REDUCTIVE DEHALOGENATION OF CHLOROPHENOLS BY ANAEROBIC MICROBIAL CONSORTIA

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A THESIS SUBMITTED FOR

THE DEGREE OF MASTER OF ENGINEERING

DIVISION OF ENVIRONMENTAL SCIENCE

AND ENGINEERING

NATIONAL UNIVERSITY OF SINGAPORE

2009

ACKNOWLEDGEMENTS

It was only this afternoon that Dr. He Jianzhong returned me my first complete draft of this dissertation and I am nothing short of embarrassed with the countless scribbles and circles she has penciled down upon the manuscript, identifying the errors and asking me to clarify and justify the statements I have made. Goodness knows how many more silly mistakes and juvenile errors may lurk and dwell within these pages yet, but it is thanks to her and all those I am about to mention that there are not many hundreds more. I simply cannot begin to thank adequately those who helped me in the preparation of this but I shall try all the same.

To Dr. He, I am especially indebted to you for having faith in me, for taking me under your wings and for guiding me to where I am today since day one, or rather, since $t = 0$.

To Dr. Liang Dawei and Dr. Chow Wai Ling, who were both equally generous and displayed the most heroic reserves of patience in answering my one simple, endlessly repeated question: "I am sorry but can you explain that to me again?"

For the good times, the bad times, to my fellow peers, colleagues, friends in the laboratory – Cheng Dan, Caian, Jason, Sandhi, Shiva, Shanquan, Ding Chang, Dave and Sidek, thanks for your help, thanks for the camaraderie, thanks for the memories.

Above all and always, my most profound gratitude to my incomparable parents back home in Penang.

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SUMMARY

The pressures of an escalating population growth and industrial advancement have led to the addition of a large variety of different xenobiotic compounds into the environment. Public concern about the possible hazardous effects of these chemicals on humans and the environment has focused largely on a few classes of compounds. Of these compounds, chlorophenols are one of the most publicized. Chlorophenols are recognized to be carcinogenic to rats, potentially carcinogenic to humans and are especially resistant to degradation due to the stability induced by their chlorine substituents. However, anaerobic microorganisms can sequentially remove these chlorine constituents from these compounds through the process of *reductive dehalogenation*, which renders them more amenable to subsequent aerobic degradation and ultimate mineralization. These microorganisms are able to utilize halogenated compounds for energy synthesis by coupling reductive dehalogenation to energy metabolism.

In this research, 20 samples from both natural (i.e. soils and sediments) and engineered (i.e. sludge from treatment plants) systems were collected from various locations in Singapore, China, Malaysia and Indonesia and were used as inocula for studies on their capability to dechlorinate pentachlorophenol (PCP) and 2,4,6-trichlorophenol (2,4,6-TCP). Of the 20 samples, only a bacterial consortium, D12, grown in defined medium and pyruvate as the carbon source exhibited the capability of dechlorinating PCP to 4-chlorophenol (4-CP). PCP was completely *meta*-dechlorinated to 2,4,6-TCP which was then further dechlorinated to (2,4-dichlorophenol) 2,4-DCP and finally 4-CP as the final dechlorination product. On the other hand, under similar conditions, all of the samples demonstrated the ability to dechlorinate 2,4,6-TCP. However, the extent which 2,4,6-TCP

was dechlorinated varied - with some being able to completely dechlorinate 2,4,6-TCP while others could not. The rates with which 2,4,6-TCP were converted also differed from one microcosm to another. Nevertheless, the dechlorination pathways for all 20 microcosms were observed to be identical. Only the *ortho* chlorine atoms from 2,4,6-TCP were removed to generate 4-CP as the end product via 2,4-DCP.

3 of the 2,4,6-TCP dechlorinating cultures, designated LWN, RIV and SC, were selected for further studies due to their capabilities to completely and rapidly dechlorinate 2,4,6-TCP. Both cultures LWN and RIV completely dechlorinated 2,4,6-TCP to 4-CP in 5 days while culture SC took 12 days. Cultures LWN, RIV and SC were then tested for the presence of possible dehalogenators within the 3 bacterial consortia. A common chlorophenol-dechlorinating bacterium from the genus *Desulfitobacterium* was discovered in culture RIV while culture SC contained *Dehalococcoides*-like bacteria, which was never reported to have been able to completely dechlorinate 2,4,6-TCP. DNA sequencing results showed an even more interesting finding with the predominance of *Sedimentibacter*-like bacteria in culture LWN since *Sedimentibacter* have never been previously shown to dehalogenate any form of halogenated compounds.

Halogenated compounds other than chlorophenols were also subjected to reductive dechlorination by cultures LWN, RIV and SC. Extensive debromination of polybrominated diphenyl ethers (PBDE) was shown to be possible by culture RIV. Meanwhile, culture SC has also shown a broad range of dehalogenating potential as it successfully dechlorinated trichloroethene (TCE) to *cis* and *trans*-dichloroethene (DCE).

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CHAPTER 1

INTRODUCTION

1.1 Background

1.1.1 Halogenated Organic Compounds

The ever increasing pressures of an escalating population growth and industrial development have led to the addition of an array of manmade chemicals into the environment. Halogenated organic compounds constitute one of the largest groups of environmental chemicals and are made up of the following 2 classes:

i. Aliphatic (e.g. chlorinated ethenes)
ii. Aromatic (e.g. chlorinated phenols, polybrominated diphenyl ethers, chlorinated biphenyls).

Their use and misuse in the industry and agriculture represent a large entry of these chemicals into the environment, resulting in widespread dissemination and oftentimes detrimental conditions especially to the environment. The ability of halogenated organic compounds, to impart toxicity, bioconcentrate and persist and subsequently, their ubiquitous distribution into the biosphere has caused a major concern over their potential effects on the quality of life.

1.1.2 Chemistry of Halogenated Organic Compounds

In many respect, the chemistry of halogenated organic compounds is due to the unique physiochemical properties of their halogen substituent (F, Cl, Br, or I) (Haggblom and Bossert, 2003). At the start of the series, the carbon-fluorine bond is very strong with high polarity. With increasing molecular weight of the halogen, carbon-halogen bond energies decrease markedly, i.e. F > Cl > Br > I. Other characteristics, such as the electron-withdrawing effect on the halogen substituent impact chemical reactivity of the molecule and its heat transfer and dielectric properties. The physical size and shape of the halogen substituent may also affect reactivity, due to steric constraints and may also hinder uptake into cells and enzymatic attack during biodegradation. In addition, the halogen moiety of an organic compound generally reduces its water solubility and conversely increases lipid solubility. The biological consequence of increased lipophilicity may be reduced biodegradation due to decreased bioavailability, and/or biomagnifications in the food chain as the non-degraded haloorganic compounds sequester in the fatty tissues of higher animals.

In halogenated aromatic compounds, biodegradability of depends on the number and position of substituents on the aromatic ring. As example, chlorophenols are found to be less readily biodegradable than phenol and their rate of biodegradation decreases with increasing numbers of chlorine substituents on the aromatic ring. In terms of the position of the chlorine (halogen), it has also been proposed that the relative order of biodegradability for chlorophenols was found to be *ortho* > *meta* > *para* in aquifer sediments and in digested but in the order of *ortho* > *para* > *meta* in anoxic natural marine sediments and in soil (Annachhatre and Gheewala, 1996).

Finally, the halogen substituent and its potential organohalide metabolites may alter, oftentimes increasing, the inherent toxicity of the molecule (Haggblom and Bossert, 2003). Although microorganisms can adapt to remove many toxic substances when these compounds are fed into similar and relevant pathways that are already present for the biodegradation of natural compounds, the great variety of halogenated organic compounds used today may disrupt the balance of the ecosystem. Microorganisms are challenged to develop new pathways by altering their own preexisting genetic information as a result of either mutation(s) in single structural and/or regulatory genes or perhaps recruitment of single silent genes when the encounter these compounds. One should recognize that it may take microorganisms a long time to acquire the ability to degrade all the new synthetic chemicals introduced into the environment by modern technology. In future, it will be necessary to develop microbial systems that can speed the evolution of degradation traits since the naturally existing microbial systems cannot cope with the high and rapid influx of the numerous and new kinds of anthropogenic halogenated organic compounds (Chaudry and Chapalamadugu, 1991).

Resistance to both chemical and biological degradation is one of the qualities that has made many organohalides useful in industrial applications (see section 1.1.3 for more information) but it is also the reason for many of the environmental problems related to the use of these compounds. The persistence of organohalides in the environment varies from days to several decades, depending on the chemical structure and the prevailing environmental conditions and can play a major role in their overall global impact.

1.1.3 Uses of Halogenated Organic Compounds

The discovery of chlorine and other halogens and the elucidation of their unique chemistry were followed by their synthesis and large-scale industrial production and application. The scale of production (past and present) of these organohalides has had direct implications for their occurrence and fate in the global environment. Organohalides are integral to a variety of applications, including use as solvents, degreasing agents, biocides, pharmaceuticals, plasticizers, hydraulic and hear transfer fluids, intermediates for chemical synthesis and numerous other industrial functions. Other halogenated compounds are produced as by-products during combustion, chlorine bleaching of pulp or disinfection of water and wastewater. As a result, many halogenated organic compounds, including aliphatic, aromatic and heterocyclic derivatives have been produced and used in the vast quantities over the last 50 to 80 years. The majority of these compounds are chlorinated, but brominated, fluorinated and iodinated compounds also have industrial applications (Stringer and Johnston, 2001).

1.1.4 Fates of Halogenated Organic Compounds in the Environment

Introduction of industrial halogenated compounds into the environment occurs through terrestrial, aquatic and atmospheric discharges. Therefore, their impact is on all major environmental compartments, i.e. soils sediments, water and air. Depending on their ultimate fate, organohalides may be degraded to harmless byproducts or they may exert harmful effects through toxicity, biomagnification and/or persistence in the environment. Their harmful impact on the biota may be direct, i.e. toxicity, or indirect, such as by

destruction of the protective ozone layer in the stratosphere by atmospheric halocarbons. Owing in part to their often xenobiotic origin and persistent character, many industrial organohalides are resistant to biodegradation, and therefore accumulate and exert their harmful effects in the environment (Haggblom and Bossert, 2003).

1.1.5 Strategies for the Treatment of Halogenated Organic Pollutants

The biological treatment of halogenated organic pollutants is possibly the most economical and efficient treatment technology available for use by environmental engineers. The biological treatment can be either aerobic or anaerobic or combination thereof. These processes have effectively demonstrated their capability in the treatment and removal of halogenated organic compounds (Chaudhry and Chapalamadugu, 1991).

The aerobic process is more effective in degrading halogenated organics with a low degree of chlorination (i.e. 3 or less halogen substituent) as these halogenated organics are more reduced in their oxidation state (Figure 1.1). It has been reported aerobic degradation of the lower order halogenated compounds is mainly due to co-metabolic reaction in which the halogenated compounds were fortuitously dechlorinated during metabolism of the primary organics (Haggblom and Bossert, 2003). The oxygenase enzyme systems in the aerobic system were often responsible for these fortuitous degradation (Haggblom and Bossert, 2003). However, these enzymes were only induced by the primary organics and the halogenated compounds had to often compete with the former, leading to a reduction in the degradation of the halogenated compounds.

Today, anaerobic biotreatment is one of the most widely used biological processes especially for the treatment of industrial wastewaters containing both primary and highly halogenated organics (Speece, 1996). The preference for anaerobic biotreatment is because the process can be very cost competitive in terms of its lower sludge handling and lower energy requirements compared to the aerobic process. An end-product of the anaerobic process, CH_4 can also be used as fuel for the generation of electricity and hence supplementing the energy needs of the treatment plant.

In addition to that, for halogenated compounds with 3 or more halo-functional groups, anaerobic treatment is preferred since highly halogenated compounds such as 2,4,6-trichlorophenol and pentachlorophenol are more reactive in a reductive environment. Due to the oxidized nature of highly halogenated organic compounds, these compounds are more susceptible to dehalogenation in the anaerobic environment (Figure 1.1) (Armenante et al., 1999). And as such, applying strategies involving a highly energy intensive oxidative process (i.e. aerobic) to treat such compounds is perhaps not the best measure.

Reductive dehalogenation, the essential and predominant process in the anaerobic transformation of halogenated compounds, is briefly introduced in the following section.

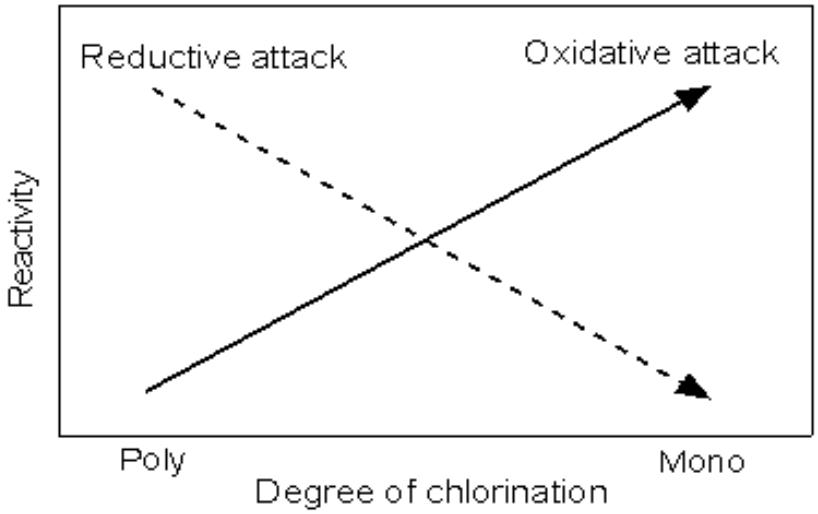


Figure 1.1: Relative trends of oxidative and reductive dechlorination as a function of dehalogenation.

1.1.6 Reductive Dehalogenation by Anaerobic Bacteria

Degradation of halogenated compounds under anoxic conditions was first studied in the 1950s and the 1960s when the fate of halogenated pesticides in agricultural soils was investigated (Allan, 1955; Guenzi and Beard, 1967). Only 15 or 20 years later, the anaerobic degradation of halogenated compounds has become a matter of special concern due to the almost ubiquitous presence of chlorinated compounds that resist aerobic degradation, such as tetrachloroethene and polychlorinated biphenyls, are transformed under anoxic conditions be reductive reactions (Parsons et al., 1984; Quensen et al., 1988).

Reductive dehalogenation reactions have a large potential for application in treatment processes for materials contaminated with halogenated compounds such as industrial wastes, soils, sediments and groundwater. Aerobically persistent polyhalogenated

compounds can be transformed by anaerobic mechanisms into harmless compounds that are further degradable by aerobic microorganisms (Holliger and Schraa, 1994).

Reductive dehalogenation under anoxic conditions has been reported for many different compounds such as chlorobenzoates, chlorophenols, chlorobenzenes, chloromethanes, chloroethanes and chloroethenes. Although abiotic processes might be involved in some of the reductive transformations observed in environmental samples, recent evidence has been presented to show that the majority of these reactions are biologically catalyzed. Several reviews are available that summarize the knowledge of reductive dehalogenations catalyzed by mixed and pure cultures (Mohn and Tiedje, 1992; El Fantroussi et al., 1998; Holliger et al., 1999). Long acclimation periods, substrate specificity, high dehalogenation rates and the possibility to enrich for the dehalogenation activity by sub-cultivation in media containing a selective organic or inorganic electron donor indicate that many of the reductive dehalogenation activities in the environment are catalyzed by specific bacteria (Holliger and Schumacher, 1994). Despite the strong evidence for the involvement of biological processes in reductive dehalogenation processes, a biological activity can be unambiguously assigned only to a few of the reductive dehalogenation reactions observed in environmental samples. The availability of pure cultures catalyzing reductive dehalogenations allows for more detailed investigations on the metabolic function of these types of reactions.

1.2 Problem Statement

From the previous sections, it is clear that the anaerobic reductive dehalogenation is the preferred treatment method for halogenated organic pollutants. However, studies of

reductive dehalogenation of this particular compound have been limited. Most of these studies have used mixed cultures and not many stable enrichments or pure cultures of dehalogenating anaerobes are known to exist. Only a few microbes capable of reductive dehalogenation have been documented and isolated as pure cultures. Even so, most isolates as well as enriched mixed cultures have shown only partial or incomplete dehalogenation of these compounds. Some of these pure cultures isolated require a co-metabolic process in order for reductive dehalogenation to take place. Furthermore, many of these cultures were grown in undefined medium (i.e. require yeast extract for growth).

The severity of the problems halogenated organic compounds can potentially pose to the environment coupled with the lack of knowledge pertaining to the biotreatment of these compounds, it is therefore crucial that more studies on reductive dehalogenation are carried out in an attempt to give both engineers and scientists alike a better and more profound understanding in this area.

1.3 Objectives

Using chlorophenols as the representative of halogenated organic compounds, the overall goal of this study is to cultivate and characterize novel anaerobic chlorophenol-dechlorinating cultures in order to develop an advanced understanding of the reductive dehalogenation process and the microbes involved as well as their correlation with each other.

Specific objectives of this study are:

i. To optimize analytical methods as well as to increase detection and measurement sensitivity of chlorophenols and the potential degradation products.

ii. To cultivate bacterial consortia responsible for the degradation of chlorophenol;

iii. To identify the microbes responsible for chlorophenol dechlorination by employing molecular biological techniques;

iii. To investigate the feasibility of the application of the chlorophenol dechlorinating cultures on the reductive dehalogenation capability on other halogenated organic compounds.

CHAPTER 2

LITERATURE REVIEW

2.1 Chlorinated Organic Compounds and Chlorophenols

Chlorinated organic compounds constitute one of the largest group of halogenated compounds and are among the most toxic and hazardous compounds found in the environment. As a result to their widespread use, they are extraneously added in large quantities and have been found to persist in the lakes, rivers, groundwater systems, sediments and soils due to their inherent resistant to both chemical and biological degradation (Stringer and Johnston, 2001).

Chlorophenols are a group of toxic, colorless, weakly acidic organic aromatic compounds in which one or more of the hydrogen atoms attached to the benzene ring of phenol have been replaced by chlorine atoms. They constitute a series of 19 toxic compounds consisting of monochlorophenols, dichlorophenols, trichlorophenols, tetrachlorophenols, and pentachlorophenol. Chlorophenols are produced by the direct chlorination of phenols using a variety of catalysts and reaction conditions and are extensively used as biocides because of their broad spectrum of anti-microbial properties.

The physical and chemical information of some important chlorophenols in this manuscript, namely 4-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol are shown in Table 2.1.

Table 2.1: The physical and chemical information of 4-CP, 2,4-DCP, 2,4,6-TCP and PCP.

Characteristics	**4-Chlorophenol**	**2,4-Dichlorophenol**	**2,4,6-Trichlorophenol**	**Pentachlorophenol**
Abbreviation	4-CP	2,4-DCP	2,4,6-TCP	PCP
Chemical formula	C_6H_5ClO	$C_6H_4Cl_2O$	$C_6H_3Cl_3O$	C_6HCl_5O
Molecular weight	128.56	163.00	197.45	266.35
Melting point	43.2 – 43.7 ºC	45 ºC	69 ºC	190 ºC
Boiling point	220 ºC	210 ºC	246 ºC	309 – 310 ºC
Density	1.306 g/cm^3	1.38 g/cm^3	1.49 g/cm^3	1.987 g/cm^3
Solubility:				
• Water at 25ºC	27,000 ppm	4,500 ppm	434 ppm	14 ppm (at 20 ºC)
• Organic solvent	Alcohol, glycerol, ether, chloroform, fixed and volatile oils, benzene	Ethyl alcohol, carbon tetrachloride, ethyl ether, benzene, chloroform	Acetone, benzene, carbon tetrachloride, diacetone alcohol, methanol, Stoddard solvent, touene, turpentine, ether	Alcohol, ether, benzene, slightly soluble in cold petroleum ether
pK_a	8.85	7.68	7.42	4.7
Partition coefficient				
• Log K_{OW}	2.4	3.2	3.69	5.01
• Log K_{OC}	1.2 – 2.7	2.42 – 3.98	1.94 – 3.34	4.5

2.1.1 Usages of Chlorophenols

All the chlorophenols have been used as biocides. The monochlorophenols have been used as antiseptics (ASTDR, 1999), although in this role they have largely been replaced by other chemicals (WHO, 1989). Specifically, 4-CP has been used as a disinfectant for home, hospital, and farm uses (WHO, 1989) and as an antiseptic in root canal treatment (Gurney and Lantenschlager 1982). 2,4-DCP has been used for mothproofing and as a miticide (WHO, 1989), while the higher chlorophenols have been used as germicides, algicides, and fungicides.

The principal use of the monochlorophenols has been as intermediates for the production of higher chlorinated phenols (WHO, 1989). The largest uses for 2,4-DCP and 2,4,5-TCP have also been used as an intermediate, especially in the production of the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (WHO, 1989). In the United States, 2,4-D is still in use, while 2,4,5-T was taken off the market in 1985. 2,4,6-TCP has been used as an intermediate in the production of higher chlorinated phenols especially 2,3,4,6-TeCP and pentachlorophenol (WHO, 1989).

2,4,6-TCP and the tetrachlorophenols have also been used directly as wood preservatives (ASTDR, 1999). In this role, the tetrachlorophenols are generally used as a mixture and are applied to lumber in an aqueous solution (WHO, 1989). Commercial pentachlorophenol, which is more frequently used as a wood preservative, also contains about 4% tetrachlorophenols and 0.1% trichlorophenols (Vainio et al., 1990). North America and Scandinavia are the main regions of the world where chlorophenols have been used as wood preservatives. The use of these compounds has been banned in Sweden since 1978, and production was banned in Finland in 1984 (Vainio et al., 1990).

Pentachlorophenol was one of the most widely used biocides in the United States. It was registered for use by EPA as an insecticide (termiticide), fungicide, herbicide, molluscicide, algicide, disinfectant, and as an ingredient in antifouling paint (Rao, 1978), but it has been a restricted-use pesticide since July 1984 (ASTDR, 2001). The principal use of pentachlorophenol is as a wood preservative (registered by EPA for power-line poles, cross arms, fence posts, and the like). The treatment of wood for utility poles represents 80% of the U.S. consumption of pentachlorophenol (ASTDR, 2001). However, pentachlorophenol is no longer contained in wood preserving solutions or insecticides and herbicides available for home and garden use since it is a restricted-use pesticide. Pentachlorophenol is used for the formulation of fungicidal and insecticidal solutions and for incorporation into other manufactured pesticide products. These non-wood uses account for no more than 2% of U.S. pentachlorophenol consumption (ASTDR, 2001). This wide spectrum of uses was partially attributed to the solubilities of the non-polar pentachlorophenol in organic solvents, and the sodium salt in water.

2.1.2 Environmental Fates of Chlorophenols

The majority of known environmental releases of chlorophenols were to surface water. The principal point source of water pollution by chlorophenols is industrial waste discharge; another point discharge is the leaching of chlorophenols from landfills. Chlorophenols enter the atmosphere through volatilization, with mono- and dichlorophenols being the most volatile. The primary nonpoint source pollution of chlorophenols comes from the application of pesticides that are made from chlorophenols and the chlorination of waste water containing phenol (ASTDR, 1999).

Once released to the environment, chlorophenols are subject to a series of physical, chemical, and biological transformations. Sorption, volatilization, degradation, and leaching are the primary processes governing their fate and transport. The pH in water and in soil and sediment is a major factor affecting the fate and transport of chlorophenols in these media, since the degree to which the compounds ionize increases with increasing pH. In addition, physiochemical properties of chlorophenols such as water solubility, Henry's law constant, organic carbon sorption coefficient, volatilization rate, and photolysis rate determine transport processes. Important environmental parameters influencing these processes include organic matter content and clay content in soil, sediment, and water, as chlorophenols are in general preferentially adsorbed to these soil constituents. In general, as the number of chlorine molecules increase, there is a reduction in vapor pressure, an increase in boiling point, and a reduction in water solubility of the chlorophenols. Therefore, increasing chlorination increases the tendency of these compounds to partition into sediments and lipids and to bioconcentrate. Chlorophenols are subject to abiotic and biotic degradation and transformations. However, compounds containing chlorine in the *meta* positions show greater resistance to microbial attack (ASTDR, 1999).

The general population may be exposed to chlorophenols through ingestion of chlorinated drinking water and food contaminated with the compounds and inhalation of contaminated air. Exposure to 4-CP could also occur through its use as a root canal packing. Populations with potentially unusually high exposure to chlorophenols generally include employees of facilities that manufacture or use chlorophenols and their

derivatives and those who live in the vicinity of chlorophenol-containing waste disposal sites and waste incinerators (ASTDR, 1999).

2.2 Utilization of Chlorinated Compounds by Microorganisms

The biological destruction of toxic and hazardous chemicals is also based on the principles that support all ecosystems. These principles involve the circulation, transformation, assimilation of energy and matter (Cookson, 2005). Microorganisms convert complex organic compounds, via their central metabolic routes, to CO_2 or other simple organic compounds. The oxidation yields energy and reducing equivalents that are used for conversion of a part of the intermediates to cell mass (assimilation), enabling growth of the organisms that carry out the degradation process (Bhatt et al., 2007).

Degradation of compounds of natural origin is usually easy to achieve, and organisms that bring about their degradation can be easily isolated from their natural environments. However, in general, compounds having a structure that is different from naturally occurring compounds (xenobiotics, most of which are toxic and hazardous) are more difficult to degrade (Leisinger et al., 1981). Nevertheless, in the recent past, an array of microorganisms has been identified that use xenobiotics such as chlorinated alkanes, chlorinated halohydrins, polychlorinated biphenyls, and chlorobenzenes for their survival.

2.2.1 Biodegradation of Chlorinated Compounds

Biodegradation of chlorinated compounds follows two pathways namely “aerobic degradation” or “anaerobic degradation.” However, irrespective of the pathway followed,

the extent of degradation depends on the structure of the compound, the number of chlorine substituents, and the position of chlorine in the molecules. Depending on the structure, chlorinated compounds can be either oxidized or reduced. Reduction is possible because of their electronegative character, which makes them highly electron deficient (Bhatt et al. 2007).

Aerobic Biodegradation - During aerobic degradation of chlorinated compounds by microorganisms, molecular oxygen serves as the electron acceptor.

Anaerobic Biodegradation - In the anaerobic mode of degradation the electron acceptor is a molecule other than O^2. This could be NO_3^-, SO_4^{2-}, Fe^{3+}, H^+, S, fumarate, trimethylamine oxide, an organic compound, or CO_2 (Cookson, 1995). The term "dehalorespiration" has been coined for anaerobic bacteria that couple the reductive dehalogenation of chlorinated aliphatic and aromatic compounds to ATP synthesis via an electron transport chain (Wohlfarth and Diekert, 1997). Reductive dechlorination or reductive dehydrogenolysis is a common biotransformation pathway for chloroaliphatics containing one or two carbon atoms, under methanogenic conditions (Semprini, 1997).

Sequential Degradation - Although degradation of chlorinated aliphatic and aromatic compounds has been reported both under aerobic and anaerobic conditions, sequential use of these processes always has an advantage over using them individually for complete mineralization of heavily chlorinated compounds. It is generally implied that aerobic microbes often fail to metabolize heavily chlorinated compounds. Therefore, it has been suggested that detoxification and complete mineralization of chlorinated wastes can be easily achieved by using a sequential treatment process, that is, anaerobic followed by

aerobic treatment. A sequential treatment step will ensure total mineralization of these chlorinated toxic compounds.

2.2.2 Biodegradation of Chlorophenols

Chlorinated phenols may be removed from a water body via various processes, namely, volatilization, photodegradation, adsorption onto suspended or bottom sediments and microbial degradation. In microbial degradation of chlorophenols, certain microbial communities showed chlorophenols degrading capability under limited anaerobic conditions; degradation is usually initiated by *microbial reductive dehalogenation* followed by ring cleavage (Annachhatre and Gheewala, 1996). Chlorophenols, like many chlorinated aromatic compounds, are amenable to reductive dehalogenation and are biotransformed in anaerobic soils, sediments and sewage sludge. As mentioned, anaerobic bacteria initiate degradation of chlorophenols by reductively removing chlorine from the aromatic ring. In this transformation, chlorine atoms are replaced with hydrogen atoms.

While abiotic and biotic natural attenuation processes may reduce the threats associated with these contaminants, the efficiency of these processes varies greatly and depends on the properties of these compounds as well as on environmental conditions. Halogenated compounds, many of which are very toxic and carcinogenic, are especially resistant to degradation due to the stability induced by their halogen (e.g. chlorine, bromine) substituents. However, anaerobic microorganisms can sequentially remove halogenic constituents from these compounds through the process of reductive dehalogenation,

which renders them more amenable to subsequent aerobic degradation and ultimate mineralization (Pavlostathis, 2002).

Thus, identification and application of novel microbes for biodegradation of these chemicals and the optimization of the process have become an essential area of research today.

2.3 Microbial Reductive Dehalogenation

Reductive dehalogenation involves the removal of a halogen substituent from a molecule with concurrent addition of a electrons to the molecule. Essentially, two processes have been identified. The first process, *hydrogenolysis*, is the replacement of a halogen substituent of a molecule with a hydrogen atom (Figs. 2.1A and 2.1B). The second process, *vicinal reduction* or *dihaloelimination*, is the removal of two halogen substituents from adjacent carbon atoms with the formation of an additional bond between the carbon atoms (Fig. 2.1C). Hydrogenolysis can transform alkyl or aryl halides, whereas vicinal reduction can transform only akyl halides. Both processes require an electron donor (reductant). In all reported examples of biologically catalyzed reductive dehalogenation, the halogen atoms are released as halide anions (Mohn and Tiedje, 1992).

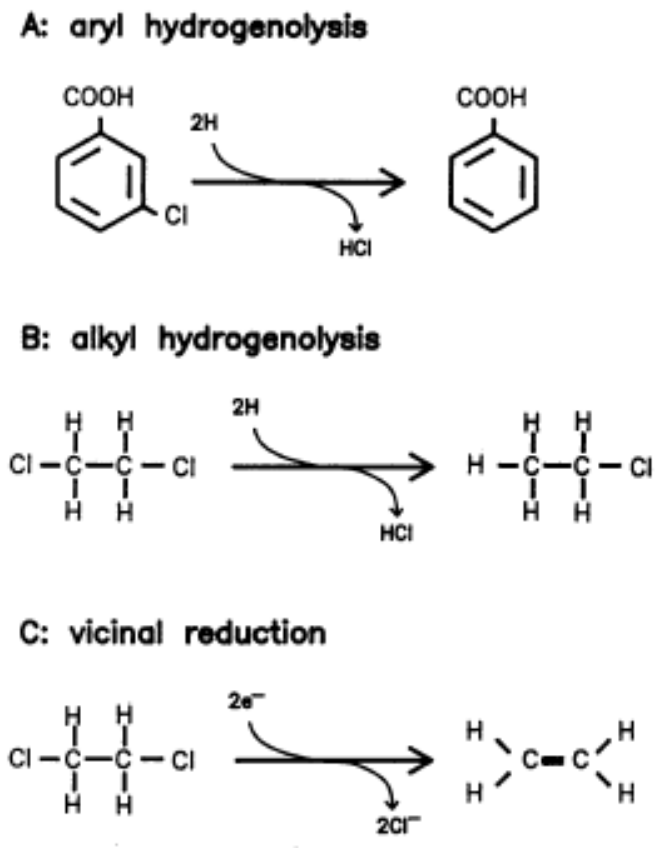


Figure 2.1: Examples of dehalogenation: (A) Aryl hydrogenolysis of 30chlorobenzoate to benzoate; (B) alkyl hydrogenolysis of 1,2-dichloroethane to chloroethane; (C) vicinal reduction of 1,2-dichloroethane to ethene.

2.3.1 Oxidative vs. Reductive Transformation

Anthropogenic compounds can be degraded in the environment as part of the natural biogeochemical processes. Many of these natural attenuation processes are microbially mediated and occur when organic compounds are oxidized for energy and growth, using oxygen as the terminal electron acceptor in coupled redox reactions. Polyhalogenated organic compounds, however, tend to be resistant to biodegradation in aerobic environments. These compounds are more oxidized than their non-halogenated

counterparts due to the presence of the highly electronegative halogen substituents, which provide stability to the molecule. As a result, reduction of these compounds is more likely to occur than oxidation as the degree of halogenation increases. However, polyhalogenated compounds can be used as electron acceptors in thermodynamically favorable reactions. The microbial reductive dechlorination of a large number of polychlorinated compounds (e.g., polychlorinated biphenyls (PCBs), halogenated alkanes, alkenes, phenols, benzenes, benzoates, etc.) has been well documented (Dolfing and Beurskens, 1995; El Fantroussi *et al.*, 1998; Fetzner and Lingens, 1994; Fetzner, 1998; Middeldorp *et al.*, 1999).

2.3.2 Mechanisms and Reactions in Microbial Reductive Dehalogenation

There are two basic mechanisms by which reductive dehalogenation can occur: *hydrogenolysis* (i.e., displacement of a halogen substituent with hydrogen) and *dihaloelimination* (i.e., replacement of two halogen-carbon bonds with a carbon-carbon bond). Both types of reactions require the transfer of electrons from an external donor and produce protons (acid). Due to the predominance of hydrogenolysis in environmental systems, the term reductive dehalogenation has been used synonymously with the term hydrogenolysis. Dehalogenation reactions form organic products which tend to be less hydrophobic, more volatile, and more soluble than the parent compounds by many orders of magnitude. Thus, dehalogenation leads to increased contaminant mobility. As halogens are sequentially removed, however, dehalogenation reactions tend to slow considerably once compounds are transformed to a di- or monohalogenated state (Pavlostathis, 2002).

2.3.3 Role of Electron Donors in Microbial Reductive Dehalogenation

Reductive dehalogenation reaction, whether catalyzed by a transition metal, bacterial cofactors, or an enzyme, requires two electrons. Therefore, a source of electrons must be available for the reaction to take place. (Bhatt et al., 2007) The source of electrons (or electron donor) for a dechlorination reaction is usually a reduced substrate provided for microbial growth.

2.3.4 Role of Electron Acceptors in Microbial Reductive Dehalogenation

All energy-yielding reactions are oxidation–reduction reactions. The reduction reaction, that is, the reaction involving the electron acceptor, establishes the metabolism mode (McCarty, 1987). Microbes preferentially utilize electron acceptors that provide the maximum free energy during respiration (Stumm and Morgan, 1981). Among the common electron acceptors used by microorganisms, O_2 typically provides the maximum free energy during electron transfer, followed by nitrate, Mn(IV), Fe(III), SO_4^{2-} , and CO_2 (Cobb and Bouwer, 1991).

Chlorinated compounds are stronger oxidants than nitrate (Vogel et al., 1987). On the basis of such thermodynamic considerations, chlorinated hydrocarbons have been shown to act as terminal electron acceptors in a respiratory process (Dolfing and Gibbs, 1992).

2.3.5 Reductive Dehalogenation in the Energy Metabolism of Anaerobic Bacteria

Several anaerobic bacteria have been isolated which are able to dechlorinate chlorinated aliphatic and aromatic compounds at catabolic rates. For some of these bacteria, it has been shown that the reductive dechlorination is coupled to energy conservation, a process designated as '*dehalorespiation'* (Holliger et al., 1999). In other reports, the terms `*halorespiration*' or `*chlororespiration*', which suggest that a halogen serves as terminal electron acceptor (in analogy to e.g. fumarate or nitrate respiration), have been used. Since this is not the case, the term `dehalorespiration' is preferable, as it indicates that the dehalogenation process is coupled to ATP synthesis via a chemiosmotic mechanism.

Principally microorganisms couple only those half-reactions that yield the maximum free energy for the synthesis of ATP. The energy released from the reaction is comparable to that of nitrate reduction and much higher than either methanogenesis or sulfate reduction under identical physiological conditions (Bhatt et al., 2007).

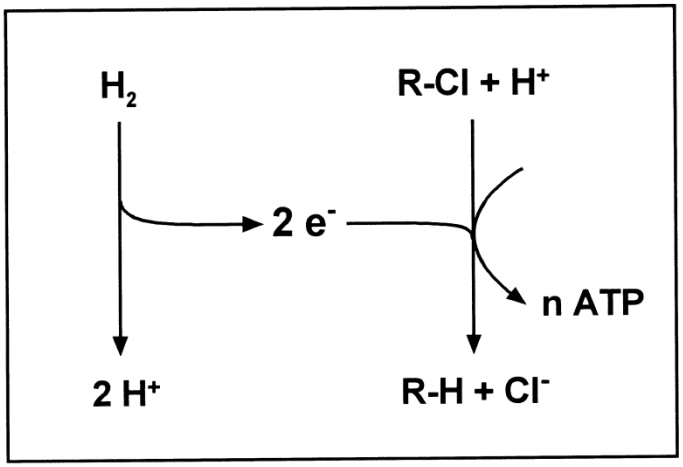
During energy metabolism, energy available from all reductive dechlorination reactions is of a similar order of magnitude, irrespective of the parent compound and the number or position of chlorines, since most of the energy becomes available due to the change in the oxidation state of chlorine (Cl^+ or Cl^-). Thus, free energy from each chlorine atom removed for a host of chlorinated organics has been calculated to vary between −130 and −171 kJ per chlorine atom removed (Dolfing and Harrison, 1992).

A prerequisite for the coupling of energy conservation to reductive dechlorination is that the following reaction is thermodynamically favorable:

$$R\text{-}Cl + 2[H] \rightarrow R\text{-}H + H^+ + Cl^-$$

It has been demonstrated that this reaction is usually exergonic, as shown for tetrachloroethene or 3-chlorobenzoate in Fig. 2.22. For most halogenated compounds, the standard redox potential for the couplet R-Cl/R-H lies between approximately +250 and +600 mV (Vogel et al., 1987; Dolfing and Harrison, 1992). Therefore, these compounds are thermodynamically favorable as electron acceptors under anaerobic conditions. This is also true of monohalogenated compounds, some of which, for example, vinyl chloride and monochlorobenzene, are often dechlorinated slowly, if at all.

Anaerobes capable of growth on a defined medium with the chlorinated substrate as the electron acceptor and an electron donor like H_2 or formate, the oxidation of which cannot be coupled to the synthesis of ATP (Fig. 2.2), have to gain their energy from dehalorespiration. For those organisms which require an electron donor yielding ATP via substrate level phosphorylation during oxidation, dehalorespiration is difficult to prove unambiguously, even if the organism depends on the presence of the chlorinated compound as an electron acceptor. It is feasible that the organohalogen merely serves as a favorable and/or necessary electron sink for reducing equivalents generated upon oxidation of the electron donor (Holliger et al., 1999).



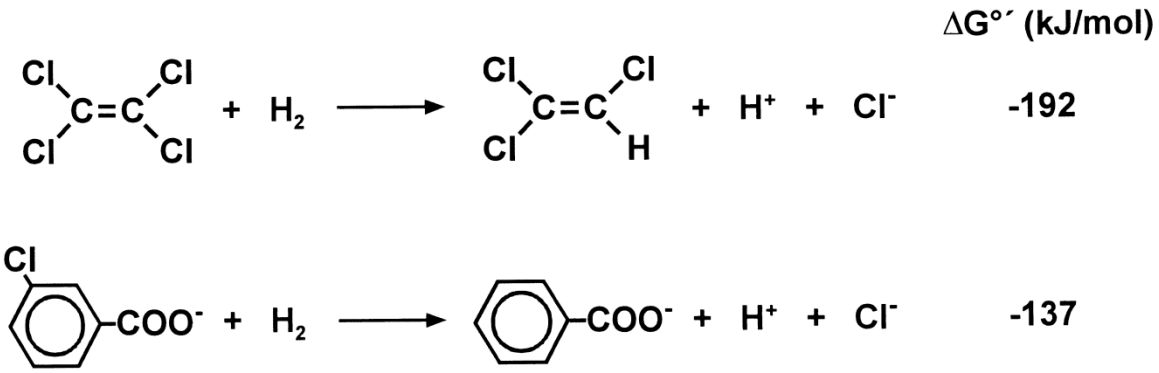


Figure 2.2: Scheme of dehalorespiration with H_2 as electron donor and tetrachloroethene or 3-chlorobenzoate as electron acceptor (Holliger et al., 1999).

2.4 Pure Isolates Capable of the Reductive Dehalogenation of Chlorophenols

To date, only a few anaerobic bacteria that can reductively dechlorinate chlorophenols have been isolated. These dechlorinators generally belong to the genus, *Desulfitobacterium*. The mainly use highly chlorinated chlorophenols as their electron donors for growth and normally thrive under neutral pH conditions. The following section describes all known pure isolates that are capable of dechlorinating chlorophenols with 3 or more chlorine substituents.

2.4.1 *Desulfomonile tiedjei* DCB-1

The *Desulfomonile tiedjei* DCB-1 is the earliest and best described dechlorinating culture to date (Mohn and Kennedy, 1992). This dechlorinating bacterium is a gram-negative, obligate anaerobe with a unique 'collar' surrounding the cell and was enriched and isolated from a stable methanogenic consortium from sewage sludge. 3-Chlorobenzoate is required for the dechlorination of chlorophenols and serves as an inducer. Neither PCP nor 3-CP could induce dehalogenation. Strain DCB-1 dechlorinates pentachlorophenol and other chlorophenols only at the *meta* position (e.g. parent compound PCP to 2,4,6-TCP as the end product). This bacterium however could not dechlorinate 3-CP. The maximum rate of PCP dechlorination observed was 54 µmol of Cl^- h^- g of $protein^{-1}$. PCP concentration of greater than 10 µM (approximately 1.7 mmol g of $protein^{-1}$ inhibits the growth of strain DCB-1.

2.4.2 *Desulfitobacterium frappieri* PCP-1

Desulfitobacterium frappieri PCP-1 was isolated from a methanogenic consortium which originated from a mixture of anaerobic sewage sludge and soil samples that had been contaminated with PCP (Bouchard et al., 1996). Anaerobic bacterium strain PCP-1 is the only known pure isolate capable of dechlorinating pentachlorophenol to monochlorophenol. This organism is a spore-forming, rod-shaped bacterium that is non-motile, assacharolytic and Gram stain negative but Gram type positive as determined by electron microscope observation. In organic electron acceptors such as sulfite, thiosulfate and nitrate (but not sulfate) stimulate growth in the presence of pyruvate and yeast

extract. The dechlorination pathway for strain PCP-1 is PCP → 2,3,4,5-TeCP → 3,4,5-TCP → 3,5-DCP → 3-CP. This bacterium dechlorinates several different chlorophenols at *ortho, meta* and *para* positions with the exceptions of 2,3-DCP, 2,5-DCP, 3,4-DCP and the monochlorophenols. The time course of PCP dechlorination suggests that two enzyme systems are involved in dehalogenation in strain PCP-1. One system is inducible for *ortho* dechlorination and the second system is inducible for *meta* and *para* dechlorinations.

2.4.3 *Desulfitobacterium dehalogenans* JW/IU-DC1

Strain JW/IU-DC1 was isolated from a methanogenic lake sediment (Utkin et al., 1994; Utkin et al., 1995) . This organism, an anaerobic, motile, Gram-type-positive, rod-shaped bacterium requires the presence of yeast for growth. This strain of dehalorespiring bacterium dechlorinates a broad range of chlorophenols (PCP, TeCP, 2,3,4-TCP, 2,3,6-TCP, 2,4,6-TCP, 2,3-DCP, 2,4-DCP and 2,6-DCP) but only at the *ortho* position (Refer to Figure 2.3). 3-chloro-4-hydroxyphenylacetate (3-Cl-4-OHPA) is required to act as the inducer for the reductive dehalogenation of chlorophenols. Pyruvate, lactate, formate, or hydrogen can serve as the electron donor for strain JW/IU-DC1.

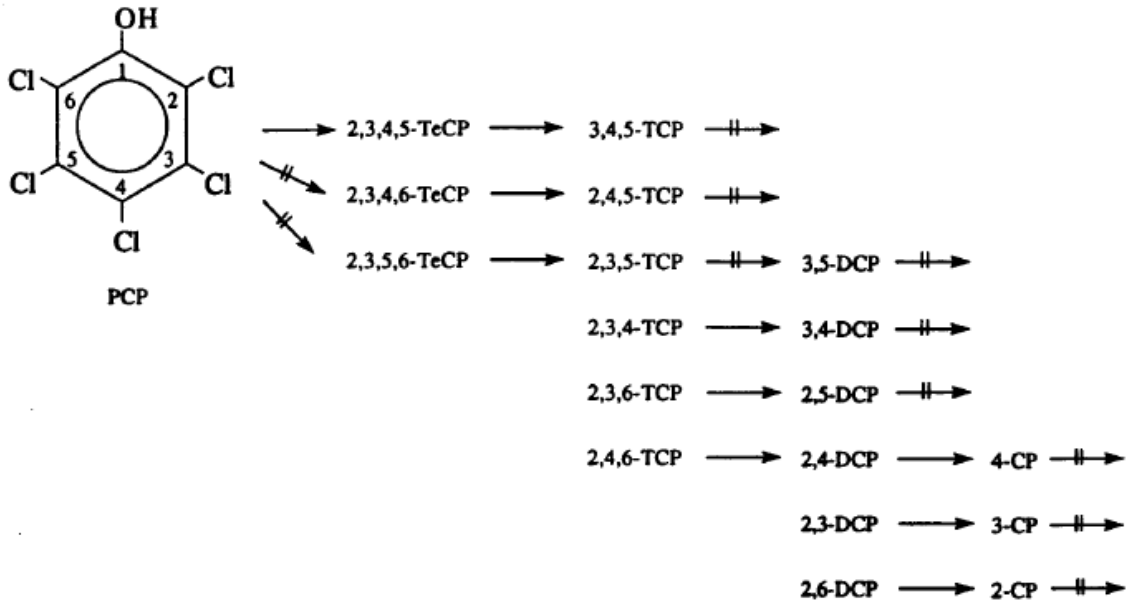


Figure 2.3: General scheme of reductive *ortho* dehalogenation with *D. dehalogenans* JW/IU-DC1 cultures grown in 0.1% yeast extract medium containing 20 mM pyruvate and 10 mM 3-Cl-4-OHPA as the inducer.

2.4.4 *Desulfitobacterium hafniense* DCB-2

Strain DCB-2 is an obligately anaerobic, spore-forming bacterium that is capable of reductive dechlorination of chlorophenols (Madsen and Licht, 1992; Christiansen and Ahring, 1996). The strain is a curved, rod-shaped organism whose cells occur singly, in pairs and in small chains. Strain DCB-2 is motile and normally has one terminal flagellum although, occasionally, two flagella can be observed too. The strain was grown in pyruvate and required yeast extract for growth. DCB-2 exhibited only *ortho*-dechlorination in chlorophenols while *meta*-dechlorination is observed only when 3,5-DCP was used as the electron acceptor. Slow and incomplete dechlorination was observed when PCP was used as the electron acceptor.

2.4.5 *Desulfitobacterium chlororespirans* Co23

Strain Co23 is an obligately anaerobic, spore-forming microorganism enriched and isolated from a compost soil that is capable of reductively dechlorinates chlorophenols (Sanford et al., 1996). The cells are slightly curved, motile rods and stain Gram negative but phylogenetically, this organism is within the Gram-positive *Desulfotomaculum* group. Terminally located spores appear in late growth. This strain is capable of *ortho* dechlorinating the following range of chlorophenols with their respective dechlorination products given in parentheses: 2,3-DCP (3-CP), 2.6-DCP (2-CP) and 2,4,6-TCP (4-CP). Pyruvate, lactate, butyrate and H_2 are used as electron donors.

2.4.6 *Desulfitobacterium dehalogenans* PCE1

A strictly anaerobic bacterium, strain PCE1, was isolated from a tetrachloroethene-dechlorinating enrichment culture (Gerritse et al., 1996). Cells of the bacterium were motile curved rods with approximately four lateral flagella and possess Gram-positive type cell walls. Yeast extract is required to support growth. With lactate or pyruvate as electron donors, several *ortho*-chlorinated phenolic compounds were utilized as electron acceptor and dechlorinated by strain PCE1. 2,4,6-TCP was reductively dechlorinated via 2,4-DCP to 4-CP and 2-CP to phenol. Dechlorination of PCP was not observed.

2.4.7 *Desulfitobacterium frappieri* TCP-A

Desulfitobacterium frappieri TCP-A is an anaerobic, chlorophenol dehalogenating microorganism enriched and isolated from the river sediments in Germany (Breitenstein et al., 2001). Strain TCP-A are slightly curved, rod-shaped rods that exhibits high motility and stained weakly Gram-positive. This strain can dechlorinate chlorophenols at chlorine substituents at both *ortho* positions and one chlorine substituent at the *meta* position. No dechlorination of chlorophenols at the *para* position was observed. PCP and 2,3,4,5-TeCP were only partially dechlorinated. 2,4,6-TCP is required to induce dechlorination for several chlorophenols. Table 2.2 describes the case. No dechlorination was recorded when yeast extract was used.

2.4.8 *Dehalococcoides* strains CBDB1 and 195

Strains CBDB1 and 195 were the first two strains of microbes from the genus *Dehalococcoides* described to dechlorinate chlorophenols (Adrian et al., 2007). Strain CBDB1 showed dechlorination capability with a wide range of chlorophenols. This strain can dechlorinate PCP, all 3 isomers of TeCP, all 6 isomers of TCP and 2,3-DCP. Figure 2.4 describes in detail. Chlorophenols were found to be preferentially dechlorinated at the *ortho* position.

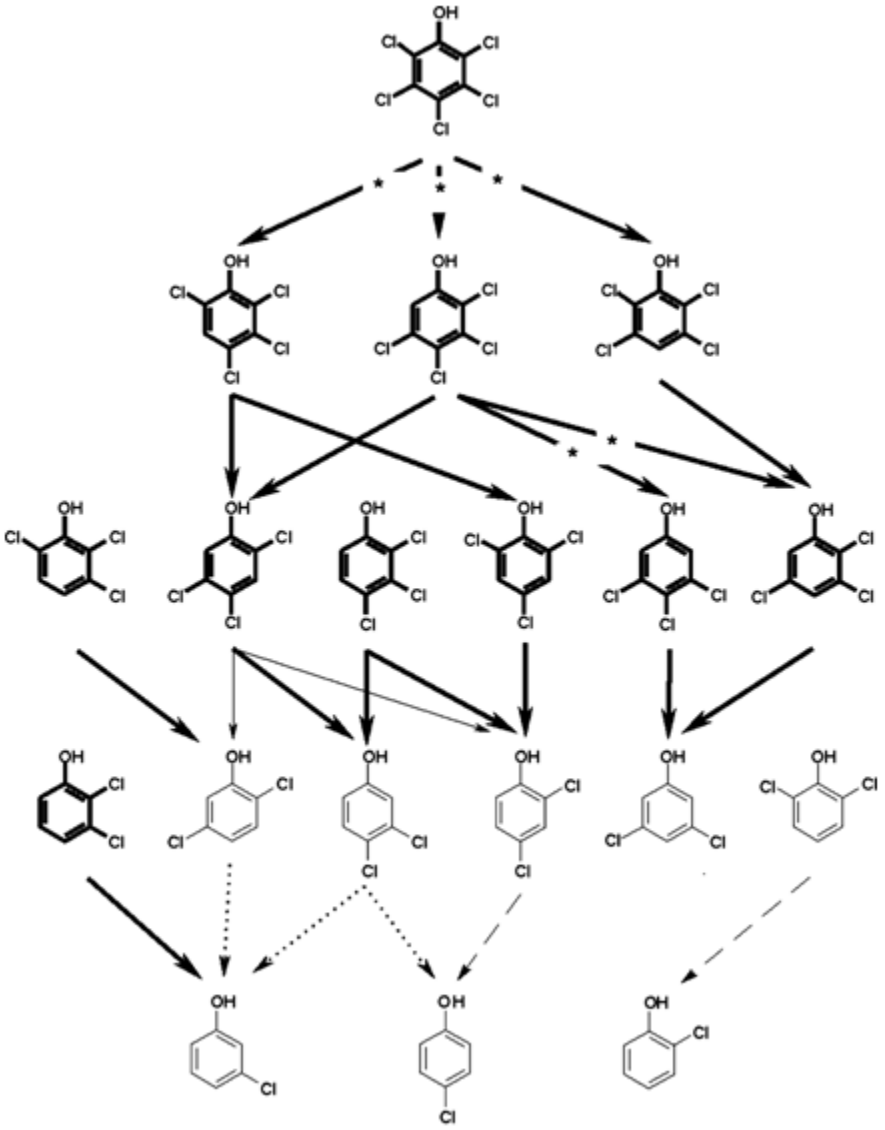
Dehalococcoides strain 195 dechlorinated a smaller spectrum of chlorophenols, all in the *ortho* position and only if a chlorine substituent was present in the flanking *meta* position. Dechlorination was detected with 2,3-DCP, 2,3,4-TCP and 2,3,6-TCP but not with other

di- and trichlorophenols or pentachlorophenol. Like strain CBDB1, strain 195 could not dechlorinate monochlorophenols.

Table 2.2: Transformation of chlorophenols by strain TCP-A.

Substrate	2,4,6-TCP as inducer	Dechlorination Products
PCP	+	2,3,4,5-TeCP[a,b]
	-	2,3,4,5-TeCP[a,b]
2,3,4,5-TeCP	+	3,4,5-TCP[a,b]
	-	No dechlorination[b]
2,3,5,6-TeCP	+	3-CP → phenol[c]
	-	3,5-CP → 3-CP → phenol[c]
2,3,5-TCP	+	3,5-CP → 3-CP
	-	3,5-CP → 3-CP; 2,5-DCP
2,4,6-TCP	+	2,4-DCP → 4-CP
	-	2,4-DCP → 4-CP
3,5-DCP	+	3-CP
	-	3-CP
2,3-DCP	+	3-CP
	-	No dechlorination
2,4-DCP	+	4-CP
	-	4-CP
2-CP	+	Phenol[a]
	-	Phenol[a]
3-CP	+	No dechlorination
	-	No dechlorination
4-CP	+	No dechlorination
	-	No dechlorination

Note: [a] The conversion of the parent compound was not complete; [b] Growth was strongly inhibited in the presence of PCP, 2,3,4,5-TeCP or respective dechlorination products; [c] By the end of study period, the molar ratio of phenol and 3-CP was 1:3 and 1:6 for induced and non-induced cells respectively.



Note: Bold compounds: compounds that are completely converted if added as a sole electron acceptor; bold arrows: main pathway; thin arrows: side pathway; dashed arrows: slow and incomplete reactions; dotted arrows: reactions that occurred only if the respective dichlorophenol was formed from a higher chlorinated phenol in the same culture. The asterisks mark reaction where the pathways could not be distinguished.

Figure 2.4: Summary of chlorophenol dechlorination reactions catalyzed by *Dehalococcoides* strain CBDB1.

CHAPTER 3

MATERIALS AND METHODS

3.1 Cultures' Origins and Descriptions

A summary of the origins and the sources' description of the cultures used in this study are tabulated below.

Table 3.1: Information summary of the cultures used in this study.

Name	Origin	Description of Source
D1	Hubei, China	Contaminated soil collected at drainage discharge
D2	Hubei, China	River sediments
D3	Hubei, China	River sediments
D4	Hubei, China	River sediments
D5	Hubei, China	Soil from old industrial zone
D6	Hubei, China	Sludge from wasterwater treatment plant
D7	Hubei, China	Sludge from wasterwater treatment plant
D8	Hubei, China	Soil in the vicinity of wastewater treatment plant
D9	Hubei, China	Soil in the vicinity of wastewater treatment plant
D10	Hubei, China	Soil in the vicinity of wastewater treatment plant
D11	Hubei, China	Soil in the vicinity of wastewater treatment plant
D12	Paya Lebar, Singapore	Sludge from wasterwater treatment plant
PA	Penang, Malaysia	Motor oil contaminated soil from car workshop
ORC	Penang, Malaysia	Soil from orchard
PY	West Java, Indonesia	Soil from paddy field

Name	Origin	Description of Source
RIV	West Java, Indonesia	River sediments
LWN	West Java, Indonesia	Soil from field
BNN	West Java, Indonesia	Soil banana plantation
TEA	West Java, Indonesia	Soil from tea plantation
SC	Jurong Island, Singapore	Sludge from wasterwater treatment plant

The selection of the cultures here were to represent a wide range of significantly different origins in terms of the degree of contamination (or non-contamination) from different locations. For example, culture LWN (collected from a field) and RIV (collected from the bed of a river) represent samples collected from sources whereby there are no (or extremely low) levels of anthropogenic contamination. Meanwhile, cultures ORC, BNN and TEA which have been collected from orchards and plantations represent samples collected from sites which were exposed to mild to medium levels of contamination due to higher levels exposure to human activities and from applications of pesticides on sampling site. Samples such SC, D6, D7 and D12 that have been collected from wastewater treatment plants represent cultures sampled from locations with very high levels of contamination from industrial and domestic waste.

On top of that, some of the selections of culture origins were based on previous studies indicating possible existence of dechlorinators within certain sites. This will help to increase the chances of discovering dehalogenation activities from the cultures selected. For instance, culture PY which has been collected from the soils of rice fields was selected as researchers in Japan have reported on numerous occasions that chlorophenols can be biodegraded in the flooded soils of paddy fields (Kim et al, 2004; Yoshida et al.,

2007). Cultures D6, D7, D12 and SC (all collected from sludge of treatment plants) on the other hand, have been selected because several pure isolates of chlorophenol dehalogenating bacteria have been have been reported to originate from municipal sludge from treatment plants (Madsen and Licht, 1992; Mohn and Kennedy, 1992). As another example, cultures D2, D3, D4 and RIV which have been collected from river sediments were chosen following reports of the discoveries of anaerobic bacteria isolated from rivers and freshwater ponds (Utkin et al., 1994; Breitenstein et al., 2001).

3.2 Preparation of Sterile Vials

All vials for experiment purposes were sterilized before use. The new vials were rinsed with Milli-Q ultra water several times. The openings of the washed vials were wrapped with aluminium foil. Vials were autoclaved in autoclave machine for 20 mins, at 121 °C, 210 kPa. The sterile empty vials were left to dry in oven after the autoclaving process.

3.3 Preparation of Anaerobic Media

1 L of trace element solution, Se/W solution and salt solution were prepared as per Tables 3.2, 3.3 and 3.4 respectively. 1 L of the medium solution was prepared accordingly as per Table 3.5. Under the continuous flushing of N_2 (minimum flow rate), medium solutions were brought to boil. Upon boiling, the process was allowed to continue for another 20 minutes. The medium solutions were cooled to room temperature under higher flow rate of N_2. The reductants and buffering agents in Table 3.6 were added to the medium solutions quickly so as to prevent the introduction of O_2 into the medium. Medium

solutions were stirred to fully dissolve chemicals using a magnetic stirrer. The media should turn colorless indicating no O_2 contamination. pH of media was allowed to stabilize in the range of 7.2 – 7.3. Medium solutions were dispensed into vials/serum bottles (continuously flushed with N_2/CO_2 in the ratio of 9:1) with a syringe. The vials were crimp sealed with black rubber stopper with aluminium caps to ensure no leakages and autoclaved. The medium solutions should be clear after autoclave. Media that have turned pink after autoclave were discarded.

Table 3.2: Trace element solution.

Reagents	**Amount (1 L)**	
	ml	**g**
HCl (25% solution, w/w)	10	-
$FeCl_2 \cdot 4H_2O$	-	1.5
$CoCl_2 \cdot 6H_2O$	-	0.19
$MnCl_2 \cdot 4H_2O$	-	0.1
$ZnCl_2$	-	0.07
H_3BO_3	-	0.006
$Na_2MoO_4 \cdot 2H_2O$	-	0.036
$NiCl_2 \cdot 6H_2O$	-	0.024
$CuCl_2 \cdot 2H_2O$	-	0.002

Table 3.3: Se/W solution.

Reagents	Amount (1 L)	
	ml	g
$Na_2SeO_3 \cdot 5H_2O$	-	0.006
$Na_2WO_4 \cdot 2H_2O$	-	0.008
NaOH	-	0.5

Table 3.4: Salt solution.

Reagents	Amount (1 x g/L)	Amount (100 x g/L)	Amount (g/100 mL)
NaCl	1.0	100.0	10.0
$MgCl_2 \cdot 6H_2O$	0.5	50.0	5.0
KH_2PO_4	0.2	20.0	2.0
NH_4Cl	0.3	30.0	3.0
KCl	0.3	30.0	3.0
$CaCl_2 \cdot 2H_2O$	0.015	1.5	0.15

Table 3.5: Medium Solution.

Reagents	Amount (1 L)	
	ml	g
100 x salt solutions	10	-
Trace element	1	-
Se/W Solution	1	-
TES (10mM)	-	2.292
Resazurin (0.1% solution)	0.25	-
Sodium pyruvate (5mM)	-	0.6804
Milli-Q ultra pure water	987.75	-

Table 3.6: Reductants and buffering agents.

Reagents	Amount (1 L)	
	ml	g
0.2mM L-cysteine	-	0.0242
0.2mM $Na_2S \cdot 9H_2O$	-	0.048
0.5mM DL-dithiothreitol (DTT)	-	0.0771
30mM $NaHCO_3$	-	2.52

3.4 Preparation of Vitamins

The final concentrations of vitamins added to the medium solution are given in Table 3.7.

Table 3.7: Final concentration of vitamins added.

Vitamins	Final Concentration (mg/L)
Biotin	0.02
Folic acid	0.02
Pyridoxine hydrochloride	0.10
Riboflavin	0.05
Thiamine	0.05
Nicotinic acid	0.05
Pantothenic acid	0.05
p-aminobenzoic acid	0.05
Thioctic acid	0.05
Vitamin B12	0.001

3.5 Substrate Chemicals

All substrate chemicals used – pentachlorophenol, 2,4,6-trichlorophenol, trichloroethene, commercial pentabromodiphenyl ether mixture and 2,2',4,4',6,6'-hexachlorobiphenyl were purchased from Sigma Aldrich (U.S.A) and were of analytical grade with purity of ≥ 98%. The chlorophenols were dissolved in hexane, the commercial pentabromodiphenyl ether mixture in ethyl acetate and 2,2',4,4',6,6'-hexachlorobiphenyl in isooctane as stock solutions. The chemicals were crimp sealed with black rubber stopper with aluminium caps in serum bottles and wrapped in aluminium foil. All stock solutions were stored in the refrigerator at 4 ºC.

3.6 Detection of Halogenated Compounds

Chlorophenols in the aqueous phase were subjected to a simultaneous derivatization and liquid-liquid extraction procedure. 1 mL of the liquid samples were mixed with 5 mL of potassium carbonate solution (5% w/v), acetylated with 200 μL acetic anhydride and extracted with 1 mL of hexane. Samples were then vortexed and shaken for 2 hours prior to analysis. Chlorophenols were analyzed using a gas chromatograph/mass spectrometer (GC-MS) model QP2010 (Shimadzu Corporation, Japan) equipped with a HP 5 capillary (J&W Scientific, U.S.A.) column (Length: 30 m; i.d.: 0.32 mm; 0.25 μm).

Chlorinated ethenes were measured with a gas chromatograph (GC-6890, Agilent Technologies, U.S.A.) equipped with a flame ionizing detector (GC-FID) and a GS-GasPro (J&W Scientific, U.S.A.) capillary column (Length: 30 m; i.d.: 0.32 mm). 100 μL of gas from the headspace of sample bottles were drawn and injected into the GC-FID for analysis.

PBDEs and PCBs were subjected to liquid-liquid extraction before analysis. 1 mL of liquid samples were removed and added with an equal volume of isooctane. Samples were mixed thoroughly by vortexing the mixture and shaken for 2 hours prior to analysis.

PBDEs were analyzed using a gas chromatograph/mass spectrometer (GC-MS) model GC 6890/MSD 5975 (Agilent Technologies, U.S.A.), which was installed with a Restek Rxi-5ms (Restek Corporation, U.S.A.) column (Length: 15 m; i.d.: 0.25 mm; film thickness: 0.25 μm).

PCBs were measured with a gas chromatograph (GC-6890, Agilent Technologies, U.S.A.) equipped with a electron capture detector (GC-ECD) and a HP 5 capillary (J&W Scientific, U.S.A.) column (Length: 30 m; i.d.: 0.32 mm; 0.25 μm).

Gas chromatography settings for the detection of the halogenated compounds are summarized in Tables 3.8, 3.9, 3.10 and 3.11.

Table 3.8: GC-MS settings for the detection of chlorophenols.

Parameter	**Operation Settings**
Injection Mode	Splitless
Injection Port Temperature	250 °C
Carrier gas	Helium
Column Flow Rate	1.92 mL min^{-1}
Oven Program:	
Initial Temperature	40 °C
Rate	15 °C min^{-1}
Final Temperature	200 °C (hold 3 minutes)

Table 3.9: GC-FID settings for the detection of chlorinated ethenes.

Parameter	Operation Settings
Injection Mode	Split (Ratio 2:1)
Injection Port Temperature	220 ºC
Carrier gas	Helium
Column Flow Rate	3 mL min^{-1}
Oven Program:	
Initial Temperature	50 ºC (hold 2 minutes)
Rate	30 ºC min^{-1}
Final Temperature	220 ºC (hold 1 minute)

Table 3.10: GC-MS settings for the detection of PBDEs.

Parameter	Operation Settings
Injection Mode	Splitless
Injection Port Temperature	300 ºC
Carrier gas	Helium
Column Flow Rate	1.2 mL min^{-1}
Oven Program:	
Initial Temperature	110 ºC
Rate	15 ºC min^{-1}
Final Temperature	310 ºC (hold 5 minutes)

Table 3.11: GC-ECD settings for the detection of PCBs.

Parameter	Operation Settings
Injection Mode	Splitless
Injection Port Temperature	250 ºC
Carrier gas	Helium
Column Flow Rate	1.2 mL min^{-1}
Oven Program:	
Initial Temperature	170 ºC
Rate	5 ºC min^{-1}
Temperature	260 ºC (hold 5 minutes)

3.7 Extraction of Bacterial Genomic DNA

Cells (1 mL) used for DNA extraction were withdrawn from cultures and then centrifuged immediately at 31,500 g for 15 minutes at 4°C. After removing the supernatant, the cell pellets were stored at -20 ºC until further processing.

Genomic DNA was extracted from frozen cell pellets by using the DNeasy Tissue Kit (QIAGEN GmbH, Germany). The instruction manual was followed closely with minor modifications.

3.8 Polymerase Chain Reaction (PCR)

The list of reagents used and their respective final concentrations for each PCR reaction are listed in Table 3.8. The details of the genus specific primer pairs used are given in Table 3.9. Polymerase chain reactions (PCR) were carried out in an Eppendorf Master Cycler ep

gradient S thermocycler (Eppendorf AG, Hamburg, Germany). PCR conditions are summarized in Table 3.10.

Table 3.12: The list of reagents used and their respective final concentrations for PCR.

Reagents	**Final Concentration**	
Sterile PCR water	na	
10x PCR buffer	1x	
MgCl2 (25 mM)	2.5	mM
BSA (10 mg/ml)	0.13	mg/ml
dNTP mix (1:1:1:1, 10mM)	0.25	mM ea
Forward Primer (5 µM)[a,b]	0.1	µM
Reverse Primer (5µM)[a]	0.1	µM
Taq DNA polymerase	na	
Template (250 ng/100 µl reaction)	25	ng/µl

Note: a: Refer to primer details in Table 3.9. b: 8F-Cy5 primers were used instead of 8F primers for TRFLP analysis

Table 3.13: The details of the genus specific primer pairs used in study.

Specificity	Primer Name	Primer Sequence (5' – 3')	Annealing Temperature (°C)	Amplicon Length (bp)
Universal bacteria primer	8F	AGA GTT TGA TCC TGG CTC AG	55	1384
	1392R	ACG GGC GGT GTG T		
Anaeromyxobacter sp.	60F	CGA GAA AGC CCG CAA GGG	56.5	401
	461R	ATT CGT CCC TCG CGA CAG T		
Desulfomonile sp.	85F	CGG GGT RTG GAG TAA AGT GG	62	1369
	1419R	CGA CTT CTG GTG CAG TCA RC		
Desulfitobacterium sp.	406F	GTA CGA CGA AGG CCT TCG GGT	60	225
	619R	CCC AGG GTT GAG CCC TAG GT		
Dehalococcoides sp.	730F	GCG GTT TTC TAG GTT GTC	58	620
	1350R	CAC CTT GCT GAT ATG CGG		
Dehalobacter sp.	179F	TGT ATT GTC CGA GAG GCA	53	828
	1007R	ACT CCC ATA TCT CTA CGG		
Desulfovibrio sp.	691F	CCG TAG ATA TCT GGA GGA ACA TCA G	63	135
	826R	ACA TCT AGC ATC CAT CGT TTA CAG C		
Desulforomonas sp.	205F	AAC CTT CGG GTC CTA CTG TC	58	815
	1020R	GCC GAA CTG ACC CCT ATG TT		
Acetobacterium sp.	572F	GGC TCA ACC GGT GAC ATG CA	59	212
	784R	ACT GAG TCT CCC CAA CAC CT		

Table 3.14: PCR conditions.

Process		Temperature (°C)	Duration
Initial Denaturation		94	2'10"
Denaturation	30 cycles	94	30"
Annealing		Refer Table 3.9	45"
Extension		72	2'10"
Final Extension		72	6'

3.9 Agarose Gel Electrophoresis

TAE buffer was prepared by mixing 40 mL of TAE solution in 1960 mL of Milli-Q ultra pure water. 1 gram of Agarose powder (SeaKem® LE Agarose, BioWhittaker Molecular Applications, USA) was mixed with 100 mL of 1xTAE mixture in a conical flask. The Agarose powder was thoroughly dissolved by heating up in microwave for about 60 seconds to 80 seconds. Dissolved Agarose was poured into a casting tray to allow solidification. The gel casting tray was first leveled using a bubble level and its well comb placed securely before the gel was poured in and allowed to harden. Trapped air bubbles in the hardening gel were removed using a pipette tip so that they would not affect DNA migration during electrophoresis. Comb was removed once gel is hardened. Gel was then transferred, together with the tray, into the electrophoresis unit. 1xTAE buffer was poured into the tray to cover the gel completely. Thawed extracted DNA samples were vortexed and centrifuge in micro-centrifuge tubes. 5 µL of 100 bp or 1-kb DNA ladder (Promega, Madison, US) was then loaded into the first well. 1 µL of 6xblue/green loading dye (Promega, USA) was mixed with 5 µL of a DNA sample and then loaded into a well. Electrophoresis (Bio-Rad, U.S) was done at 90V for 90 minutes when all DNA samples

had been loaded. Gels were removed from tray and proceed for 1 hour of staining process in 5% ethidium bromide (30 µL of ethidium bromide in 600 mL of 1xTAE buffer). Another hour of destaining process was allowed to be carried out in another holding well filled with water. The bands in gel were visualised by UV excitation and pictures were taken with a digital camera (Gel Doc, Bio-Rad, USA).

3.10 TRFLP Analysis

Prior to TRFLP analysis, DNA (PCR products) were subjected to restriction enzyme digestion. The preparation of a 20 µL sample for restriction enzyme digestion is summarized in Table 3.11. The products were then incubated in a water bath at 35 ° for 3 hours. The enzymes were then deactivated by incubation at 65 ℃ for 10 minutes.

0.5 µL of samples were mixed with 40 µL of Sample Loading Solution (SLS) and 0.2 µL of DNA size standard (600 bp) and loaded onto a 96- well plate and overlaid with 1 drop of mineral oil before analysis. TRFLP analyses were carried out with a Beckman CEQ 8000 DNA analysis system.

Table 3.15: Reagents for restriction enzyme digestion.

Reagents	**Volume (µL)**
DNA free water	7.3
Enzyme Buffer 4	2
BSA (10 mg/ml)	0.2
Enzyme (HhaI or MspI or RsaI)	0.5
DNA (PCR product)	10

CHAPTER 4

RESULTS

4.1 Derivatization and Extraction of Samples for Analytical Determination

Following the run of standards in the GC-MS, the quality of the chromatograms for both the derivatized and non-derivatized forms of chlorinated phenols (4-chlorophenol; 2,4-dichlorophenol; 2,4,6-trichlorophenol and PCP) were studied and compared. The preparation of standards, derivatization-extraction procedure and the GC-MS operating conditions used are as described previously in Section 3.5.

Apart from the obvious variation in the order of appearance and retention time of samples, the quality of the peaks from the chromatogram differed significantly as well.

For the underivatized samples, the peak of 2,4-DCP appeared first following injection after 5.826 minutes. This is then followed by 4-CP after 6.097 minutes and finally 2,4,6-TCP after 7.605 minutes. Meanwhile, the derivatization of the chlorinated phenols causes the change of retention time in the following order – 4-CP (6.461 minutes), then 2,4-DCP (7.552 minutes) and finally 2,4,6-TCP at (8.414 minutes). PCP elutes last amongst the congeners tested for both the underivatized and derivatized cases at 10.96 and 11.44 minutes, respectively.

In general, the derivatization of chlorophenols yielded much higher and sharper peaks with higher areas under the peaks. The non-derivatized chlorophenol standards, on the

other hand, gave broad and tailed peaks which is not favorable for detection and quantification of samples. While underivatized forms of 2,4,6-TCP, 2,4-DCP and 4-CP normally resulted in acceptable response from the chromatography analyses in higher concentrations, however, at concentrations of lower than that of 20 µM, the peaks from the chromatograms were very poor and were barely readable or distinguishable from the detector noise. Chromatography analyses of underivatized forms of PCP were generally very poor even with concentrations as high as 50 µM. Figures 4.1, 4.2 and 4.3 illustrate the cases as discussed above in greater detail.

Figure 4.1: Chromatogram showing low peak of underivatized 4-CP sample.

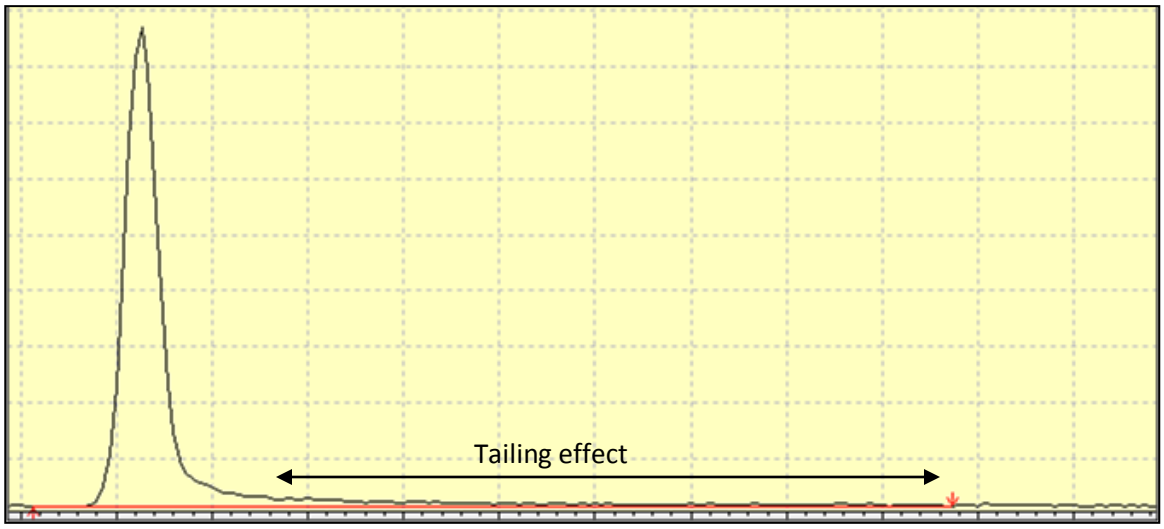


Figure 4.2: Chromatogram showing tailing effect on the peak of an underivatized 4-CP sample.

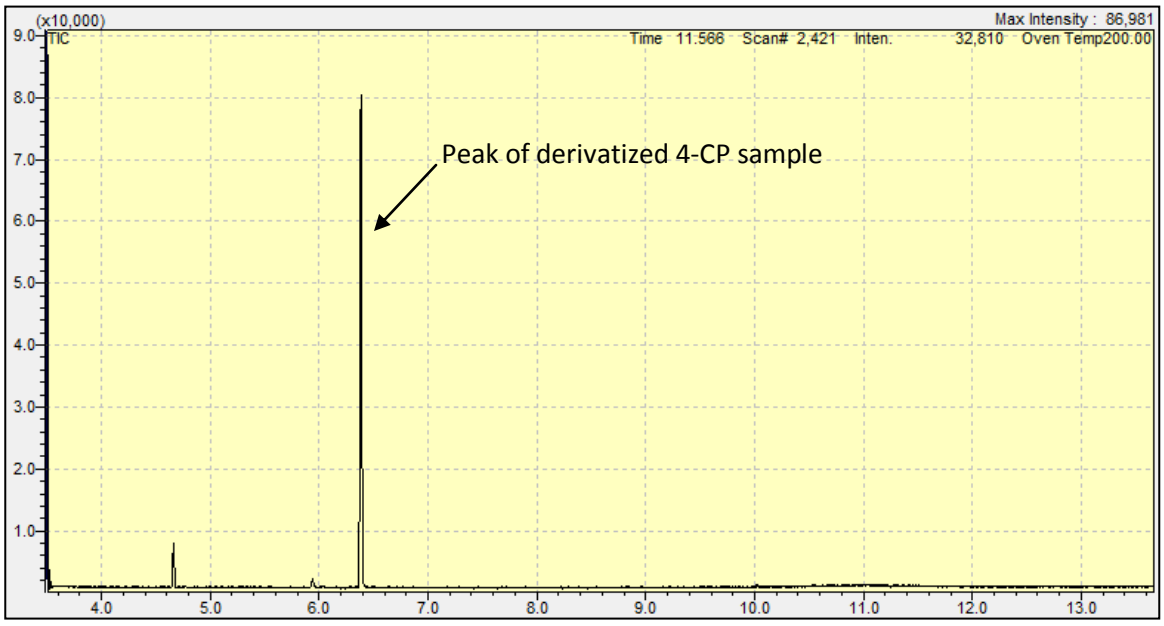


Figure 4.3: Chromatogram showing the significantly higher and sharper peak of a derivatized 4-CP sample.

4.2 Microcosm Studies: Dechlorination of Chlorophenols

Samples collected from the 20 sites were used as inocula for microcosm studies on their capability to dechlorinate 2,4,6-TCP and PCP (Table 3.1). The microcosms established were amended with 30 mL bicarbonate buffered defined mineral salts medium and 10 mM of pyruvate. The medium were reduced by L-cysteine (0.2 mM), $Na_2S.9H_2O$ (0.2 mM) and DL-dithiothreitol (0.5mM). Wolin solution and vitamin B_{12} (25 mg/L) were also added to all samples. 2,4,6-TCP and PCP were spiked into the microcosms with the final concentration of 50 µM and 25 µM respectively.

4.2.1 Dechlorination of 2,4,6-Trichlorophenol

For microcosms spiked with 2,4,6-TCP, all samples demonstrated the ability for dechlorination. The samples were tested on a weekly basis for 8 weeks to detect for possible dechlorination products as an indication of dehalogenating activities within the microcosms. Degradation products such as 2,4-dichlorophenol and 4-chlorophenol were generated following dechlorination. The dechlorination pathways for all 20 microcosms were similar. 2,4,6-TCP were degraded to 4-CP as the end product via 2,4-DCP. Degradation of 4-CP to phenol did not take place throughout the duration of the study. Figure 4.4 illustrates the degradation pathway of 2,4,6-TCP to 4-CP. The rates of dechlorination of 2,4,6-TCP for the 20 samples, however, differed greatly with some being able to degrade the spiked substrates to the end product, 4-CP, significantly faster than others. Figures 4.5a to 4.5t are examples of GC-MS chromatograms showing the dechlorination products of 2,4,6-TCP by each of the 20 cultures tested after 33 ± 3 days to

show the varying dechlorination rates of each samples as well as their similar dechlorination pathways. In particular, cultures LWN and RIV demanded greater attention as they displayed excellent capabilities to dechlorinate 2,4,6-TCP. Cultures LWN and RIV were found to be able to dechlorinate 2,4,6-TCP to 4-CP in less than 7 days. Culture SC stands out too among the microcosm tested as it was able to completely dechlorinate 2,4,6-TCP and its intermediate product, 2-4-DCP, to its end product 4-CP in a relatively quick fashion (about 14 days).

Microcosms containing promising cultures that were able to achieve effective and rapid dechlorination of 2,4,6-TCP to 4-CP, i.e. complete degradation of 2,4,6-TCP to its end product within 14 days, were singled out and sequentially transferred for further enrichment and studies. A total number of 3 cultures were identified and these cultures are, in no particular order, LWN, RIV and SC.

With each transfer, the concentration of 2,4,6-TCP was gradually increased and its inhibition of growth was observed. No dechlorination products were observed when culture SC was spiked with 2,4,6-TCP of a final concentration of 150 µM while culture RIV seemed to be prohibited by 250 µM of the substrate. Culture LWN exhibited high tolerance to 2,4,6-TCP dechlorination. At 1,000 µM, reductive dechlorination activities were still detected and dechlorination products like 2,4-DCP and 4-CP were detected.

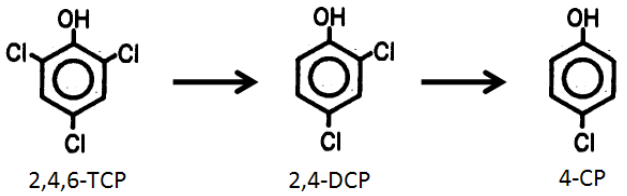


Figure 4.4: Degradation pathway of 2,4,6-TCP to 4-CP via 2,4-DCP.

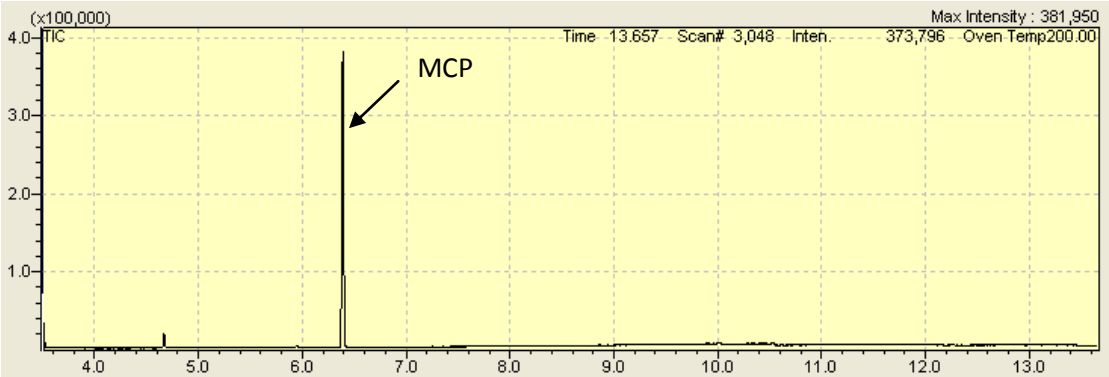


Figure 4.5a: Chromatogram showing degradation product(s) of D1 after 33 ± 3 days

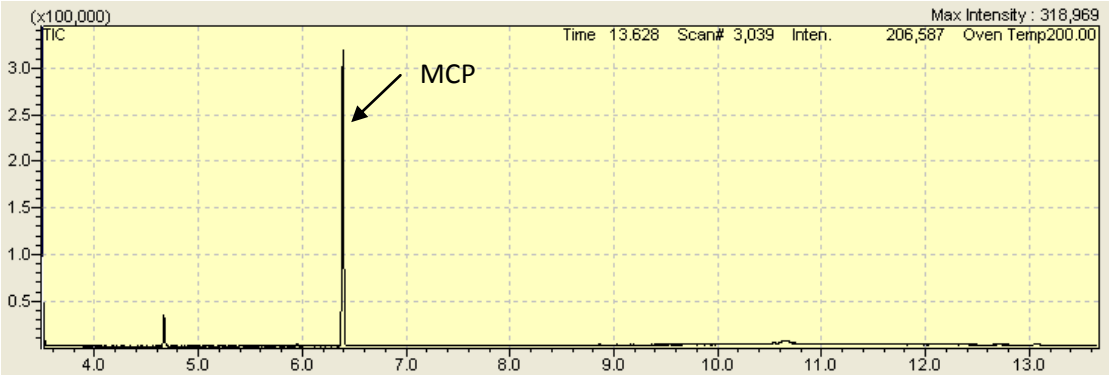


Figure 4.5b: Chromatogram showing degradation product(s) of D2 after 33 ± 3 days

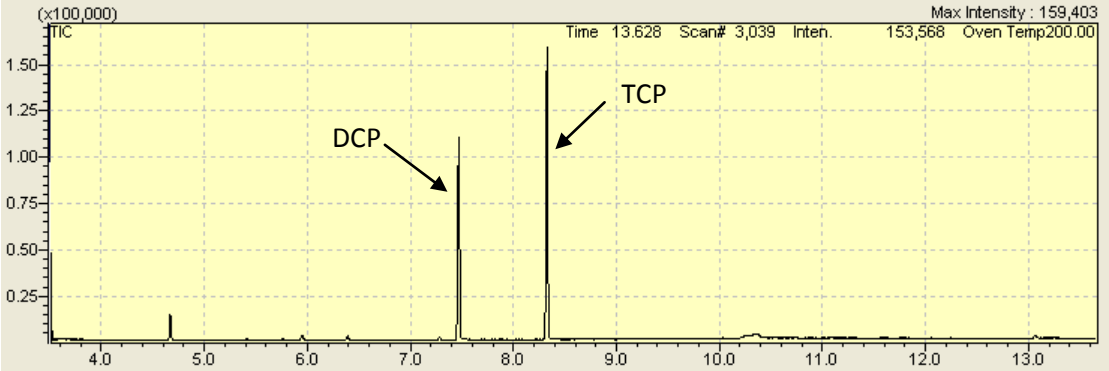


Figure 4.5c: Chromatogram showing degradation product(s) of D3 after 33 ± 3 days

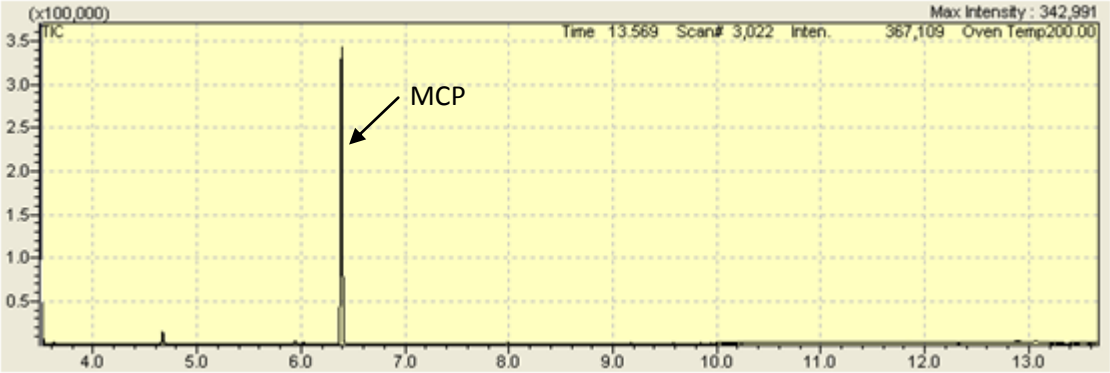


Figure 4.5d: Chromatogram showing degradation product(s) of D4 after 33 ± 3 days

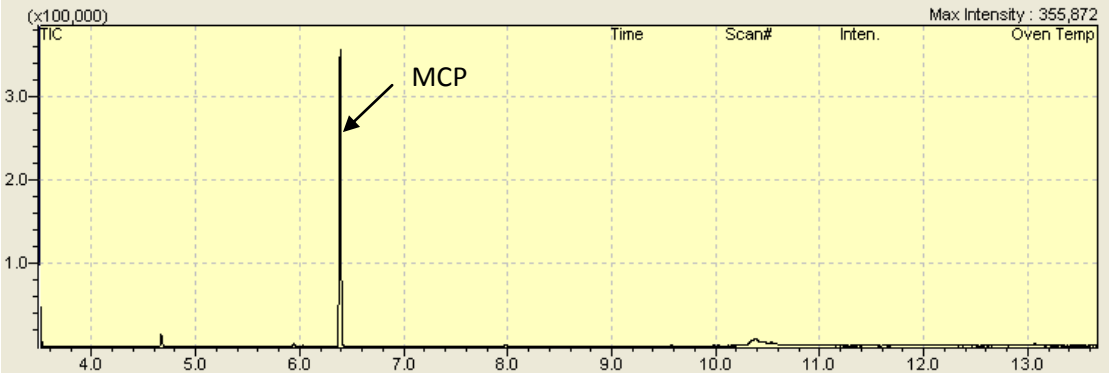


Figure 4.5e: Chromatogram showing degradation product(s) of D5 after 33 ± 3 days

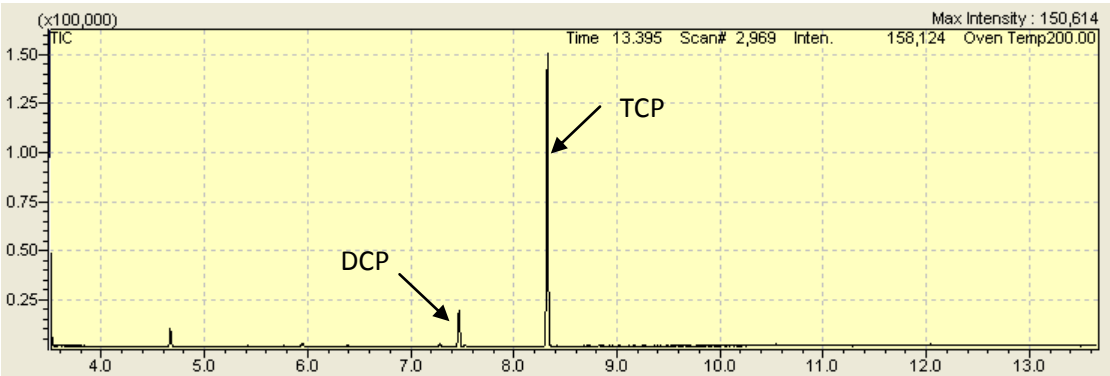


Figure 4.5f: Chromatogram showing degradation product(s) of D6 after 33 ± 3 days

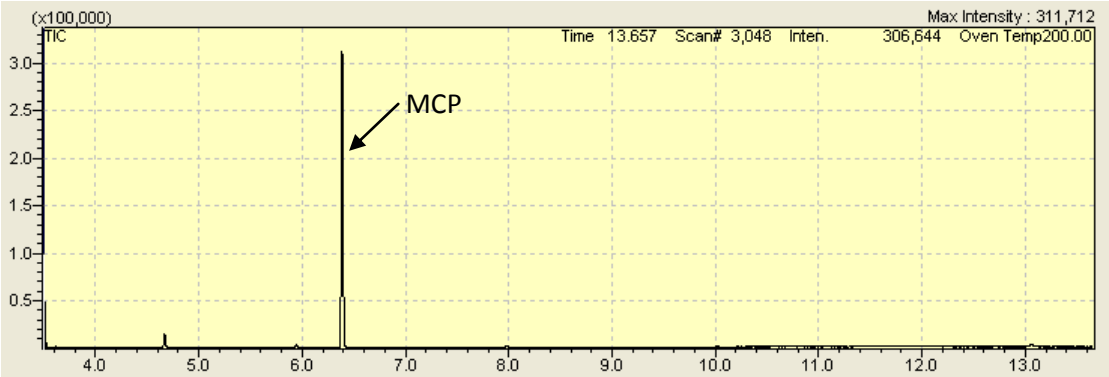


Figure 4.5g: Chromatogram showing degradation product(s) of D7 after 33 ± 3 days

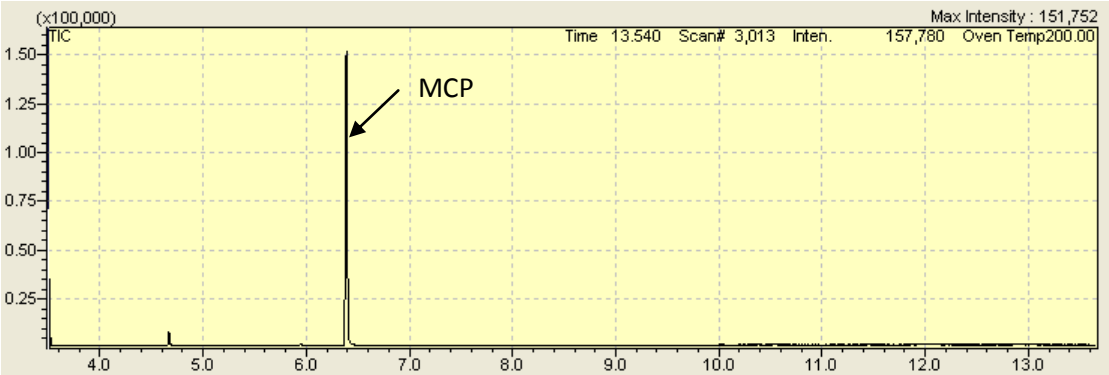


Figure 4.5h: Chromatogram showing degradation product(s) of D8 after 33 ± 3 days

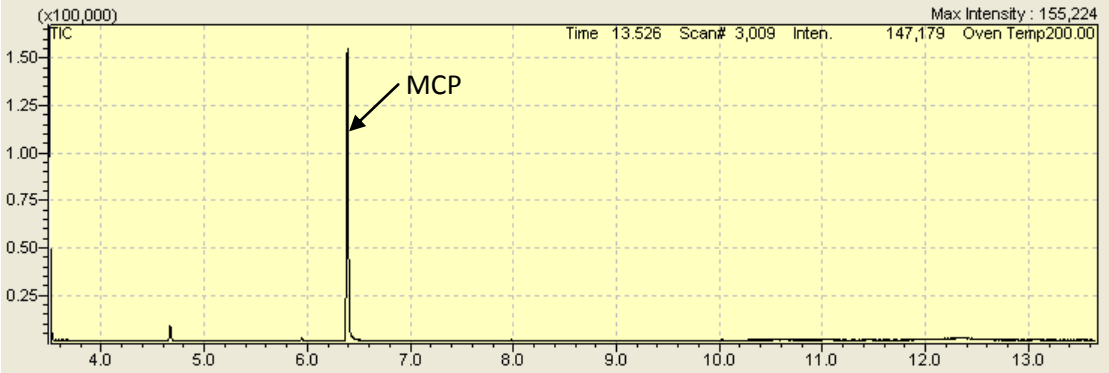


Figure 4.5i: Chromatogram showing degradation product(s) of D9 after 33 ± 3 days

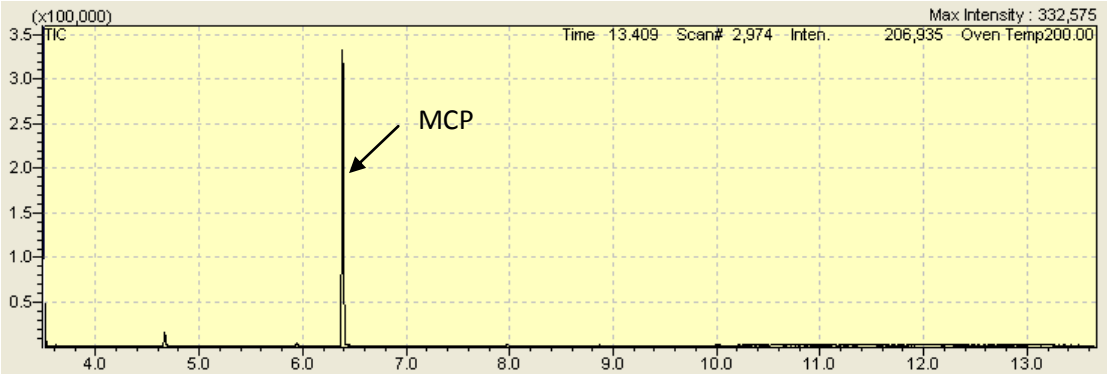


Figure 4.5j: Chromatogram showing degradation product(s) of D10 after 33 ± 3 days

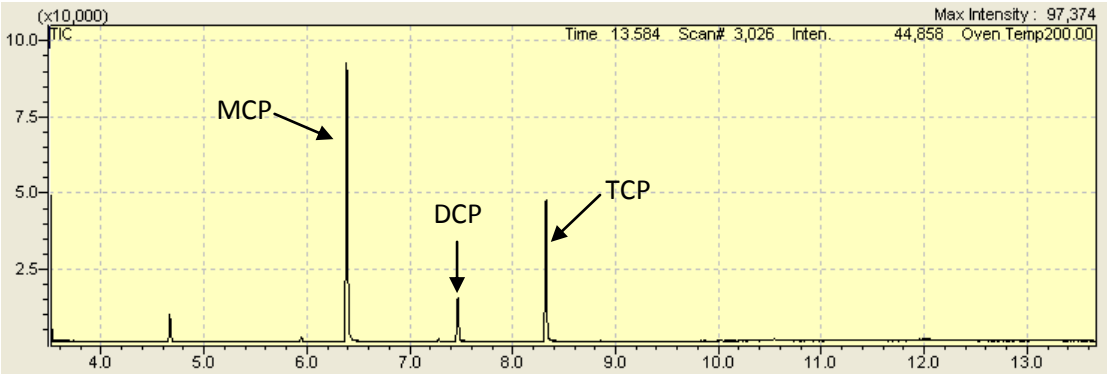


Figure 4.5k: Chromatogram showing degradation product(s) of D11 after 33 ± 3 days

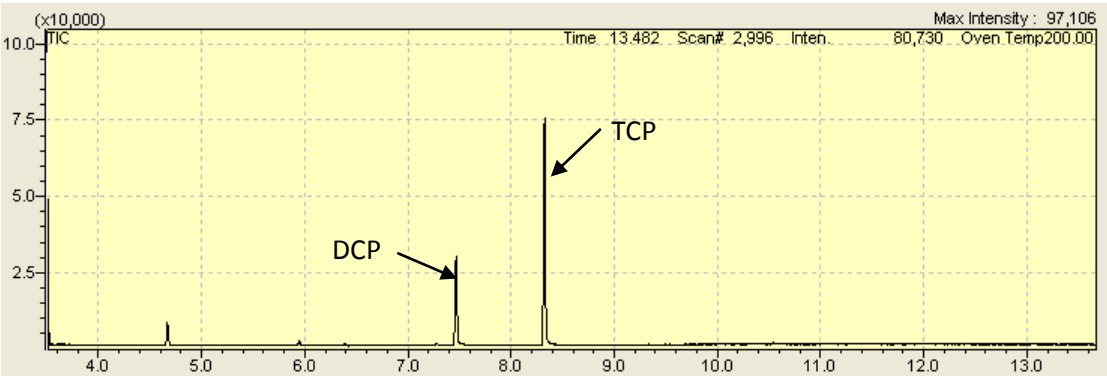


Figure 4.5l: Chromatogram showing degradation product(s) of D12 after 33 ± 3 days

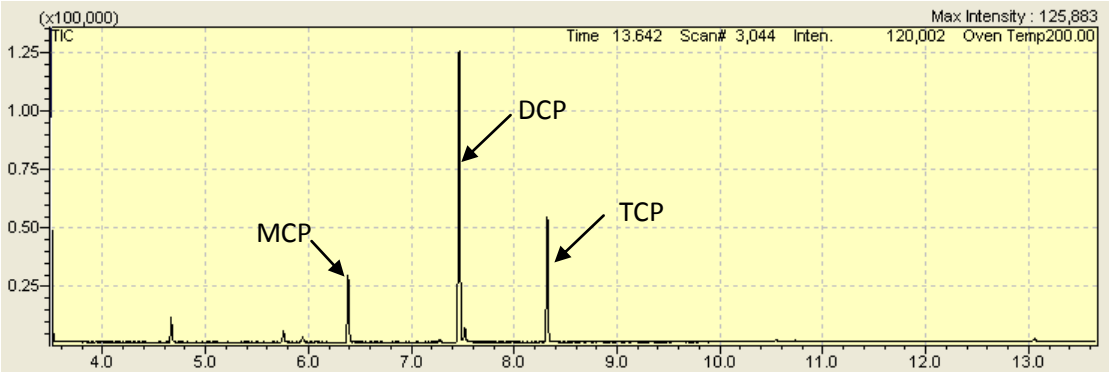


Figure 4.5m: Chromatogram showing degradation product(s) of PA after 33 ± 3 days

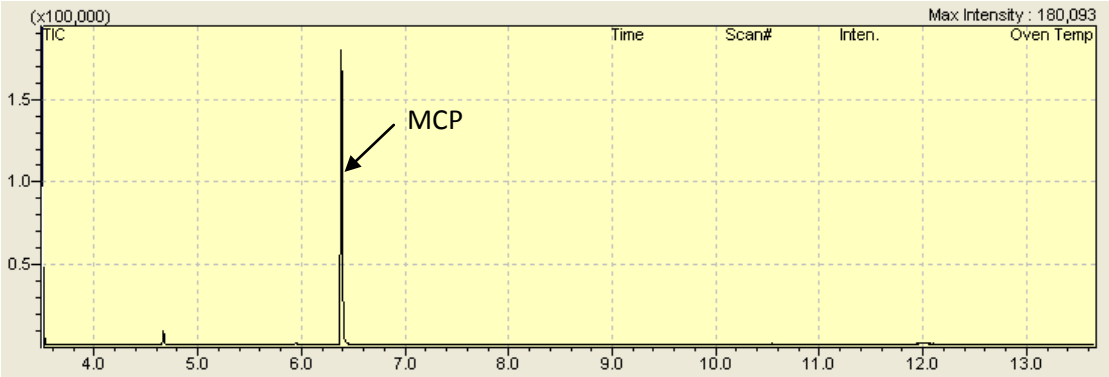


Figure 4.5n: Chromatogram showing degradation product(s) of PY after 33 ± 3 days

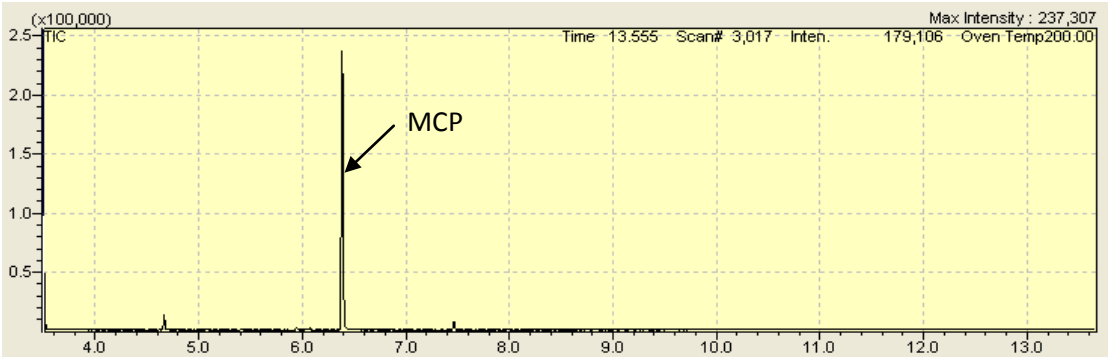


Figure 4.5o: Chromatogram showing degradation product(s) of SC after 33 ± 3 days

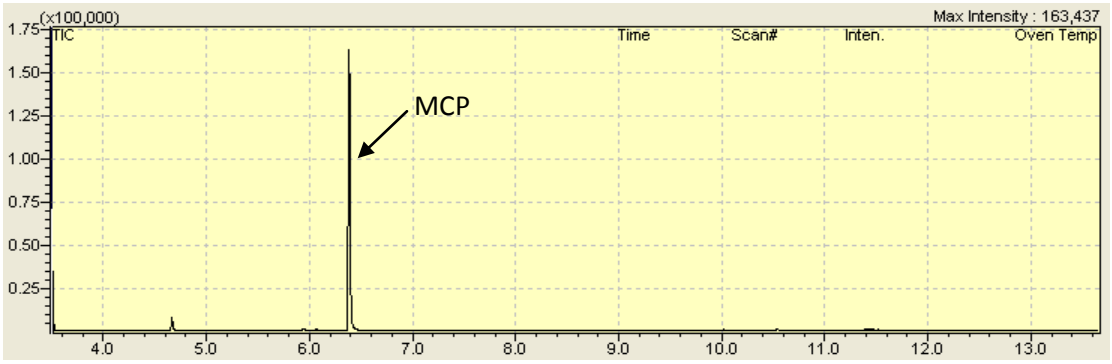


Figure 4.5p: Chromatogram showing degradation product(s) of BNN after 33 ± 3 days

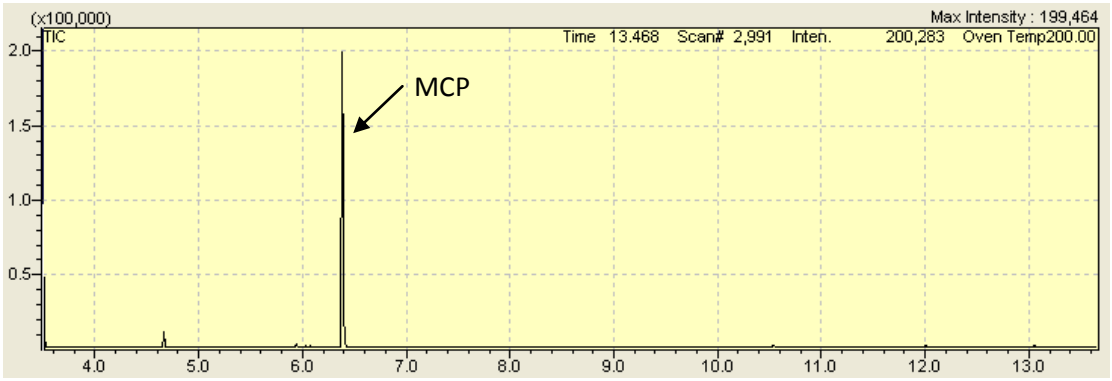


Figure 4.5q: Chromatogram showing degradation product(s) of LWN after 33 ± 3 days

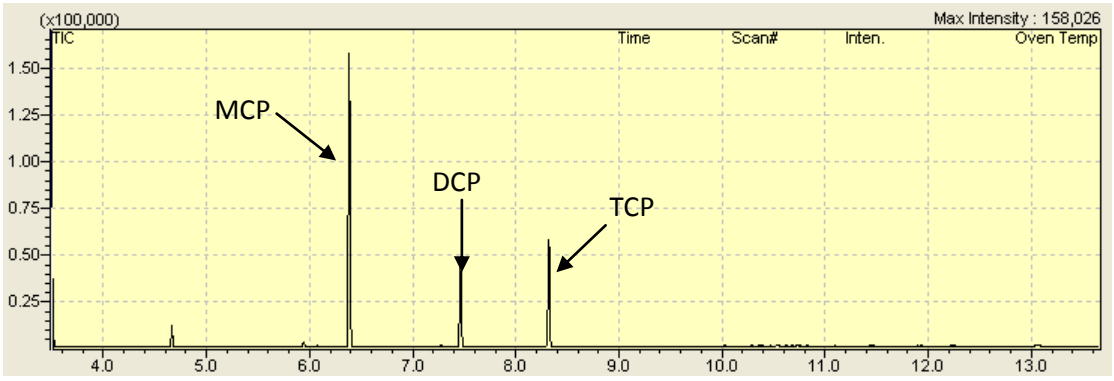


Figure 4.5r: Chromatogram showing degradation product(s) of ORC after 33 ± 3 days

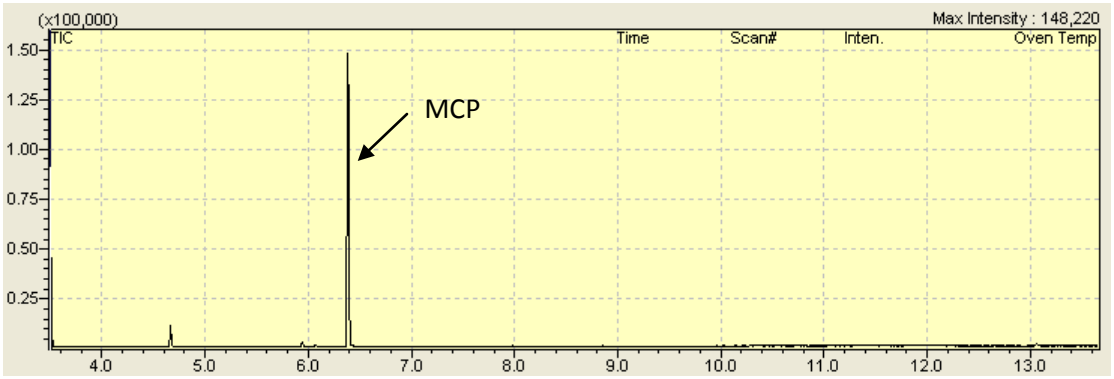


Figure 4.5s: Chromatogram showing degradation product(s) of RIV after 33 ± 3 days

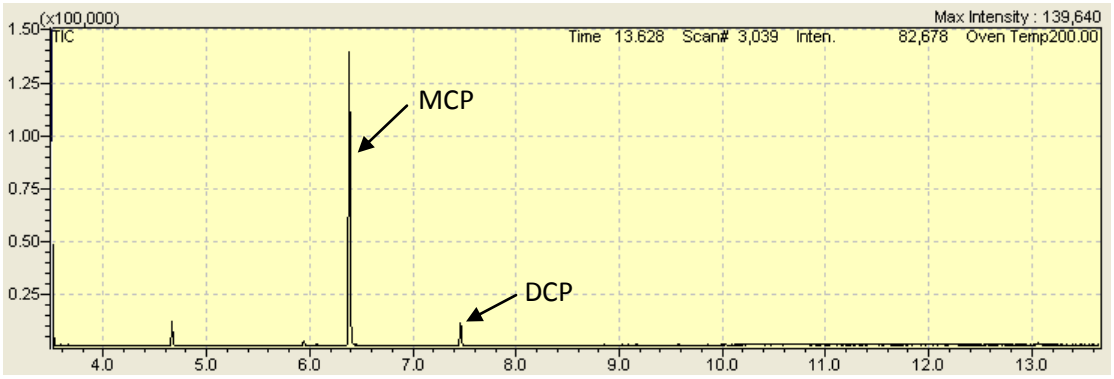


Figure 4.5t: Chromatogram showing degradation product(s) of TEA after 33 ± 3 days

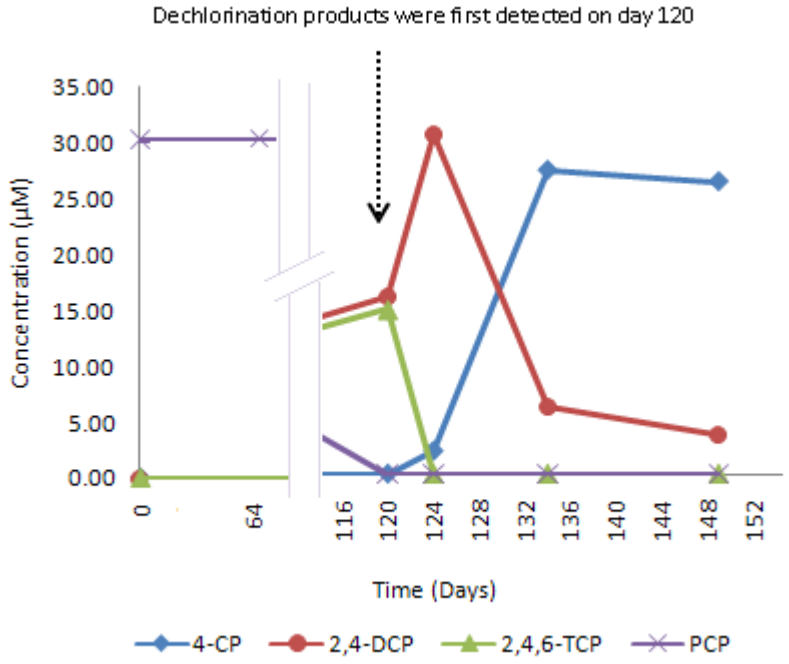
4.2.2 Dechlorination of Pentachlorophenol

Microcosms containing PCP were tested for dechlorination activities on a bi-weekly basis for the first 4 weeks and randomly but less frequently (e.g. once every 4 or 6 weeks) for a period of 20 weeks. Once dechlorination products were detected, tests were conducted more frequently (i.e. once a week). None of the cultures in the microcosms mentioned above showed any hint of PCP dechlorinating capability except for culture D12. Degradation products of PCP were first detected on the 120th day and this indicates the occurrences of dechlorination activities. By then, PCP were found to have been

completely dechlorinated and dechlorination products, 2,4,6-TCP and 2,4-DCP were detected. Dechlorination continued and both 2,4,6-TCP and 2,4-DCP were then completely dechlorinated to 4-CP within the next 30 days. However, dechlorination could not proceed beyond 4-CP after 20 weeks of incubation and thus, making 4-CP the dechlorination end product of culture D12 when fed with PCP.

Culture D12 is capable of the dechlorination of PCP and its intermediates, 2,4-6,TCP and 2,4-DCP to its end product, 4-CP. Figures 4.6A and 4.6B describe the time course study of PCP dechlorination and the dechlorination pathway by culture D12 respectively.

(A)



(B)

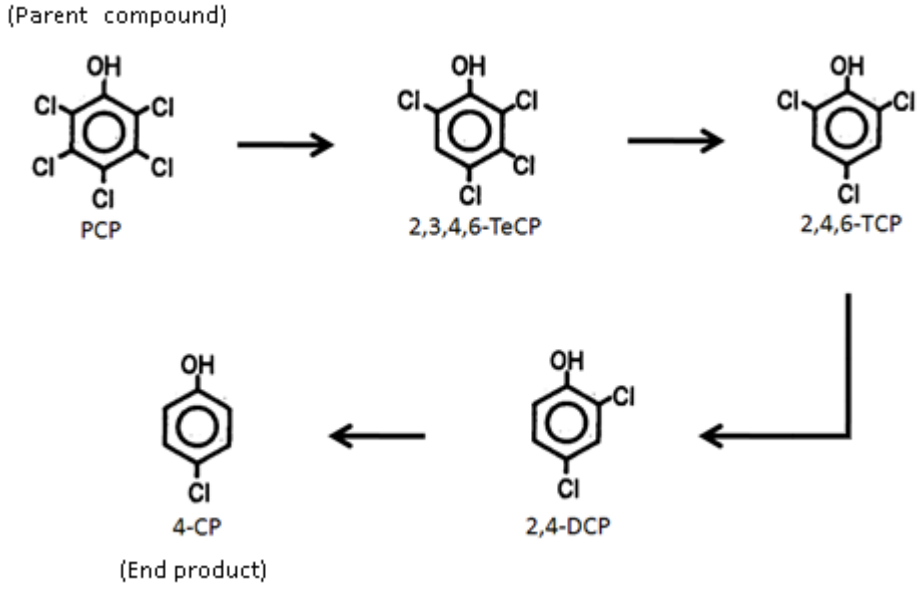


Figure 4.6: (A) Time course study of PCP dechlorination by culture D12. (B) Degradation pathway of PCP to 4-CP by culture D12.

4.3 Kinetics of Dehalogenation of Chlorophenols

Three of the cultures showing most effective and fastest dechlorination rate, LWN, RIV and SC as well as 3 other selected cultures, PY, D3 and D12, were subjected to an advanced study on their dechlorination capabilities. These cultures were sequentially transferred and enriched until sediment-free cultures were obtained before proceeding with the kinetic studies of chlorophenol dechlorination. For this purpose, 2 mL of the active cultures (cultures in exponential or stationary phase) were inoculated into 98 mL of liquid medium in 160 mL sterile bottles. For all the 6 cultures studied, pyruvate (10 mM) was added as the electron donor while 2,4,6-TCP (ca. 50 - 60 mM) was used as the electron acceptor.

The dechlorination kinetics of 2,4,6-TCP of sediment-free cultures, LWN, RIV, SC, PY, D3 and D12, after 6 sequential transfers are represented in the graphs below.

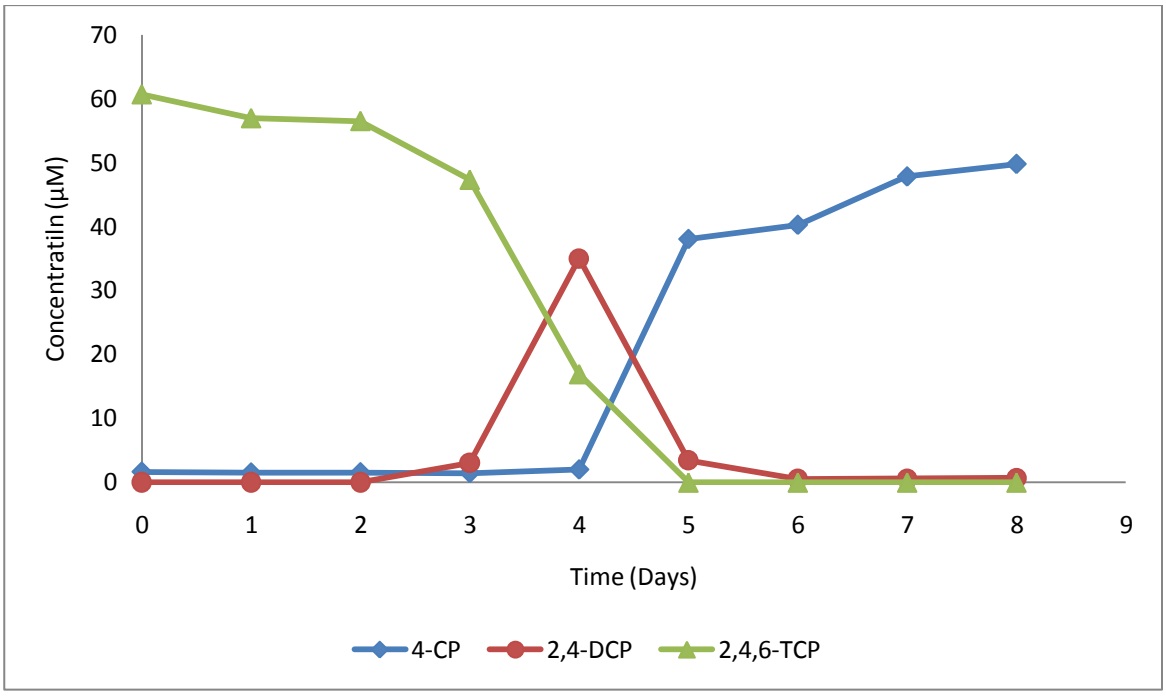


Figure 4.7: 2,4,6-TCP dechlorination kinetics of culture LWN.

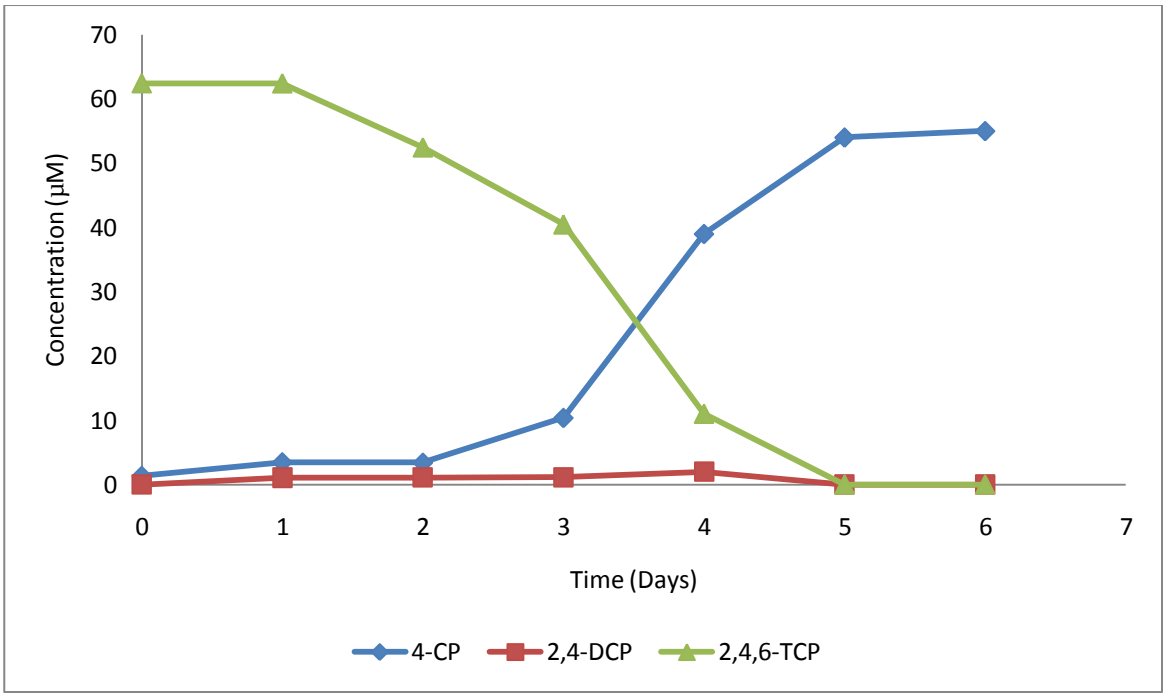


Figure 4.8: 2,4,6-TCP dechlorination kinetics of culture RIV.

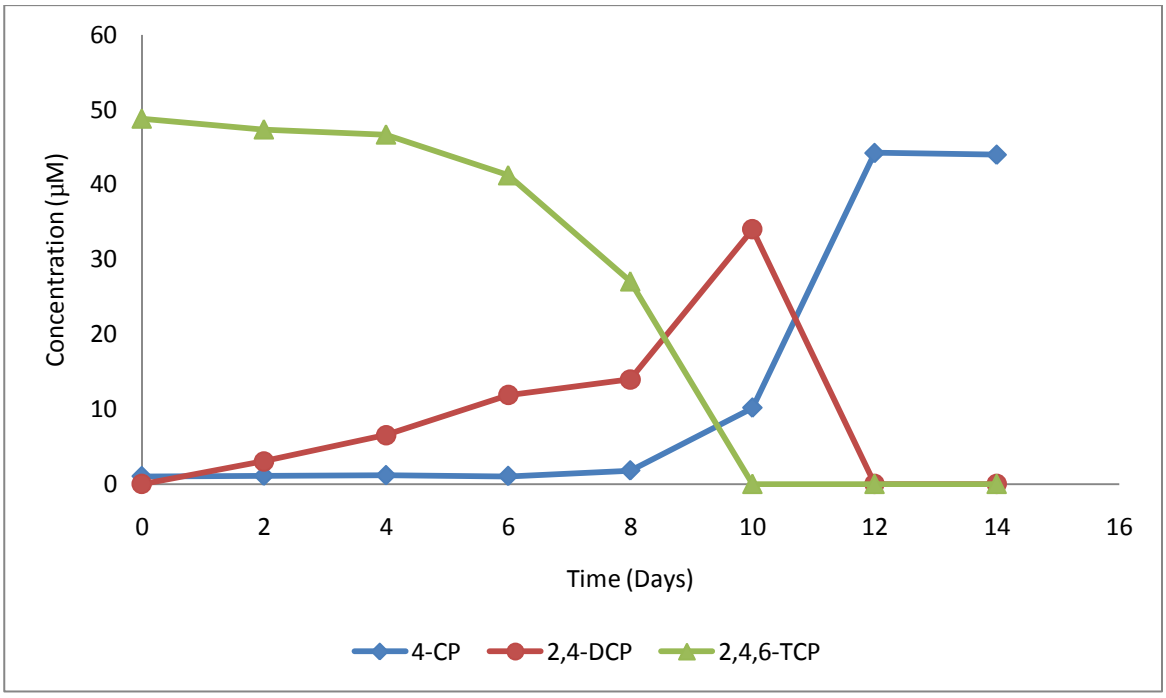


Figure 4.9: 2,4,6-TCP dechlorination kinetics of culture SC.

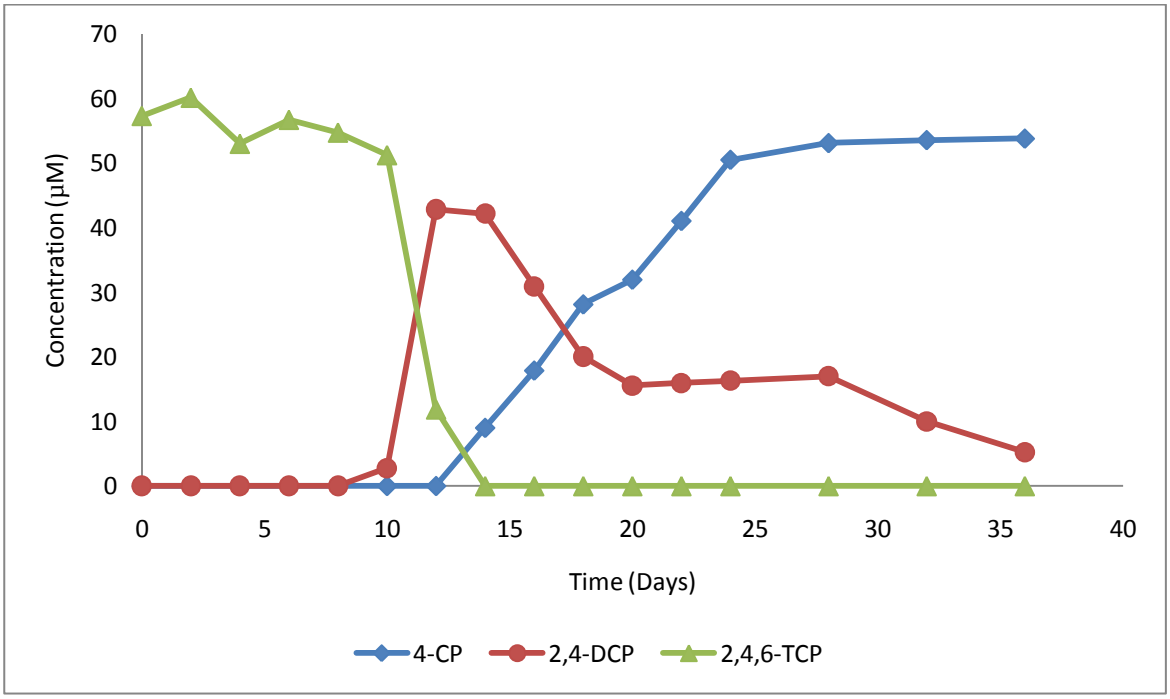


Figure 4.10: 2,4,6-TCP dechlorination kinetics of culture PY.

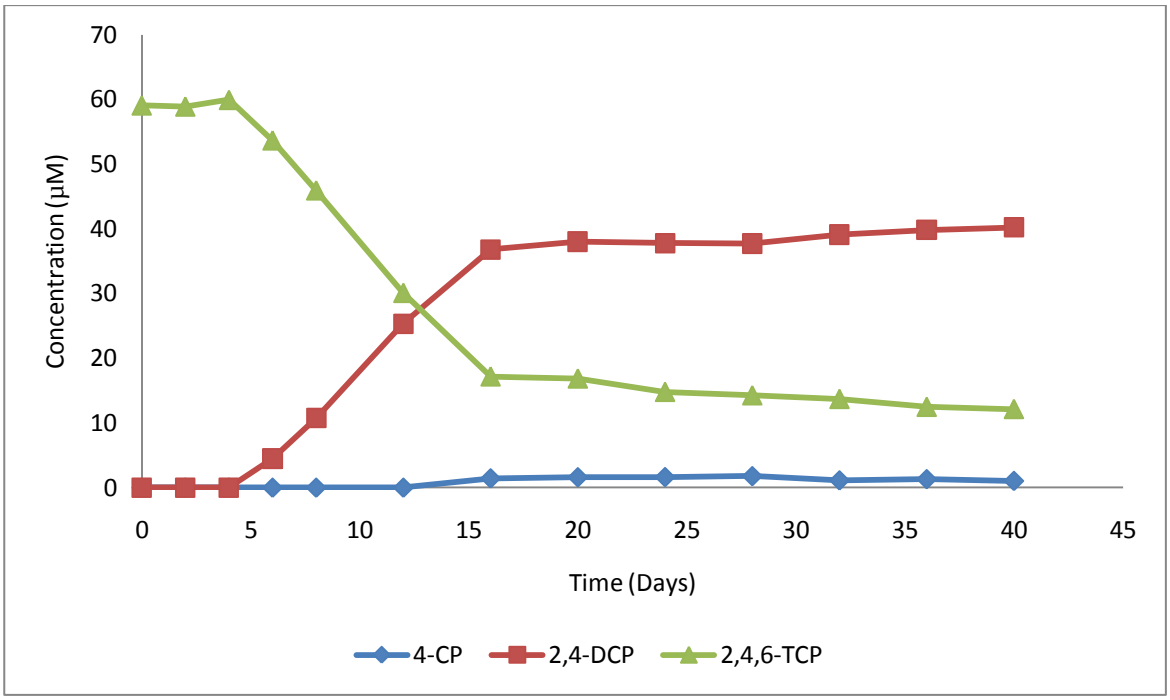


Figure 4.11: 2,4,6-TCP dechlorination kinetics of culture D3.

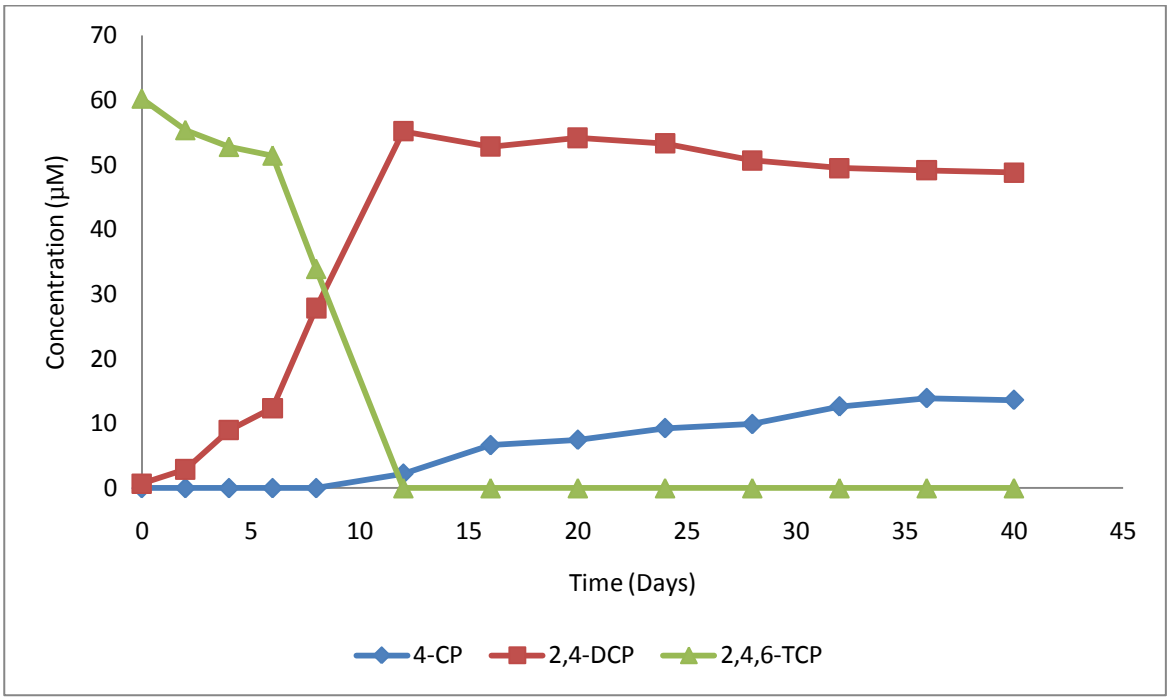


Figure 4.12: 2,4,6-TCP dechlorination kinetics of culture D12.

4.4 Effects of Different Electron Donors on Reductive Dehalogenation

The effects of electron donors on the fate of 2,4,6-TCP following reductive dehalogenation were studied for cultures LWN, RIV and SC and were compared to the previous studies when pyruvate was used as the electron donor. The types of electron donors used for this purpose were:

i. lactate (10 mM)

ii. acetate (10 mM)

iii. acetate (10 mM) + hydrogen (6 mL - added using a sterilized disposable plastic syringe at partial pressure of 3.4×10^4 Pa)

The study showed similar outcomes to the results of the earlier studies when pyruvate was used as the electron donor for all cultures. 2,4,6-TCP was dechlorinated via 2,4-DCP producing 4-CP as the end product. The rate of which 2,4,6-TCP was dechlorinated did not differ much either.

Cultures LWN, RIV and SC can utilize the type of electron donors as listed above for the dechlorination of 2,4,6-TCP and will not affect their effectiveness and degradation rate.

4.5 Reductive Dehalogenation with Different Electron Acceptors

Cultures LWN, RIV and SC were tested on their abilities to degrade other halogenated organic compounds as electron acceptors. Using 10 mM of pyruvate as the sole electron donor, halogenated compounds like chlorinated ethenes, polybrominated diphenyl ethers

(PBDE) and polychlorinated biphenyls (PCB) were used in place of chlorophenols and tested for dehalogenation activities. The types of electron acceptors used here were:

i. Trichloroethene (TCE)

ii. Commercial Pentabromodiphenyl Ether mixture (Penta-BDE)

iii. 2,2',4,4',6,6'-Hexachlorobiphenyl (PCB 155)

The cultures were incubated for a period of 9 weeks and tested for dehalogenation activities.

4.5.1 Dechlorination of Chlorinated Ethenes

Trichloroethene (TCE), a suspected human carcinogen, commonly used as solvent for a variety of organic materials was used as substrate for dechlorination by cultures LWN, RIV and SC.

Over the course of 9 weeks, cultures LWN and RIV were unable to reductively dehalogenate TCE. Culture SC however was successful in dechlorinating ~20 µmoles of TCE to *trans*- and *cis*-dichloroethene (DCE) as the final products. TCE were completely dechlorinated to *trans*- and *cis*-DCE in a ratio of 3:1. Figure 4.13 shows the dechlorination pathway of TCE to *trans*- and *cis*-DCE by culture SC.

Culture SC was then sequentially transferred for further studies and investigation on its chloroethene dechlorinating ability.

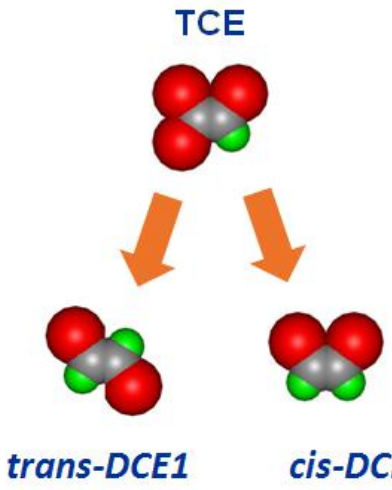


Figure 4.13: Dechlorination pathway of TCE to *trans*- and *cis*-DCE in a ratio of 3:1 by culture SC.

4.5.2 Debromination of Polybrominated Diphenyl Ethers

The commercial mixture of Penta-BDE dissolved in ethyl acetate (consisting of Tetra-, Penta and Hexa-BDE), a known endocrine disruptor which is commonly used as additives for flame retardants, was used as substrate and checked for debromination activities by cultures LWN, RIV and SC.

Of the 3 cultures used during the entire period of study, only culture RIV was able to show signs debromination. Chromatogram from GC-MS analysis (see Figure 4.14) revealed a significant decrease in the peaks of the parent compounds, Hexa- and Penta-BDE, with a corresponding increase in Tetra-BDE peaks as the degradation products after 63 days.

Culture RIV was then sequentially transferred for further studies on its PBDE debrominating capabilities.

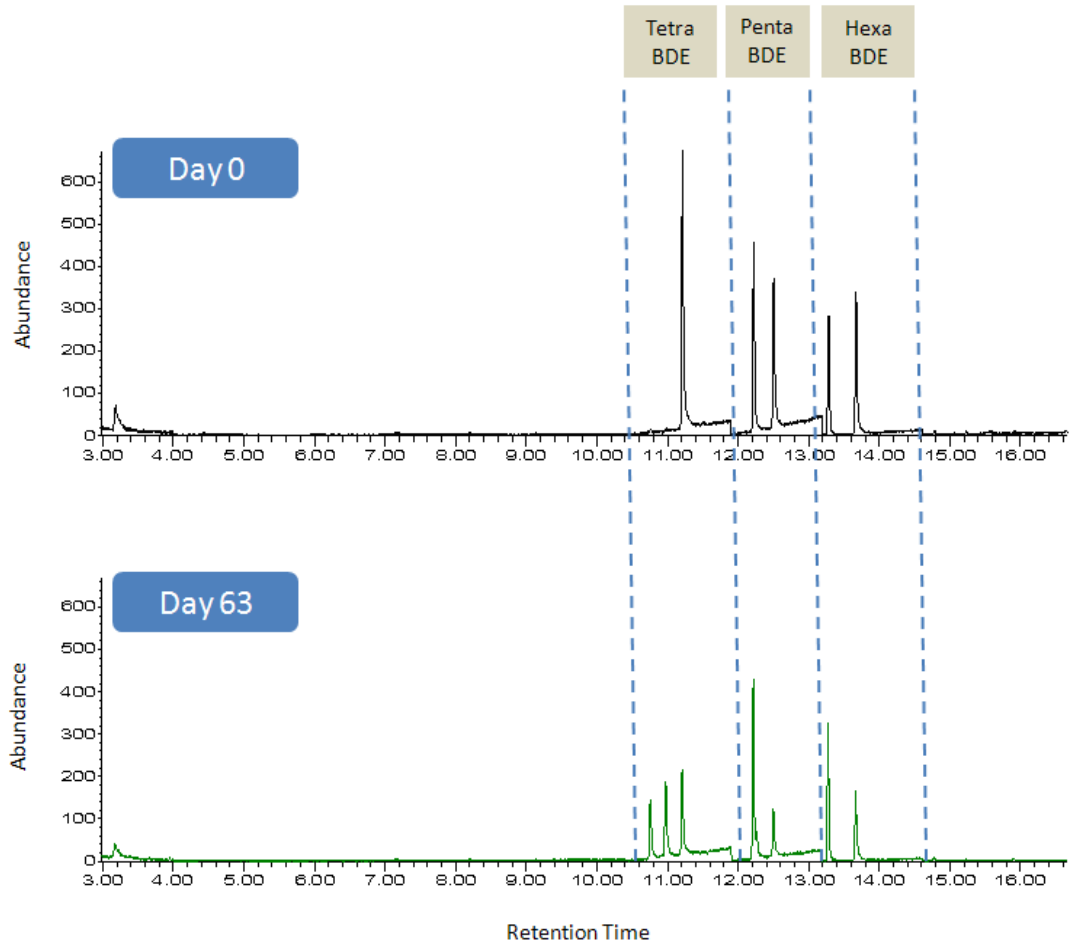


Figure 4.14: Chromatogram showing degradation of parent compound (Day 0) to debromination product (Day 63).

4.5.3 Dechlorination of Polychlorinated Biphenyls

2,2',4,4',6,6'-Hexachlorobiphenyl or simply PCB 155, classified as a persistent organic pollutant and also another recognized carcinogen found in commercial mixtures of PCB was tested to determine if it can be dechlorinated by cultures LWN, RIV and SC. Unfortunately, during the study period of 9 weeks, no traces of dechlorination products

were found. Cultures LWN, RIV and SC are incapable of dechlorinating PCB 155 for the duration of the study.

4.6 Presence of Possible Chlorophenol Dechlorinating Microbes in Cultures

DNA extracted from cells of cultures showing positive response to chlorophenol degradation were collected and amplified using the polymerase chain reaction technique. The cultures selected here were LWN, RIV, SC and PY for their rapid dechlorinating capability as well as their ability to completely dechlorinate 2,4,6-TCP.

To identify and acquire information of possible dehalogenating microbes present in the cultures, genus specific primer pairs were used to target the 16S rRNA gene of genomic DNA extracted from the 4 culture samples as given above. The primer pairs used were that of those belonging to the genera of *Dehalococcoides* (DHC), *Desulfitobacterium* (DST), *Anaeromyxobacter* (AMB), *Desulfovibrio* (DSV), *Desulforomonas* (DSF), *Desulfomonile* (DSM), *Acetobacterium* (ACE) and *Dehalobacter* (DEB). These were selected based on previous reports of microorganisms belonging to the aforementioned with abilities to dechlorinate chlorophenols such as DHC, DST, AMB and DSV as well as those capable of degrading other halogenated compounds (e.g. PBDE, PCB, PCE etc.) such as DSF, DSM, ACE and DEB. The products of the cultures' DNA targeted with the genus specific primer pairs were stained and subjected to gel electrophoresis. Subsequently, the bands on the gel were viewed. Figures 4.15 and 4.16 show the results of the work carried out.

When the genomic DNA of culture RIV were targeted with *Desulfitobacterium* primers, clear bands with high intensity were observed on the gel showing presence of amplicons with the size of 225 base pairs (Figure 4.15, Lane 18). No other amplicons from the other genera tested were observed.

Meanwhile, amplicons from the genera *Dehalococcoides* and *Desulfovibrio* were detected in culture SC. The band indicating the presence of the amplicons belonging to the genus, *Dehalococcoides* was found to be very bright and clear (Figure 4.15, Lane 10). On the other hand, the amplicon band from the *Desulfovibrio* genus paled in comparison and was found to be significantly lower in terms of the intensity of the band's brightness (Figure 4.16, Lane 18). Amplicons for the other 6 genera were not detected.

Attempts to target extracted DNA from cultures PY and LWN with the 8 genus specific primers known for their dehalogenating abilities yielded negative results with no bands of amplicons detected in the gel following electrophoresis.

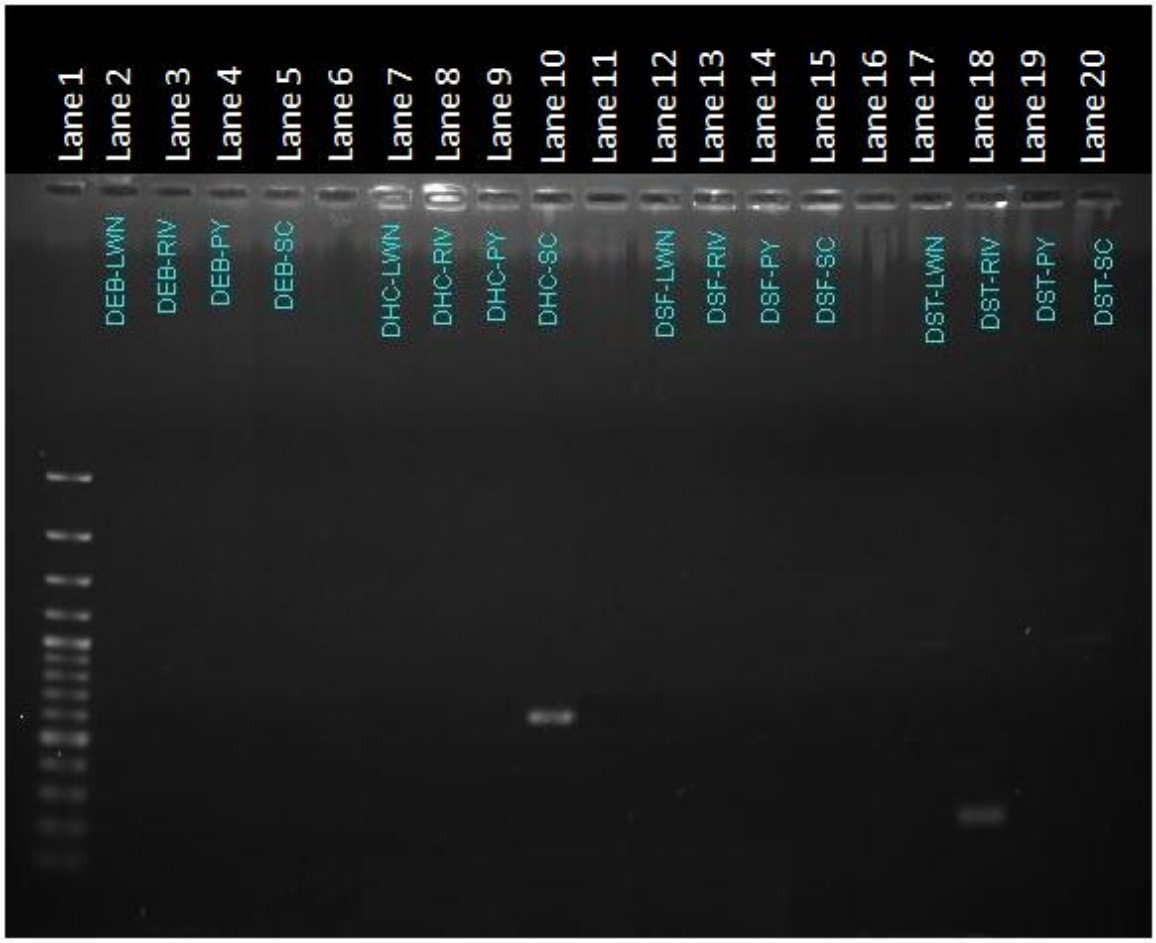


Figure 4.15: Gel electrophoresis results for cultures' DNA targeted with genus specific primers (DEB, DHC, DSF and DST) using the direct PCR approach.

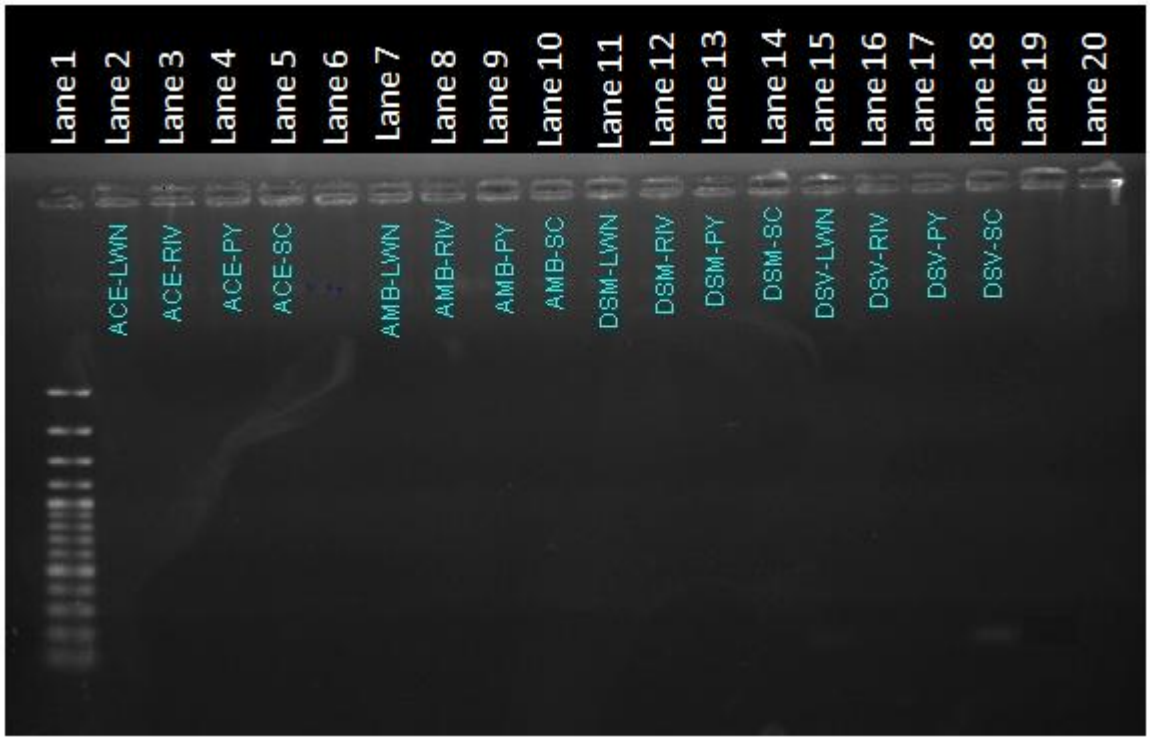


Figure 4.16: Gel electrophoresis results for cultures' DNA targeted with genus specific primers (ACE, AMB, DSM and DSV) using the direct PCR approach.

The nested PCR approach was then carried out and the 16S rRNA gene of genomic DNA extracted from the same cultures, LWN, RIV, SC and PY, were again targeted with similar genus specific primers used in the direct PCR approach discussed above.

Similar to that from the direct PCR approach, amplicons from the genus *Desulfitobacterium* was detected for culture RIV while amplicon bands from the genera *Dehalococcoides* and *Desulfovibrio* were again detected for culture SC. This time around however, amplicon bands from the genera *Dehalococcoides* and *Desulfitobacterium* were detected for culture PY. In addition to that, amplicons belonging to the genera *Acetobacterium* and *Desulfovibrio* were found in all 4 cultures tested. These amplicons, however, were present in bands with weak intensity in terms of their brightness as

compared to the others. Figures 4.17 and 4.18 describe the result of the study while Tables 4.1 and 4.2 summarize both sets of experiments using direct and nested PCR approach respectively.

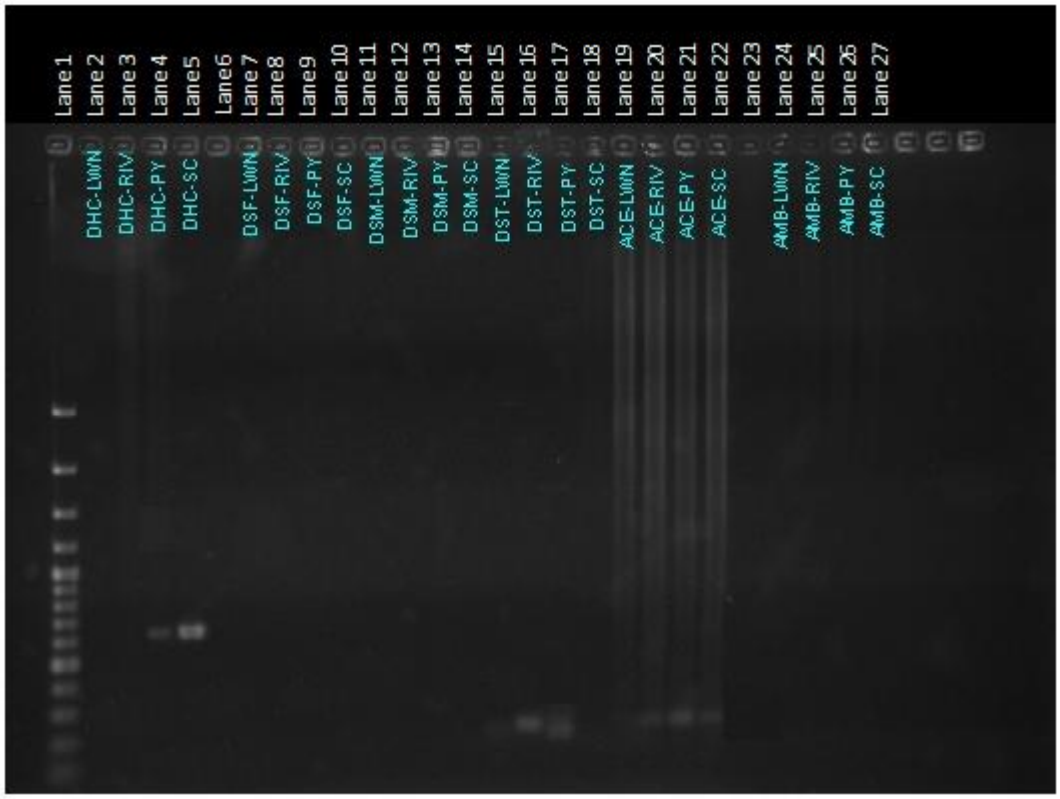


Figure 4.17: Gel electrophoresis results for cultures' DNA targeted with genus specific primers (DHC, DSF, DSM, DST, ACE and AMB) using the nested PCR approach.

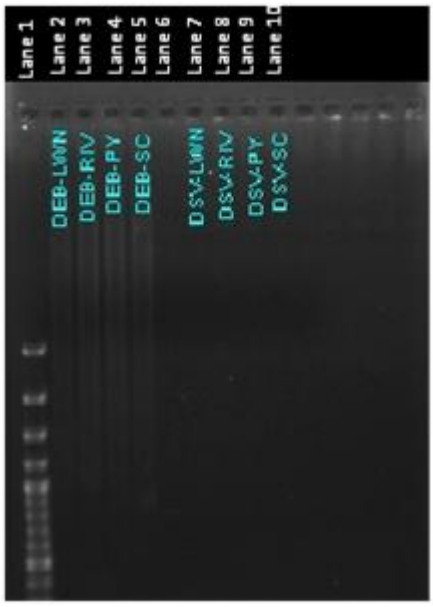


Figure 4.18: Gel electrophoresis results for cultures' DNA targeted with genus specific primers (DEB and DSV) using the nested PCR approach.

Table 4.1: Summary of results for the detection of microorganisms from specific genera using the direct PCR approach.

Culture	Genus							
	DHC	DSF	DSM	DST	ACE	AMB	DEB	DSV
LWN	×	×	×	×	×	×	×	×
RIV	×	×	×	√	×	×	×	×
PY	×	×	×	×	×	×	×	×
SC	√	×	×	×	×	×	×	√

Note: '√' denotes presence of amplicon band of corresponding genus type; '×' denotes absence of amplicon band of corresponding genus type.

Table 4.2: Summary of results for the detection of microorganisms from specific genera using the nested PCR approach.

Culture	Genus							
	DHC	DSF	DSM	DST	ACE	AMB	DEB	DSV
LWN	×	×	×	×	√	×	×	√
RIV	×	×	×	√	√	×	×	√
PY	√	×	×	√	√	×	×	√
SC	√	×	×	×	√	×	×	√

Note: '√' denotes presence of amplicon band of corresponding genus type; '×' denotes absence of amplicon band of corresponding genus type.

4.7 Microbial Community Shift in Culture LWN

TRFLP was used to the change of the microbial community composition culture LWN. 50 mM of 2,4,6-TCP was used for the earlier and more mixed generations and the concentration was gradually increased with each sequential transfer for enrichment. 3 active cultures were compared here – 1 sample, LWN3, from generation 3 spiked with 50 µM of 2,4,6-TCP and 2 samples, LWN9-500 and LWN9-1000, from generation 9 spiked with 500 µM and 1000 µM of 2,4,6-TCP respectively. Restriction enzymes, *Hha*I, *Msp*I and *Rsa*I, were used for the TRFLP analysis.

The fluorescently labeled terminal restriction fragments (TRF) from the digests revealed significant changes in community composition following enrichment of culture LWN from generation 3 to generation 9 as well as the increase of 2,4,6-TCP concentration for reductive dehalogenation from 50 µM to 1000 µM. The electrophenograms of the study are given below. The horizontal axis shows the length of the terminal restriction

fragments in nucleotide bases and the vertical axis shows the relative level of fluorescence intensity.

TRFLP revealed significant differences in the communities between the 3rd and the 9th generation of culture LWN for all the restriction enzymes used. The shifts in the community composition can be observed by the loss of many TRFs and a corresponding increase of others. Take cultures LWN3 and LWN9-1000 digested with *Msp*I restriction enzyme as examples (Figures 4.20A and 4.20C). Community profile shows the disappearance in the area of TRFs from 184 to 220 nucleotides while most of the TRFs in the area between 471 and 636 nucleotides have completely disappeared leaving only a few low and insignificant peaks. Most notably, dominant TRFs at 96 and 97 nucleotides for LWN3 have disappeared completely. On the other hand, a significant increase of TRFs at 162 nucleotides can be observed with its dye signal rising from 4,200 to 56,000.

An increase in the concentration of substrate (2,4,6-TCP) also resulted in the change of the microbial composition of culture LWN. Take Figures 4.20B and 4.20C as examples. Both are the 9th generation of culture LWN with 500 µM of 2,4,6-TCP spiked into the former and 1,000 µM of 2,4,6-TCP in the latter. Community profile shows either the complete disappearance or significant decrease for all except for the TRF in the area of 162 nucleotides whereby its dye signal's intensity increased from 17,500 to 56,000.

Dominant peaks corresponding to the terminal fragment size of digested PCR products from culture LWN9-1000 (i.e. the most enriched culture) were compared to that of known dechlorinators and no match was found. This could suggest the possibility of a new strain of TCP dechlorinating microbe.

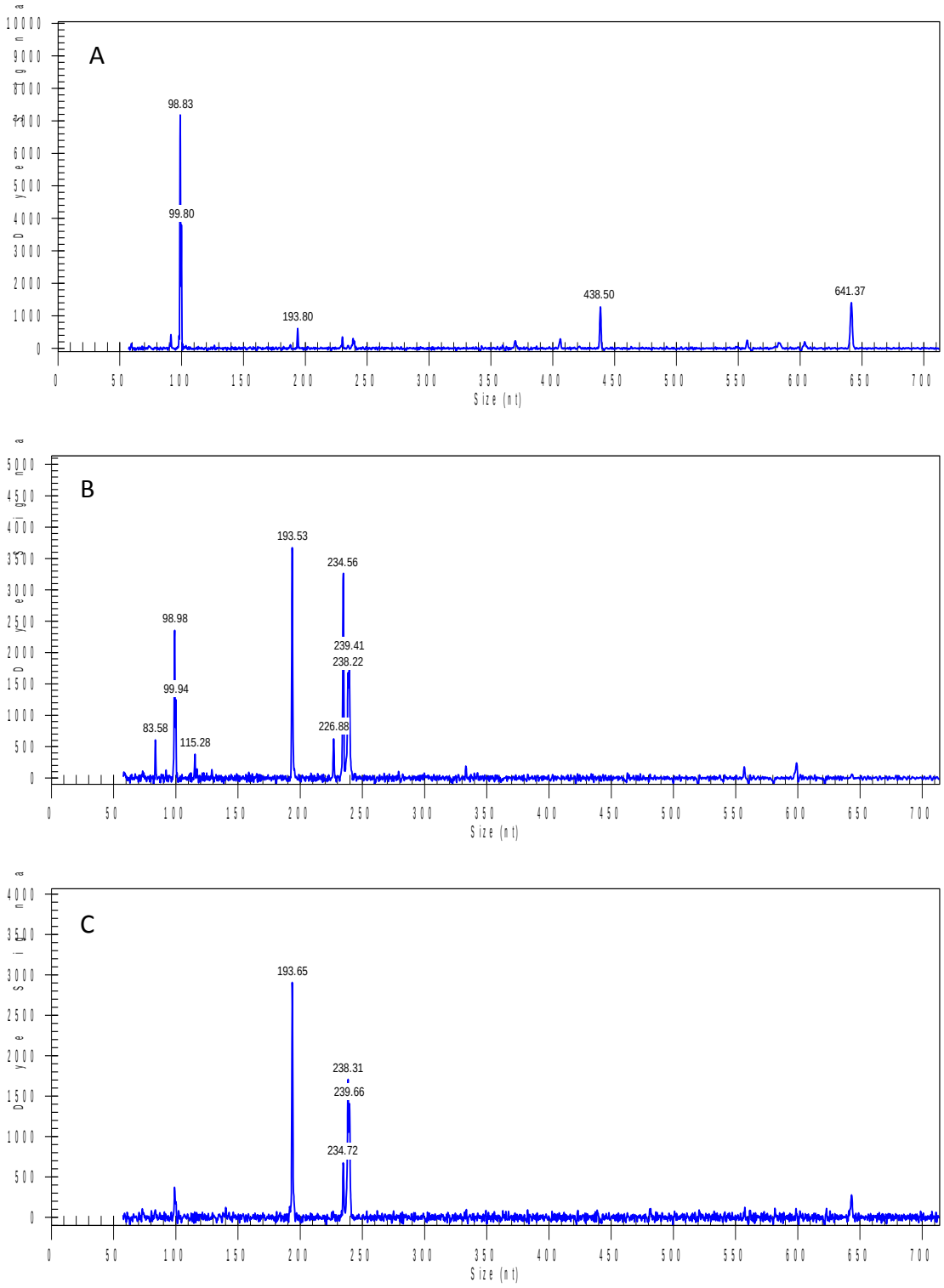


Figure 4.19: (A) Electrophenogram of culture LWN3 using the *Hha*I restriction enzyme. (B) Electrophenogram of culture LWN9-500 using the *Hha*I restriction enzyme. (C) Electrophenogram of culture LWN9-1000 using the *Hha*I restriction enzyme.

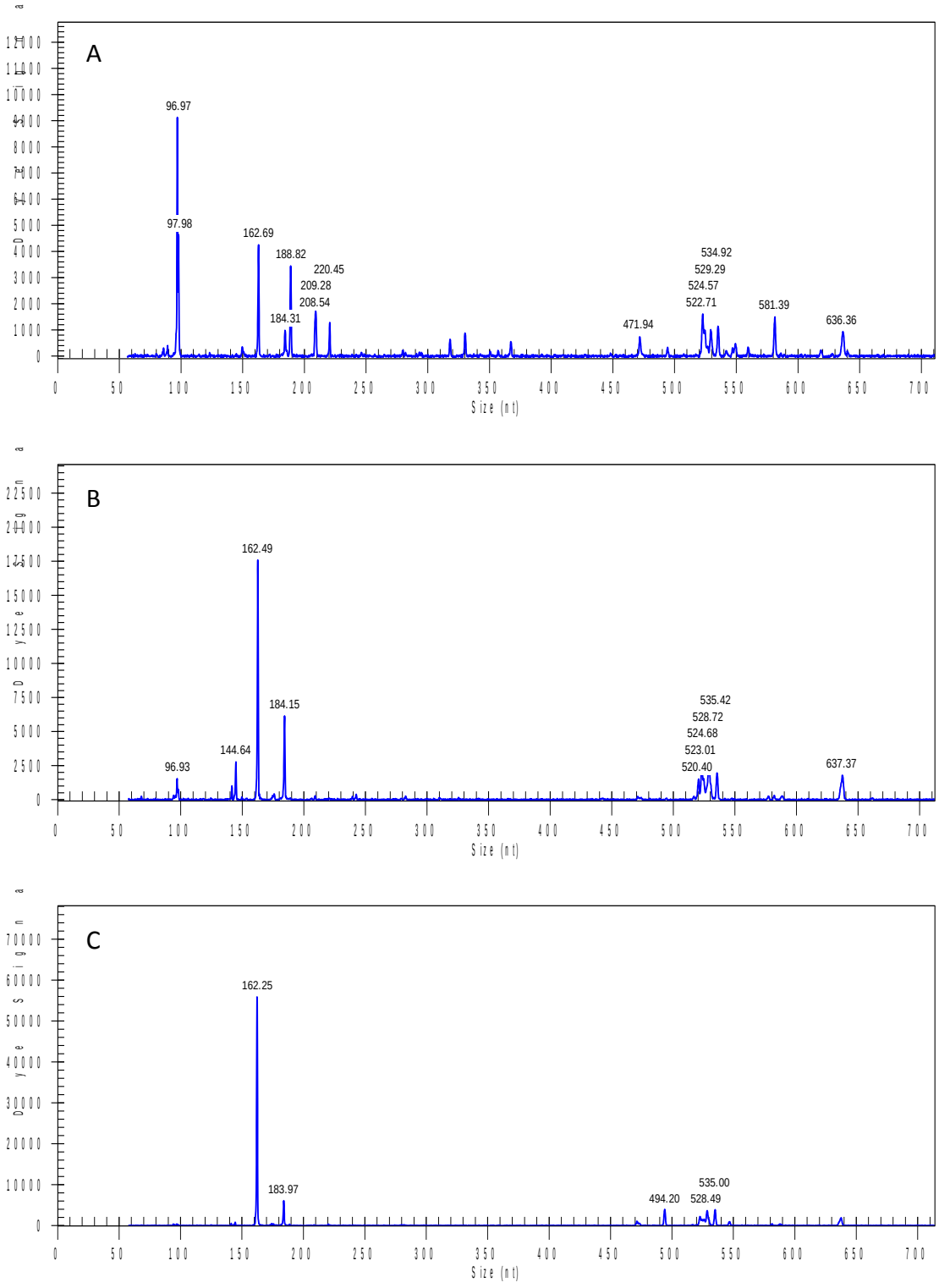


Figure 4.20: (A) Electrophenogram of culture LWN3 using the *Msp*I restriction enzyme. (B) Electrophenogram of culture LWN9-500 using the *Msp*I restriction enzyme. (C) Electrophenogram of culture LWN9-1000 using the *Msp*I restriction enzyme.

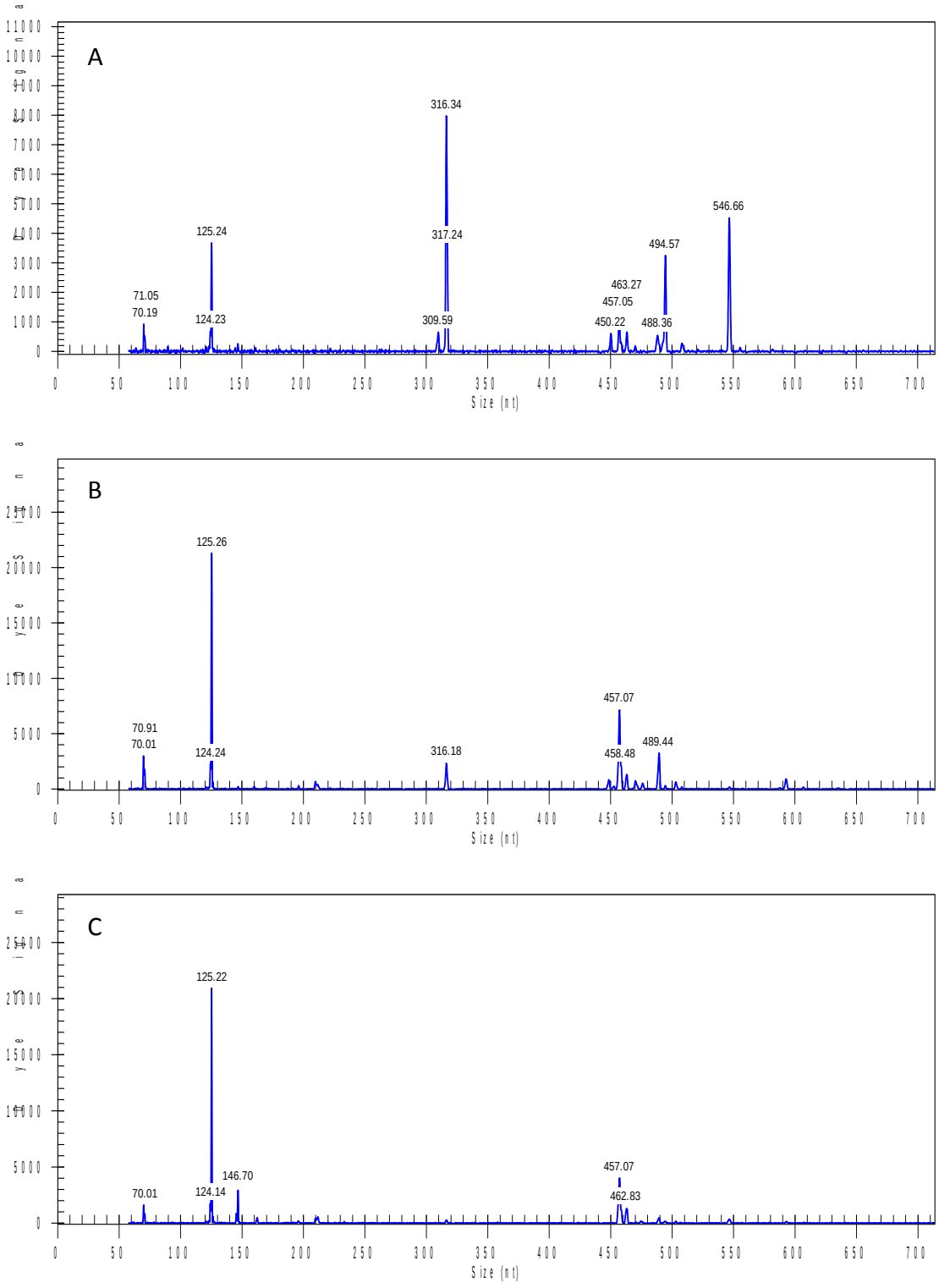


Figure 4.21: (A) Electrophenogram of culture LWN3 using the *Rsa*I restriction enzyme. (B) Electrophenogram of culture LWN9-500 using the *Rsa*I restriction enzyme. (C) Electrophenogram of culture LWN9-1000 using the *Rsa*I restriction enzyme.

4.8 Identifying the Possible Dechlorinator in Culture LWN

The analyses following TRFLP on culture LWN as described in the previous section have given us an understanding of the changes and the profile of the microbial community composition with each transfer and with the increase of chlorophenol concentration. From the electrophenogram for culture LWN9-1000 digested with the *Msp*I enzyme (Figure 4.20C), it is evident that there is a dominant strain of bacterium which is present within the culture that responds positively with the enrichment process and also with the increase of the concentration of chlorophenol.

The DNA of culture LWN9-1000 is then harvested again; amplified via PCR and digested with enzyme *Msp*I. The products were then subjected to gel electrophoresis and the amplicon band corresponding to 162 base pairs was excised from the gel. The DNA products were purified and sequenced using the 8F primer. The sequencing result is shown in Figure 4.22.

TGCTACACATGCAGTCGACGAAGTATATTTTCGGATATGCTT
AGTGGCGGACGGGTGAGTAACGCGTGAACAATCTGCCCTGT
ACACAGGAATAGCCTCGGGAAACTGGGATTAAAACCGAGA

Figure 4.22: Partial DNA sequencing results of culture LWN

A BLAST-N (Basic Local Alignment Search Tool – Nucleotide) analysis from the NCBI (National Center for Biotechnology Information) database revealed that the bacterial strain corresponding to the 162 base pairs from the TRFLP analysis for the DNA products

of culture LWN digested with the *Msp*I enzyme showed 98% homology with the 16S rRNA of several other microbes. Table 4.3 summarizes a list of these microorganisms which show some possible affiliation with reductive dehalogenation of halogenated products.

Table 4.3: List of microorganisms with strong affiliation to culture LWN.

Accession No.	Description	Max. Ident.
AM933661.1	Sedimentibacter sp. enrichment culture clone MB2_218 partial 16S rRNA gene	98%
AF349757.2	Uncultured bacterium TCE41 16S ribosomal RNA gene, partial sequence	98%
AY766466.1	Sedimentibacter sp. C7 16S ribosomal RNA gene, partial sequence	98%
AY673993.1	Sedimentibacter sp. B4 16S ribosomal RNA gene, partial sequence	98%
NR_025498.1	Sedimentibacter saalensis strain ZF2 16S ribosomal RNA, partial sequence	98%

CHAPTER 5

DISCUSSION

5.1 The Importance of Derivatization Prior to Gas Chromatographic Analysis

Due to adsorption problems and high polarity (especially with the lower congeners), chlorinated phenols tend to give broader and tailed peaks in chromatography, with the effects increase as the column ages (Mussman et al., 1994). This will, in turn, affect the quality of the chromatographic peaks and subsequently, influence the reliability of the results from the chromatographic analysis. While the extraction of chlorophenols from the aqueous phase can be achieved as either their native species (to some extent) or as less polar derivatives (Ramil Criado et al., 2004), the latter is preferred in this study since this is the critical step in analytical determination and also because chlorophenols need to be measured and quantified at low concentrations (e.g. μg/L).

One way of circumventing these shortcomings is to derivatize chlorinated phenols to less polar compounds to obtain more favorable chromatographic peaks (Ballesteros et al., 1990). Derivatization, or in this case, acetylation of chlorophenols with acetic anhydride, is one of the procedures widely employed to convert chlorophenols into less polar compounds which ultimately causes an increase in the extraction efficiency (Fattahi et al., 2007). The derivatization leads to sharper peaks and therefore to better separation and higher sensitivity.

Derivatization of chlorophenols prior to extraction is essential for yielding good and reliable results for the purpose of detection and quantification. As such, derivatization of all congeners of chlorophenols was carried out in subsequent experiments in this study.

5.2 Reductive Dehalogenation of Chlorophenols

A total of 20 microcosms made up of sediments, soils and various forms of slurry from distinctly different sources were set up to test for their ability to dechlorinate 2,4,6-TCP and PCP. These were collected from both engineered (e.g. wastewater treatment plants) as well as polluted and non-polluted natural systems (e.g. rivers, orchards etc.) from locations with different climatic conditions, topographic and geographical settings from the South East Asian region like Singapore, Malaysia and Indonesia as well as those collected from China. Cultures were grown in serum bottles in a selective fashion by paying careful attention to nutrient and incubation requirements for the desired microorganisms and counter-selective for the undesired organisms.

5.2.1 Reductive Dechlorination of 2,4,6-TCP

Under strict anaerobic conditions, using a specific medium and a set of incubation conditions as described in earlier sections, the reductive dehalogenation of 2,4,6-TCP can be achieved fairly easily. This is clearly the case as the microbes from all the microcosms established were able to show signs of dechlorination as the initial concentration of 2,4,6-TCP was shown to decrease during the period of study with the emergence of

dechlorination products with the likes of 2,4-DCP and 4-CP as confirmed following chromatography analysis.

Several notable dissimilarities can be spotted here when describing the reductive dehalogenation process by the different dehalogenation microbes from the microcosms established.

The cultures displayed varying periods of lag phase when compared to one another with some showing only a brief period of lag phase while others required longer lag phases. Culture LWN and RIV, for instances, required only a 1-day and 2-day lag phase respectively before dechlorination commences. Cultures SC, D3 and D12 have a 5-day lag phase while culture PY needed about 10 days before any signs of dechlorination can be observed.

Differences other than the period of lag phase between cultures were also apparent here. The ability (or inability) of these cultures to completely dechlorinate 2,4,6-TCP and its intermediates was also observed. The rate at which 2,4,6-TCP was dechlorinated also differed greatly. Cultures LWN, RIV, SC and PY exhibited the ability to completely dechlorinate 50 µM of 2,4,6-TCP and its degradation product, 2,4-DCP, to 4-CP completely. This was achieved in less than 5 days for both cultures LWN and RIV while culture SC required 10 to achieve this purpose. Some cultures, as examples, cultures PY and D12, were not as effective. Culture PY needed 36 days to completely dechlorinate 50 µM of 2,4,6-TCP and 2,4-DCP to 4-CP. While 2,4,6-TCP was completely dechlorinated to 2,4-DCP in 12 days, the dechlorination of 2,4-DCP to 4-CP was found to at a very low rate and was still on-going on day 40 as 2,4-DCP was still found to be in great abundance.

Some cultures like D3, on the other hand, were unable to completely dechlorinate 50 µM of 2,4,6-TCP even after 40 days.

For cultures which demonstrated complete dechlorination of 2,4,6-TCP, the dechlorination pathway and end product were found to be similar. Reductive dehalogenation proceeds via an intermediate (2,4-DCP) with 4-CP as the final degradation product. The results of the 2,4,6-TCP dechlorination study suggest that, for all the cultures tested, 2,4-DCP produced from 2,4,6-TCP was by the reductive dechlorination of the *ortho*-chlorine and subsequently *ortho*-dechlorinated 2,4-DCP to 4-CP. The fact that 4-CP was not dechlorinated further to phenol suggested that dechlorination of the chlorine substituent at the *para* position was not possible.

To date, only several strains of microorganisms have been isolated as pure cultures which are capable dechlorinating 2,4,6-TCP – all of which belong to the genus, *Desulfitobacterium* (Madsen and Licht, 1992; Utkin et al., 1994; Gerritse et al., 1996; Bouchard et al., 1996; Sanford et al., 1996; Breitenstein et al., 2001). Coincidentally, these known isolates share the similar dechlorination pathway as discovered here i.e. *ortho*-dechlorination of 2,4,6-TCP to 4-CP via 2,4-DCP. However, unlike this study where a defined was medium used, yeast extract was always present for growth of the aforementioned isolates and the growth in the absence of this additive at the expense of reductive dechlorination has never been established.

In addition to the pure *Desulfitobacterium* strains named above, several other microbes from other genera have been isolated and these too, have shown a preference in *ortho*-dechlorination of chlorinated phenol. These strains however showed a lower degree of

dechlorination capability as compared to those from the ones from the *Desulfitobacterium* genus as well as the cultures grown in this study. *Desulfovibrio dechloroacetivorans* SF3 *ortho*-dechlorinates only 2-CP and 2,6-DCP to phenol. Dechlorination of 2,4,6-TCP was not shown possible (Sun et al., 2000). Pure isolates from the *Dehalococcoides* genus have shown similar capacity. Strain CBDB1 dechlorinates 2,4,6-TCP to 2,4-DCP. Further *ortho*-dechlorination of 2,4-DCP to 4-CP was slow and incomplete. *Dehalococcoides* strain 195 dechlorinates phenols only in the *ortho* position and only if a chlorine substituent is present in the flanking *meta* position. 2,3-DCP was converted to 3-CP, 2,3,4-TCP to 3,4-DCP and 2,3,6-TCP to an equimolar mixture of 2,5-DCP and 2-CP. Dechlorination of 2,4,6-TCP was not possible (Adrian et al., 2007).

5.2.2 Reductive Dechlorination of PCP

The reductive dechlorinate of PCP was found to be relatively more difficult as compared to the dechlorination of 2,4,6-TCP using the same cultures. Throughout the entire duration of the study, only culture D12 was found to be capable of dechlorinating PCP. Inhibition of bacterial activities by PCP is commonly observed (Guthrie et al., 1984; Ruckdeschel et al., 1987). Krumme and Boyd (1988) studied the degradation of chlorophenols in anaerobic upflow bioreactors and found that there was minimal or no biodegradation of PCP.They argued that the lack of dechlorinating activities in the bioreactors may have been due to the highly toxic nature of PCP. This may have been the case as observed in this study and we can attribute the non-dechlorinating activity by the cultures tested here as the result of the substrate's toxicity level.

In culture D12, PCP and its intermediates were completely dechlorinated to the end product, 4-CP. The limiting step in the PCP dechlorination was the transformation of 4-CP to phenol. The intermediates detected in the reductive dechlorination of PCP by D12 were 2,4,6-TCP and 2,4-DCP. 2,3,4,6-TeCP, however, was not detected. This could possibly suggest that the dechlorination of PCP began by the simultaneous removal of both *meta* positioned chlorines to 2,4,6-TCP. This is then followed by the *ortho*-dechlorination of 2,4,6-TCP to form 2,4-DCP. Another *ortho*-dechlorination takes place afterwards to remove to *ortho*-chlorine from 2,4-DCP to its end product, 4-CP. Similar to that of 2,4,6-TCP dechlorination, the removal of chlorine at the *para* position was not possible throughout the experiment. The dechlorination pathway by the microbial consortium in culture D12 shows indicated the preferable dechlorination in the *meta*-position (possibly simultaneously) which is then followed the sequential dechlorination of both *ortho*-position chlorines.

An extended lag phase has been monitored here before the dechlorination took place. This is especially common when it comes to higher chlorinated compounds such as PCP (Palekar et al., 2003). During this phase, the inoculum cells are adapting themselves to active growth in the new environment and may be extended if grown previously in very different environment (pH, temperature, nutrients etc.). Once the cells have adapted themselves to the new environment, they will enter the exponential phase and this is occurs with the corresponding decrease of PCP concentration levels as observed here. Another possibility that may have caused the extended lag phase of the microbial community can be ascribed to the cells' lack of exposure or acclimatization period to chlorophenols. Culture D12 has been obtained from a domestic waste water plant where

there was no history of chlorophenol contamination. In his work, Bryant et al. (1991) investigated the dechlorination of PCP in anaerobic sediments that contained non-adapted and chlorophenol adapted microbial communities. Chlorophenol adapted sediment cultures were found to dechlorinate PCP without an initial lag phase while microbial communities that were not previously exposed to chlorophenols either did not dechlorinate PCP or did so after an extended lag phase.

Juteau et al. (1995) have cultivated an anaerobic consortium that is capable of PCP dechlorination in bioreactors. The degree of dechlorination was similar to that of culture D12 which is the dechlorination of pentachlorophenol to monochlorophenol. The dechlorination pathway however, was clearly different. The analysis of chlorophenol intermediates found in the effluent of the reactor suggested that main PCP reductive dechlorination pathway used by this consortium was successively by *para*, *ortho*, *ortho* and finally *meta* dechlorination. The main dechlorination pathway of PCP exhibited can be summarized as: PCP → 2,3,5,6-TeCP → 2,3,5-TCP → 3,5-DCP → 3-CP.

The PCP dechorinating microbial consortium obtained from the mixture of sewage sludge and soil samples from the study by Juteau et al. was subsequently enriched and this ultimately resulted in the isolation of a pure strain of PCP dechlorinating bacterium, *Desulfitobacterium frappieri* PCP-1 (Bouchard et al., 1996). This spore forming, rod shaped bacterium grows only on pyruvate and requires yeast extract to enable the reductive dechlorination of PCP. While the end product of strain PCP-1 remained 3-CP, the pathway of PCP dechlorination is neither same as that discovered by Juteau et al. nor similar to that as shown in this study by culture D12. The kinetics of dechlorination of PCP by strain PCP-1 revealed that the *ortho*-position chlorines from PCP was rapidly

dechlorinated to 3,4,5-TCP and that there was a 36-hour lag period before this compound was *para*-dechlorinated to 3,5-DCP which will be subsequently be *meta*-dechlorinated to 3-CP. From this result, Bouchard *et al*. explained that there may be two different enzyme systems involved in PCP dechlorination in strain PCP-1. The first dechlorinates PCP rapidly and dechlorinates 2,3,4,5-TeCP only at the *ortho* position to generate 3,4,5-TCP while the second system dechlorinates 3,4,5-TCP at the *para* and *meta* positions to generate 3-CP.

A search in the literatures revealed that 4 other pure isolates have been found be able to dechlorinate PCP. With the exception of *Dehalococcoides* sp. strain CBDB1, all the other cultures, however, are only capable of dechlorinating pentachlorophenol to generate various congeners of trichlorophenol as the end product. With *Dehalococcoides* sp. strain CBDB1, PCP dechlorination resulted in the production of a mixture of 3,5-DCP, 3,4-DCP, 2,4-DCP, 3-CP and 4-CP, indicating that several dechlorination pathways were catalyzed (Adrian et al. 2007). Both *Desulfitobacterium hafniense* DCB2 (Madsen and Licht, 1992) and *Desulfitobacterium dehalogenans* JW/IU-DC1 (Utkin et al., 1994) *ortho*-dechlorinated PCP to generate 3,4,5-TCP as the end product. *Desulfomonile tiedjei* DCB-1, probably one of the earliest discovered dechlorinating anaerobic bacterium, *meta*-dechlorinates PCP to 2,4,6-TCP as its end product via its intermediate, 2,3,4,6-TeCP. 3-Chlorobenzoate is required to serve as an inducer for PCP dehalogenation for strain DCB-1 (Mohn and Kennedy, 1992).

5.3 Cultures RIV, LWN, SC and their Dehalogenation Capabilities

5.3.1 An Evaluation of Culture RIV and its Dehalogenation Capabilities

Culture RIV, enriched from fresh water sediment of a river in West Java, Indonesia, was found to be able to completely dechlorinate 2,4,6-TCP to 4-CP using a defined medium with pyruvate as its sole electron donor for reductive dehalogenation. Removal of the *ortho*-chlorine substituents from the phenol ring of 2,4,6-TCP begins after a 1-day lag phase period to form its end product. No 2,4-DCP, a possible intermediate, was detected during the course of the experiment. This could suggest that both *ortho*-chlorines are removed simultaneously during the reductive dehalogenation process of culture RIV. The dechlorination of 2,4,6-TCP was efficient and rapid as about 60 µM of the parent compound was found to be completely depleted after 5 days of incubation.

Using genus specific primer pairs belonging to 8 genera of microorganisms known for their chlorophenol and halogenated compound dehalogenating capabilities, the 16S rRNA gene of the genomic DNA extracted from culture DNA was targeted to confirm the presence of possible dehalogenators from the genera as mentioned. Only microbes belonging to the genus, *Desulfitobacterium*, were found to be present in culture RIV following the direct PCR approach (see section 4.6). This finding is not surprising since most of the chlorophenol dechlorinating pure strains isolated belong to this genus group (Madsen and Licht, 1992; Utkin et al., 1994; Gerritse et al, 1996; Sanford et al, 1996; Bouchard et al, 1996; Breitenstein et al., 2001).

Other than the capability to dechlorinate chlorophenols, enrichment culture RIV which contains microbes from the *Desulfitobacterium* genus group was found to be able to

debrominate polybrominated diphenyl ethers. A commercial mixture of Penta-BDE consisting of Tetra-, Penta- and Hexa-BDE congeners were found to be debrominated after 9 weeks of incubation. Concentrations Hexa- and Penta-BDE were found to be significantly decreased and this resulted in the formation of debromination products, Tetra-BDE. The debromination pathway will not be discussed here due to the complexity of the numerous starting substrates present in the parent compound and the diversity of the products formed which causes difficulty in delineating the specific PBDE debromination pathways. Assuming the desulfitobacteria from culture RIV are responsible for the dechlorination of 2,4,6-TCP as described above, it is safe to postulate that these microbes may likely be involved in the debromination of PBDE here as well. This was supported with a study by Robrock et al. (2008) on the debromination of PBDE of several pure isolates belonging to the *Desulfitobacterium* genus. It was found that all 3 strains (all of which can extensively dechlorinate chlorophenols), namely *Desulfitobacterium hafniense* (formerly *frappieri*) PCP-1, *Desulfitobacterium chlororespirans* Co23 and *Desulfitobacterium dehalogenans* JW/IU-DC1, produced a variety of debromination congeners when exposed to octa-BDE mixture. Robrock et al. went on to explain that it is possible that the reductive dehalogenases responsible for chlorophenol degradation are involved in debromination because experiments with the representative *Desulfitobacterium* strain in which chlorophenol were not added as electron acceptor generated no detectable PBDE debromination activity. There results suggest that either the debrominating enzymes were not induced by the PBDEs alone or that the PBDE transformation by these isolates is co-metabolic, requiring concomitant presence of energy-generating electron acceptors.

Desulfitobacterium spp. are strictly anaerobic bacteria that were first isolated from environments contaminated by halogenated organic compounds. They are very versatile microorganisms that can use a wide variety of electrons including both man-made and naturally occurring halogenated organic compounds. Most of the Desulfitobacterium strains can dehalogenate halogenated compounds by mechanisms of reductive dehalogenation, although the substrate spectrum of halogenated organic compounds varies substantially from one strain to another, even with strains belonging to the same species (Villemur et al., 2006).

Because of their versatility, desulfitobacteria can be excellent candidates for the development of anaerobic bioremediation processes.

5.3.2 An Evaluation of Culture LWN and its Dehalogenation Capabilities

Through the process of reductive dehalogenation, culture LWN transforms 2,4,6-TCP to 4-CP as the end product. Culture LWN shared several similarities with culture RIV in terms of its dechlorination abilities. Dechlorination proceeds after a 2-day lag phase and dechlorination occurred only at the *ortho* position. Even though 2,4-DCP (the intermediate) was detected, it was quickly found to be quickly depleted and transformed into 4-CP. Removal of 2,4,6-TCP and its intermediate was quick with 60 µM of the parent substrate being dechlorinated completely in 5 days. Culture LWN demonstrated high tolerance level even to the toxic nature of 2,4,6-TCP and was able to resume dechlorination activities of the substrate even at 1,000 µM.

Interestingly, no microbes from the genus *Desulfitobacterium* was detected in culture LWN since in the past, only pure isolates (with the exception of *Dehalococcoides* strain CBDB1 and 195) from this genus have been able to perform reductive dehalogenation of chlorophenols with higher numbers of chlorine substituents (e.g. trichlorophenol, tetrachlorophenol and pentachlorophenol). *Dehalococcoides* strain CBDB1 have shown a wide spectrum of chlorophenol dechlorinating ability but was unable to dechlorinate 2,4,6-TCP beyond 2,4-DCP. Dechlorination of 2,4-DCP to 4-CP was reported to be very slow and incomplete (Adrian et al. 2007). *Dehalococcoides* strain 195 dechlorinates only in the *ortho* position and only if a chlorine substituent was present in the flanking *meta* position. Dechlorination was only detected with 2,3-DCP, 2,3,4-TCP and 2,3,6-TCP and not with 2,4,6-TCP and PCP (Adrian et al. 2007). Attempts to detect the presence of *Dehalococcoides* in culture LWN also yielded negative results. Other known chlorophenol dechlorinating bacteria like *Anaeromyxobater* (Sanford et al., 2002) was not found in the culture LWN either. Microbes from the genus *Desulfovibrio* were detected but was found to be in insignificant levels. Furthermore, to our knowledge, only one isolate form the *Desulfovibrio* genus have been found to possess the ability to dechlorinate chlorophenol and this strain can only utilize 2-6-DCP and 2-CP for dehalorespiration (Sun et al., 2000).

An interesting finding here is the detection of a microorganism which showed 98% similarity with a host of bacteria from the genus, *Sedimentibacter* following a partial 16S rRNA sequence analysis. This microorganism is found to be dominant in the highly enriched culture LWN. The dominance of culture LWN was shown to increase as the culture becomes more enriched with each transfer and was found to be positively

correlated with the concentration of 2,4,6-TCP. This could suggest that this strain of bacteria may actually be responsible for the reductive dehalogenation works in culture LWN. This point becomes even more convincing since microorganisms from other known 2,4,6-TCP dechlorinating genera, *Desultiftobacterium* and *Dehalococcoides*, were not present in the culture.

Sedimentibacter have been shown to grow in cultures that contain chlorophenols (Breitenstein et. al, 2001; Zhang and Wiegel, 1994) as well as other chlorinated compounds such as β-hexachlorocyclohexane (van Doesburg et al., 2005). However, microbes from the *Sedimentibacter* genus have never been documented to be directly responsible for the dechlorination of chlorinated compounds. Instead, their presence has been reported to be a requirement in dehalogenating cultures for the growth of the dechlorinators and dechlorination. Van Doesburg et al. (2005) reported the metabolic dechlorination of β-hexachlorocyclohexane by a *Dehalobacter* sp. in the presence of a strain of bacteria from the S*edimentibacter* genus. However, the role of the *Sedimentibacter* in the coculture could not be clarified. It has been hypothesized that *Sedimentibacter* stimulates the transformation of β-hexachlorocyclohexane via the excretion of growth factors like vitamins, amino acids and other compounds for the use of the *Dehalobacter* in reductive dehalogenation. When grown in coculture with *Dehalobacter*, only 10% of the bacteria present are *Sedimentibacter*. This was not the case in culture LWN as *Sedimentibacter* represent the majority of the microbial population within the culture. Another interesting and notable difference here is that, *Sedimentibacter* has been reported to grow only in the presence of yeast extract

(Breitenstein et al., 2002) but the *Sedimentibacter* containing culture LWN was grown completely in the absence of yeast extract.

Although the facts so far have heavily linked *Sedimentibacter* in culture LWN to the dechlorination of chlorophenols, but its role cannot yet be confirmed. Whether the *Sedimentibacter*-like in culture LWN is the actual dechlorinators of chlorophenols or not, we can safely postulate that it plays a very important role in the reductive dehalogenation works of chlorophenols and further, in depth studies are required to understand its function.

5.3.3 An Evaluation of Culture SC and its Dehalogenation Capabilities

A *Dehalococcoides* containing culture was enriched from an anaerobic sewage sludge obtained from a wastewater treatment plant off Jurong Island, Singapore. Culture SC, named after the company, SempCorp Industries, which has graciously permitted us the collection of sample from its wastewater treatment facility, reductively dechlorinates 2,4,6-TCP to 4-CP via 2,4-DCP. 2,4,6-TCP was seen to gradually decrease after a lag phase of 5 days with a corresponding accumulation of 2,4-DCP. This is then followed by another *ortho*-dechlorination of 2,4-DCP to generate 4-CP.

Although microbes from the genera *Acetobacterium* and *Desulfovibrio* were detected, they only represent the minority of the microbial population within the consortium. So far, no reports have shown any cases of chlorophenol degradation by the genus *Acetobacterium* and there has only been one case showing reductive dechlorination of chlorophenol by a pure strain from the genus *Desulfovibrio* (Sun et al., 2000). This strain,

however, can only dechlorinate 2,6-DCP and 2-CP. Chlorophenols with more chlorine substituents like 2,4,6-TCP and other congeners of di- and monochlorophenol inhibited growth.

The presence of *Dehalococcoides* as the dominant species and the fact that mixed and pure cultures from this group have a great potential in dehalogenation of a wide spectrum of halogenated compounds like chlorophenols, polybrominated diphenyl ethers, polychlorinated biphenyl, chlorinated ethenes (Adrian et al., 2007; Robrock et al., 2008; Bedard et al., 2007; Bedard, 2008; Field and Sierra-Alvarez, 2007; He et al., 2003a; Fennell et al., 2004) give an impression that the *Dehalococcoides* from culture SC may be responsible for the dechlorination of 2,4,6-TCP. Thus far, only Adrian et al. (2007) have described the growth of *Dehalococcoides* (strains 195 and CBDB1) with chlorophenols as electron acceptors.

Culture SC has shown great potential in dechlorinating chlorinated ethenes as well. Trichloroethene used as the sole electron acceptor was completely dechlorinated to *trans*- and *cis*-dichloroethene in the ratio of 3:1. For the same reasons stated above, we postulate that there is a high possibility of the dehalogenators to be from the *Dehalococcoides* genus as found in great abundance in culture SC. Numerous studies of degradation of chlorinated ethenes by *Dehalococcoides*, most notably strains 195, BAV1 and FL2, have been reported and extensively studied.

Aside from *Dehalococcoides*, studies have shown that under anaerobic conditions, PCE or TCE can be dechlorinated by a variety of other dehalogenating microbes e.g. *Dehalobacter*, *Desulfuromonas* and *Desulfitobacterium* (Wild et al., 1996; Krumholz,

1997; Miller et al. 1997). However, only *Dehalococcoides* are known to be able to complete reductive dechlorination of PCE or TCE beyond DCE to vinyl chloride and ethene due to their possession of the functional reductive dehalogenase (RDase) genes such as *pceA*, *tceA*, *bvcA* and *vcrA* genes (Krajmalnik-Brown et al, 2004; Magnuson et al., 1998; Muller et al., 2004; Smidt and de Vos 2004). *Dehalococcoides* species strains BAV1 and FL2 for examples are able to dechlorinate all DCE isomers to VC or ethene while strain 195 reductively dechlorinates tetrachloroethene to ethene (He et al., 2003b; He et al., 2005; Maymo-Gatell et al., 1997).

5.4 Comparisons between Cultures LWN, RIV and SC

Enrichments cultures LWN, RIV and SC have shown effective capabilities to reductively dechlorinate 2,4,6-TCP and its intermediate, 2,4-DCP, completely to 4-CP in defined media and with pyruvate as its carbon source. Only the *ortho* positioned chlorines were removed for all three cultures. Complete dechlorination and end product were achieved within ~7 days for cultures LWN and RIV whereas culture SC required ~14 days to completely dechlorinate 2,4,6-TCP and 2,4-DCP to 4-CP.

An interesting observation can be observed in the nature of the formation and loss of the intermediate product, 2,4-DCP. Cultures LWN and RIV demonstrated a quick transformation of 2,4,6-TCP to 4-CP during the reductive dehalogenation process as 2,4-DCP was not shown to be accumulated. 2,4,6-TCP was dechlorinated to 2,4-DCP which was then immediately used up by the dehalogenators to be transformed to 4-CP. Another possibility is that both *ortho* chlorines of 2,4,6-TCP may have been removed

simultaneously to form 4-CP which explains the non-accumulation of 2,4-DCP as the intermediate. Culture SC, on the other hand, exhibited a parallel increase of 2,4-DCP with the decrease 2,4,6-TCP. Upon the complete dechlorination of 2,4,6-TCP, the accumulated 2,4-DCP was then further dechlorinated to 4-CP as the end product.

Further studies showed that some of these cultures have the ability of dehalogenate halogenated compounds other than chlorophenols. While culture LWN was not able to dehalogenate the tested alternative halogenated compounds in this study, culture RIV and SC showed excellent potential in dehalogenating polybrominated diphenyl ethers and chloroethenes respectively. Culture RIV was found to be able to debrominate a commercial mixture of penta-BDE whilte culture SC effectively dechlorinated TCE completely to *trans*- and *cis*-DCE in a ratio of 3:1.

The dehalogenators responsible 2,4,6-TCP dechlorination may be different in all three cultures as well. In culture RIV, only microbes from the genus *Desulfitobacterium* were found from the genera tested. This did not come as a surprise as the *Desulfitobacterium* bacteria were commonly known to be able to dechlorinate chlorophenols. *Desulfitobacterium* was not detected at all in culture SC and LWN. Instead, *Dehalococcoides* was found in culture SC. While it has been proven that bacteria from the *Dehalococcoides* genus are popular their dehalogenating abilities (having been able to dehalogenate chloroethenes, PBDE, PCB etc.), this could be, for the first time, a report that shows a *Dehalococcoides* containing culture that can completely dechlorinate 2,4,6-TCP and 2,4-DCP to 4-CP. Meanwhile, no dehalogenators from commonly known dehalogenating genus were detected in culture LWN. Instead, DNA sequence results of the most abundant microbes in the culture revealed the presence of bacteria from the

genus *Sedimentibacter*. This could be both an equally exciting and important finding as there were no other reports which suggested a strain of bacteria from this genus that is capable of the dechlorination of chlorophenols.

CHAPTER 6

CONCLUSION

6.1 Major Findings

With the reference to the problem statement and the aims set out earlier in this report, the objectives of this study have been met. Listed below are the central findings of this study.

6.1.1 Development of Method for Chlorophenol Detection

A method involving the simultaneous derivatization and liquid-liquid extraction procedure followed by GC-MS analysis was successfully developed for the detection and measurement of all chlorophenol congeners. The derivatization or acetylation step plays an important role in ensuring sharper and 'tailless' peaks on chromatograms due to better separation and higher sensitivity.

6.1.2 Cultivation of Bacterial Consortia Capable of Chlorophenol Dechlorination

A bacterial consortium designated, D12, grown in defined medium and pyruvate as the carbon source demonstrated the capability of dechlorinating PCP to 4-CP. PCP was completely *meta*-dechlorinated to 2,4,6-TCP which was then further dechlorinated to 2,4-DCP and finally 4-CP as the final dechlorination product.

2,4,6-TCP was also observed in 3 highly enriched cultures, namely LWN, RIV and SC. These cultures were grown in defined media with pyruvate as the carbon source. Dechlorination pathways for all 3 cultures were identical i.e. 2,4,6-TCP → 2,4-DCP → 4-CP.

6.1.3 Identification of Possible Microbes Responsible of 2,4,6-TCP Dechlorination

Using molecular techniques as discussed in previous sections, 2,4,6-TCP dechlorinating cultures LWN, RIV and SC were tested for the presence of possible dehalogenators within the 3 bacterial consortia. A common chlorophenol-dechlorinating bacterium from the genus *Desulfitobacterium* was found in culture RIV while culture SC comprises of *Dehalococcoides*-like bacteria, which was never reported to have been able to completely dechlorinate 2,4,6-TCP in the past. DNA sequencing results showed an even more interesting find with predominance of *Sedimentibacter*-like bacteria in culture LWN since *Sedimentibacter* have never been previously shown to dehalogenate any form of halogenated compounds.

6.1.4 Dehalogenation of Halogenated Compounds Other than Chlorophenol

Toxic halogenated compounds other than chlorophenols such as chloroethenes, polybrominated diphenyl ethers and polychlorinated biphenyls were used as alternative substrates for reductive dehalogenation by the chlorophenol-dechlorinating cultures. In this study, extensive debromination of PBDE was shown to be possible by culture RIV.

Meanwhile, culture SC has also shown a broad range of dehalogenating potential as it was successful in complete dechlorination of trichloroethene. All cultures were grown in defined media, without the aid of yeast extract for growth.

6.2 Recommendations and Future Studies

6.2.1 A Need to Screen for and Isolate Undiscovered Dehalogenators

The diversity of reductively dechlorinating bacteria is large, almost equaling the diversity of chlorinated compounds synthesized. However, many new genera and species of dechlorinating bacteria still remain undiscovered. In addition, the beneficial, as well as potentially detrimental, interactions of dechlorinating populations with other microbial populations resulting from the presence of alternative terminal electron acceptors (e.g., nitrate, Fe^{3+}, Mn^{4+}), and the effect of such interactions on the dechlorination process need to be further explored.

Studies such as this one are required to support and complement other current research efforts on the enrichment and isolation of organisms as well as the conditions under which reductive dechlorination occurs in order to improve our understanding of the reductive dechlorination process by microorganisms in complex natural environmental and engineered systems for the development of more efficient *in situ* bioremediation and waste treatment technologies and strategies. With so many uncertainties and so many questions left unanswered coupled with the tremendous potentials microbial reductive dechlorination can offer, this certainly has opened up a field that is of great interest and importance for fundamental as well as applied research in environmental engineering.

6.2.2 Narrowing the Success Gaps between Laboratories and Site Situations

Future research should focus on both basic and applied aspects so as to be able to transfer the knowledge gained to the applied and 'real' situations. Since bioremediation is an important tool in detoxifying and eliminating environmental contaminants, a thorough understanding of microbial genetics, biochemistry, and physiology is required. Attempts should be made to bridge the gap between accomplishments at laboratory level and success of the same at a field scale. Oftentimes, laboratory testing does not accurately predict field results for many processes. The cause for the most part is ascribed to disparities in physiological conditions, concentration of the target chemical, and other physical, chemical, and microbial aspects that were either not taken into consideration or show constant variation. Studies should focus on researches that are more similar to actual field or ground conditions. The concentration of the target chemicals used for carrying out biodegradation studies in the laboratory should not be hypothetical but should relate to contamination levels present in the environment. Additionally, treatment of hazardous chemicals in the environment also presents the possibility of unknown by-products of biodegradation entering the environment. Therefore, sound knowledge of the degradation products, metabolic pathway, biochemistry, and other details relating to treatability studies should be collected before venturing into a full-scale bioremediation process. Most of the research reported on degradation of chlorinated compounds is limited to flask experiments, and there is a need to also develop suitable bioreactor systems for treatment of waste containing high concentrations of chlorinated compounds emitted from manufacturing industries.

6.2.3 Discovery of Bacteria with Wider Substrate Range

Most studies concentrate on the discoveries of bacteria with specific substrate dechlorination capabilities. This makes them only suitable to treat single-compound-containing waste streams. As such, studies should be diverted to focus on the identification and isolation of bacteria with broader substrate spectrum dechlorination capabilities to make them more suitable to treat waste streams containing mixtures of several chlorinated compounds.

6.2.4 Genetic Engineering and 'Superbugs'

It is also worthwhile to pay attention to the development and the extension of the knowledge we currently possess in the field of genetic engineering of dechlorinating microbes. Most of the xenobiotic degrading microorganisms harbor plasmids which code for the catabolic genes. With the combination of an in depth understanding of the biochemistry and genetics of plasmid-borne degradation and the applications of recombinant DNA techniques, it is possible to characterize the appropriate genes and transfer them to construct improved strains with enhanced capability for degradation of several toxic compounds. One of the objectives of genetic engineering of toxic chemical-degrading microorganisms is to develop the so-called "superbugs," capable of detoxifying or decontaminating the toxic chemicals in the natural environment. To establish the potential applications of the recombinant strains in the environment, the strains must be stable members of the indigenous microflora and the recruitment of catabolic enzymes and gene regulators with appropriate specificities, by means of natural gene transfer or

laboratory manipulation, to produce new hybrid pathways for chlorinated compounds must not significantly alter the host or the natural ecosystem. Although the risks of releasing recombinant microorganisms still remain unknown, the prospects for the construction of catabolic pathways to effect mineralization and detoxification of halogenated compounds are very encouraging.

6.3 Closing Remarks

Obviously, the most efficient means of preventing further pollution lies in restricting the usages of the recalcitrant chlorinated compounds and replacing them with non-recalcitrant alternatives. However, this cannot be implemented immediately and overnight as chlorinated compounds difficult and expensive to replace in agriculture and manufacturing industries. As such, studies of dehalorespiring microorganisms as well as the process of reductive dehalogenation itself on chlorinated compounds such as this one are vital in the controlling the generation and reducing contamination of these extremely toxic compounds into the environment.

While we acknowledge and appreciate the numerous contributions and the dedicated works that have been carried out in this field, there is so much more that needs to be accomplished in this direction.

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