP	> 300	No degradation	40 – 50%	50
4-MCP	Stepwise reduction of pH Acclimation with 3-/4-MCP	> 300	No degradation	35 – 40%	50

8.1.2 Dechlorination inhibited with inhibition of methanogenesis

Although the anaerobic sequencing batch reactor was operated at pH 5.5 for more than 2 years, the stepwise reduction of pH, long SRT (> 30 days) and re-circulation of biogas helped to preserve methanogens in the system. When the system was stabilized, maximum 60% of CH₄ could be detected in produced biogas.

During the period of inhibition by overloading with PCP, it was found that dechlorination of PCP completely ceased when methanogenesis was severely inhibited. The CH₄ composition in the biogas reduced to below 10%. But fermentation of sucrose was still found to proceed as the COD in the effluent was equal to the COD values of the VFAs detected in the effluent. During the inhibition period, about 88% of the PCP could be removed through biosorption by the sludge. A similar phenomenon was observed when pH was dropped below 2.0. In that case, dechlorination of 2-MCP was inhibited completely and CH₄ could not be detected.

In both cases, acidogenesis of sucrose did proceed as per normal while the conversion of longer VFAs to acetate and methanogenesis was affected. qPCR analysis also suggested the *Methanosarcinaceae* family was the mostly affected group. In both inhibition events, the copy number of the family reduced by over 1000 times and was below 0.01% among the total methanogens. These results suggested involvement of methanogens in dechlorination of chlorophenols under acidic environment.

8.1.3 Evidence of syntrophism in dechlorination of 2-MCP

Methanogenic specific inhibitor BES and gram-positive antibiotic vancomycin were used to investigate the role of methanogens and bacteria in dechlorination under acidic condition. The inhibitory effect of BES on dechlorination of 2-MCP was consistent no matter which electron donor (sucrose, acetate or H₂) was used. The involvement of methanogens in dechlorination could be conclusive in this case. However, in the vancomycin batches, dechlorination of 2-MCP was inhibited but to a lesser extent compared with the BES batches while methane production was not affected. This phenomenon led to the argument that dechlorination of 2-MCP could

not be completed by methanogens solely. It was possible there was cooperation between two different groups of microorganisms. More acetate was directed to methanogenesis from other acetate consuming pathways, which suggested the possible candidate working together with the methanogens was an acetate-oxidizing bacteria (including homoacetogens).

8.1.4 Evidence of cometabolic process for dechlorination

With the addition of external carbon sources, dechlorination under acidic condition was found to be more robust and the process was enhanced. Meanwhile, lack of dechlorinating bacteria identified in the community suggested the dechlorination under acidic condition was a cometabolic process. The syntrophism between methanogens and acetate-oxidizing bacteria degraded the chlorophenols.

8.2 Conclusions and Implications

This study has suggested the possible involvement of two groups of microorganisms in degrading chlorophenols under acidic condition through a syntrophic cometabolism. It is then proposed that acidogenic bacteria ferment the organic carbons to generate H_2 as the electron donors, which could be utilized for the dechlorination by syntrophic cooperation between methanogens and acetate-oxidizing bacteria. This proposed mechanism for dechlorination under acidic environment would then require the proper maintenance of the microbial community to include all groups of microorganisms involved in the process. As a result, the dechlorination reactor developed in this study was not a phase-separated acidogenic reactor. The process included acidogenesis and methanogenesis in the same reactor and it became an acidic single-stage anaerobic reactor. However, the acidic anaerobic system is different from the conventional anaerobic digester in terms of the methanogens present as only a few phenotypes could be retained in the reactor. The methanogens adapted to the toxic chlorophenols and the low pH environment could stabilize the whole process as the methanogens could remove the fermentative metabolites effectively and itself could be a participant in the dechlorination. The limited divergence of the methanogenic community would also prevent the unnecessary competition for electron donors between methanogenesis

and dechlorination. The acidic anaerobic sludge reactor would not require the extensive adjustment of pH of the influent wastewater if the wastewater was acidic. At the same time, partial dechlorination limited to removal of *ortho*-chlorine suggested the technique could possibly only serve as a well-defined pretreatment process. Downstream processes would be required to address other chlorophenols and metabolites generated from *ortho*-dechlorination.

8.3 Recommendations

In this project, it was found feasible to dechlorinate chlorophenols in an acidic reactor with the involvement of the syntrophism between acetate-oxidation bacteria and methanogens. The acidic reactor is defined as the acidic single-stage process involving acidogenic phase and methanogenic phase. This is different from the existing conclusions about dechlorination of chlorophenols by acidogenic sludge in a phase-separated acidogenic reactor (Mun *et al.*, 2008a; Mun *et al.*, 2008b; Mun *et al.*, 2008c). Although the author of this project proposes the feasibility of dechlorination under acidic condition, it is recommended to review the current acidogenic dechlorination process claimed earlier. Firstly, the acidogenic dechlorination process should be re-investigated to study the dechlorination without participation of other anaerobes. With the evidence from this project, it is suggested the acidogenic phase could not complete the dechlorination of chlorophenols. Secondly, the efficiency and stability in terms of degrading chlorophenols in acidic single-stage process and acidogenic process should be studied. The dechlorinators involved in the different process could be different. Their dechlorination capabilities and stabilities will be the one of main criteria for selection of treatment process. And lastly, the reasons and protocols to develop the different dechlorination systems under acidic condition should be understood.

Besides that, another question had remained unanswered in the project. The proposed syntrophism in dechlorination is based on tests with inhibitors. Although it was possible neither the methanogens nor the bacteria should be inhibited, this need to be further investigated and confirmed. The role of each group of microorganisms is not well defined. The question about whether the dechlorination

is coupled with acetate-oxidation or methanogenesis should be studied. In other words, the question regarding which microorganism is the “dechlorinator” and which the “supporter” needs to be answered. The knowledge will be important for optimization of the process. There is possibly complexity in the processes as the syntrophic acetate-oxidizing bacteria (homeacetogens) could perform the reverse of acetate oxidation to grow on H_2/CO_2 to produce acetate. The direction of the reaction depends on the biochemical condition the bacteria are involved in. In order to determine the roles of acetate-oxidation bacteria and methanogens, stable isotope technique could be applied. The isotope-labeled substrates, chlorophenols and carbon source, could be helpful to trace the metabolites from the substrate degradation and useful to elucidate the interactions between different metabolism pathways. Recent development of RNA-based stable isotope probing technique, which involves the incorporation of stable isotope labeled substrate into cellular biomarkers, could be used to identify organisms assimilating the particular substrate (Manefield *et al.*, 2002). The roles of acetate-oxidation bacteria and methanogens involved in the dechlorination process could then be addressed with more direct evidence.

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Appendix A

Calculation of molecular fraction of PCP as a function of pH

At equilibrium,

$$K_a = \frac{[H^+][PCP^-]}{[PCP]} \quad 2$$

Take $\log$ on both sides of the equation,

$$-\log(K_a) = -\log\left(\frac{[H^+][PCP^-]}{[PCP]}\right) \quad 3$$

$$pK_a = \log([PCP]) - \log([H^+][PCP^-])$$

$$pK_a = \log([PCP]) - \log([H^+]) - \log([PCP^-])$$

$$pK_a = \log\left(\frac{[PCP]}{[PCP^-]}\right) + pH$$

Re-arrange the equation,

$$\frac{[PCP]}{[PCP^-]} = 10^{pK_a - pH} \quad 4$$

And take consideration,

$pK_a = 4.81$ and $[PCP] + [PCP^-] = [PCP]_{\text{total}}$ and substitute into Equation 4

$$\frac{[PCP^-]}{[PCP]} = \frac{1}{1 + 10^{pH - pK_a}}$$

Appendix B

Electrophoresis patterns of digestion with *EcoR I* for positive clone identification

Appendix C

Restriction fragment length polymorphism electrophoresis pattern with *Rsa I*