CHAPTER 2

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 HCB property

HCB does not occur naturally in the environment. It is an unwanted by-product in the manufacture of solvents, chlorinated compounds, and its main source is the manufacture of pesticides. HCB is also used as a chemical intermediate in dye manufacture, in the synthesis of other organic chemicals, and as a wood preservative (ATSDR, 1999). It is one of 12 persistent organic pollutants banned by the United Nations. However, HCB may be present for years after being banned because this substance is persistent. It is reasonably anticipated to be human carcinogen based on sufficient evidence of carcinogenicity in experimental animals. There is inadequate evidence for the carcinogenicity of hexachlorobenzene in humans.

HCB is a white crystalline solid, hydrophobic, strongly sorbed to soil organic matter and tend to persist in soils. It has low water solubility (in water at 25°C: 0.006 mg/l, at 20°C: 0.005815 mg/l), moderate volatility (vapor pressure at 20°C: 1.09×10^{-5}, Henry's law constant: 5.8×10^{-4} atm-m^3/mol) and thus is likely to show low mobility in the soil environment (EHC 195, 1997).

The primary routes of potential human exposure to hexachlorobenzene are ingestion, inhalation, and dermal contact. The adverse health effects of HCB on adults include skin, neurological and orthopaedic abnormalities.

2.2 Anaerobic Degradation Process. (Tchobanoglous and Burton, 1991)

The anaerobic biological conversion of the organic matter can be divided in three steps (see in Figure.2.1). The first step in the process involves the enzyme-mediated transformation (hydrolysis) of higher-molecular-mass compounds into compounds suitable for use as a source of energy and cell carbon. One group of organisms is responsible for hydrolyzing organic polymers and lipids to basic

structural building blocks such as monosaccharides, amino acids, and related compounds.

Theoretical Stages

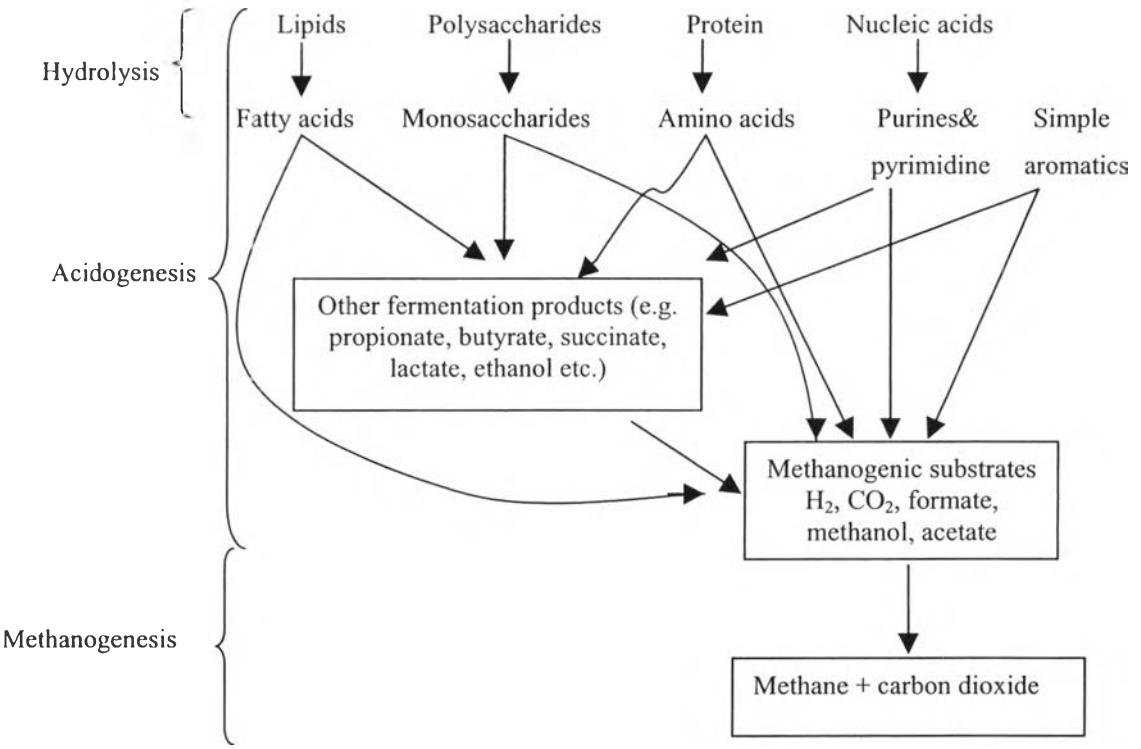

Figure2.1 Schematic diagram of the patterns of carbon flow in anaerobic

The second step (acidogenesis) involves the bacterial conversion of the intermediate compounds second group of anaerobic bacteria ferments the breakdown products to simple organic acids, the most common of which in anaerobic digester is acetic acid. The group of microorganisms, described as nonmethanogenic, consists of facultative and obligate anaerobic bacteria. Collectively, these microorganisms are often identified in the literature as "acidogens," or "acid formers."

The third step (methanogenesis) involves the bacterial conversion of the intermediate compounds into simpler end products, principally methane and carbon dioxide. Microorganisms of a third group converts the hydrogen and acetic acid formed by the acid formers to methane gas and carbon dioxide. The bacteria responsible for this conversion are strict anaerobes and are called methanogenic bacteria. They are identified as "methanogens," or "methane formers." It is important

to note that methane bacteria can only use a limited number of substrates for the formation of methane. Currently, it is known that methanogens use the following substrates: CO_2 +H_2, formate, acetate, methanol, methylamines, and carbon monoxide for their growth. The methanogens are able to utilize the hydrogen produced by the acidogens because of their efficient hydrogenase. Because the methanogens are able to maintain an extremely low partial pressure of H_2, the equilibrium of the fermentation reactions is shifted towards the formation of more oxidized end products (e.g., formate and acetate).

2.3 Dechlorination

Dehalogenation is a mechanism that removes halogen from halogenated compounds but dechlorination is specific to chlorine removal from chlorinated compounds. There are two types of microbially mediated reactions involved in the removal of halogen substituents from organic molecules. Oxidative and reductive reactions involve the transfer of electrons from or to the halogenated compound, respectively. Due to the electronegative character of halogen substituent groups, highly halogenated compounds are more oxidized than lesser-chlorinated isomers and thus are less susceptible to oxidative reactions; rather, they will undergo reductive reactions for thermodynamic reasons.

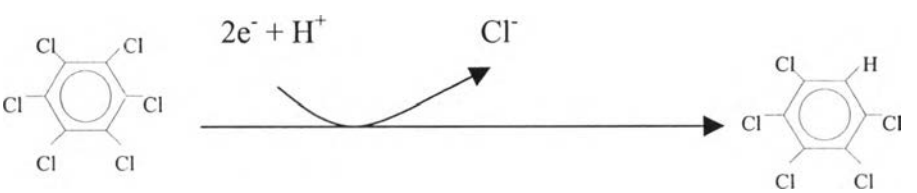

Figure 2.2 Mechanism of reductive dechlorination of chlorinated benzene

Under anaerobic conditions, reductive dehalogenation is the dominant mechanism for halogen removal. Reductive dehalogenation of chlorinated organic compounds takes place in reduced environments such as deep soils and sediments and is mediated by native microbial consortia acclimated to existing contaminants. The result of reductive dehalogenation is less chlorinated benzene congener, which is less toxic, less likely to bioaccumulate, more soluble and volatile than the polychlorinated

benzenes. Thus more mobile, and more susceptible for microbial attack are produced (Adriaens and Vogel, 1995).

In general, there are many factors affect the biodegradation of recalcitrant chemicals, which are summarized in Table 2.1 and 2.2 as chemical-specific factors and environment-specific factors of concern.

Table 2.1 Chemical-specific factors which influence the biodegradation of recalcitrant compounds.

State	• Gas, liquid or solid • Surface area
Solubility	• Aqueous
Hydrophobicity	• Ability to dissolve in hydrophobic (lipophilic) solvents, hydrophobic compounds will have relatively low water solubility
Adsorbabilty	• Ability to adsorb to and complex with organic and inorganic fractions in soil, sediments and water
Size and shape	• Can affect ability to permeate cell membrane and to interact with enzymes.
Charge	• Can affect ability to permeate cell membrane and to interact with enzymes
Toxicity	• May be a specific effect or a general one
Detailed molecular structure	• Important factors include:- a) Presence of an "easily metabolisable" structural unit (s) b) Presence of a "difficult to metabolize" structural unit (s) c) Presence of an "un-natural" (xenobiotic) structural unit (s) d) Degree of branching e) Nature of substituents f) Number of substituents g) Position of substituents
Concentration	• Too high or too low

Table 2.2 Environment-specific factors

Biotic factors • Presence of suitable or 'potentially suitable' organisms
Abiotic factors
Physical
• Temperature or pressure
Chemical
• Nutrients – minerals, growth factors
• Presence of oxygen as: i) terminal electron acceptor ii) inhibitor
• Presence of alternative electron acceptors: i) nitrate ii) sulfate iii) carbon dioxide
• pH
• Inhibitory materials
• Soil type • Moisture level • Type of water – fresh, brackish, saline

HCB has very low solubility, as it is hydrophobic compound, so it is difficult for microorganism to metabolite. It is likely to adsorb with organic fraction in sediment (log K_{ow} = 5.73). Therefore, the dechlorination of HCB has to be carried out under optimal condition such as suitable temperature, adequate nutrient and appropriate electron donor.

2.3.1 Physiology of Biodegradative Microorganisms (Adriaens and Vogel, 1995)

One of the primary variables affecting the activity of heterotrophic bacteria is the ability and availability of reduced organic materials to serve as electron donors (and energy sources). Whether a contaminant serves as an effective energy source for a given microorganism is a function of the average oxidation state of the carbon in the material. In general, highest oxidation states correspond to lower energy yields and thus provide less energetic incentive for an organism to degrade it. The number of (halogen) substituents affects the oxidation level of chlorinated organic compound. Thus, the relative oxidation state of the organic carbon present in a range of alkyl and aryl halides can be expressed as the ratio of the number of chlorines to the number of carbons in the molecule.

Table 2.3 Abbreviations, Chemical Names, and Chlorine to Carbon Ratios of Common Alkyl and Aryl Halides Contaminants

Abbreviation	Chemical Name(s)	Chlorine / Carbon
Alkyl		
PCE	Perchloroethylene, tetrachloroethylene	2
TCE	Trichloroethylene,1,1,1-trichloroethylene	1.5
c-DCE	cis-Dichloroethylene, 1,1-dichloroethylene	1
VC	Vinyl chloride, monochloroethylene	0.5
PCA	Perchloroethane, hexachloroethane	3
CF	Chloroform, trichloromethane	3
DDT	Dichloro-diphenyl-trichloroethane	0.4
Aryl		
PCB	Polychlorinated biphenyl	0.08-0.8
PCDD/F	Poly chlorinated dibenzo-p-dioxin/furan	0.08-0.7
HCB	**Hexachlorobenzene**	**1**
DCB	Dichlorobenzene	0.3
CB	Chlorobenzene	0.17
PCP	Pentachlorophenol	0.8
DCP	Dichlorophenol	0.3

Table 2.3 showed that tetrachloromethane (CT) and hexachlorobenzene (HCB) represent the most oxidized alkyl (No.Cl/No.C = 4) and aryl (No.Cl/No.C = 1) halides, respectively (Adriaen et al, 1995). Therefore, HCB is likely to be dechlorinated under reductive dechlorination rather than oxidative dechlorination.

2.3.2 Dechlorination by Halorespiring Microorganisms (Suthersan, 2001)

Halorespiration, also referred to as dehalorespiration, occurs when the organic compound acts as an electron acceptor (primary growth substrate) during reductive dechlorination. During halorespiration, the chlorinated organic compounds are used directly by microorganisms (termed halorespirators), such as an electron acceptor while dissolved hydrogen serves as an electron donor.

Halorespiration occurs as a two-step process, which results in the interspecies hydrogen transfer by two distinct strains of bacteria. In the first step, bacteria ferment organic compounds to produce hydrogen. During primary or secondary fermentation, the organic compounds are transformed to compounds such as acetate, water, carbon dioxide, and dissolved hydrogen. Fermentation substrates are biodegradable, nonchlorinated contaminants or naturally occurring organic carbon.

In the second step, the nonfermenting microbial consortia utilize the hydrogen produced by fermentation for halorespiration.

Process of PCE reductive dechlorination is shown in Figure 2.3 to be an example for the explanation of steps involving in dechlorination which PCE is an electron acceptor. The relation between various organic compounds and microorganisms with different function in dechlorination process and cause the transformation of chlorinated compound could be seen.

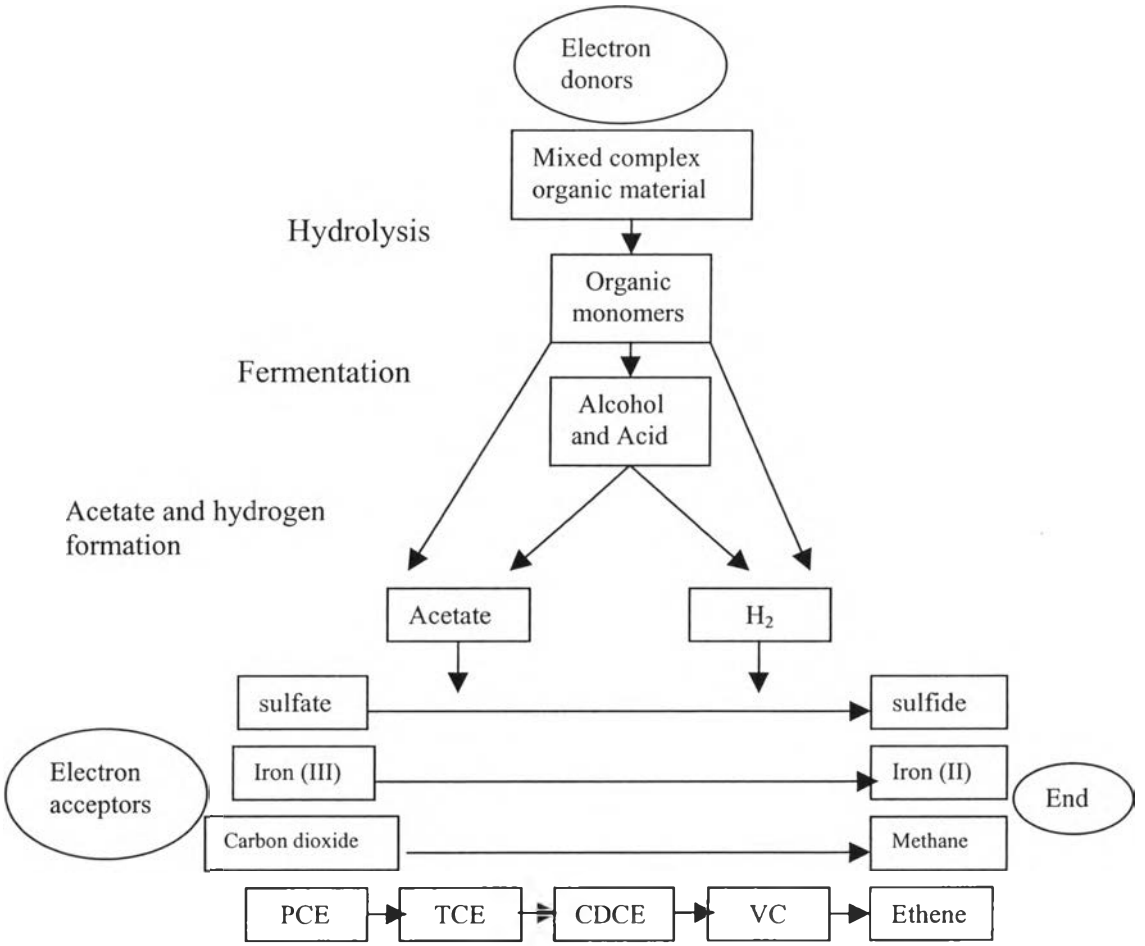

Figure 2.3 Steps In the process of biodegradation of PCE by reductive dechlorination (Suthersan, 2001).

As shown in Figure 2.3, biodegradable organic matter is required as an electron donor to initiate the process. Different types of microbes are involved at each stage. The bottom steps shows that PCE must compete for electrons with sulfate, iron, and carbon dioxide, meaning that a large amount of organic electron donors may be needed to supply enough electrons.

2.3.3 Mechanism of Anaerobic Dechlorination

Reductive dechlorination of aryl halides is thought to involve two one-electron reduction steps, resulting in the removal of halogen substituent and the formation of an intermediate aryl halide radical, which abstracts a proton from water to complete the reaction.

Though the actual mechanism of reductive dehalogenation is still unclear, factors that influence the process include the type of electron acceptors, temperature and substrate availability to the microorganisms. The most effective substrate (electron donor) is apparently dependent on the microbial population involved rather than the chemical structure of the compound to be reduced (Vogel, 1990). Electron acceptor conditions affect the population composition of microbial communities and correlated with the occurrence of reductive to dechlorinations activity. Thus, it was found that methanogenic conditions are conducive to dechlorinating activity of aryl halides, both in laboratory and in the environment. Whereas alkyl halides have been shown to be dechlorinated under methanogenic, sulfidogenic, and denitrifying conditions, aryl halides have only been demonstrate to undergo dechlorination under methanogenic and sulfidogenic conditions (Vogel, 1990). The presence of sulfate has been demonstrated to slow down dechlorinating activity on aryl halides. Thus, reductive dechlorination depends, at least in part, on the electron donor requirements and the efficiency with which electron can be directed to dechlorination.

The transformations resulting in the degradation of organic materials can be classified into two broad categories: mineralization and cometabolism. Whether a compound is mineralized or cometabolized has implications for the development of a waste treatment process for environmental remediation. Mineralization is the complete conversion of organic products. A compound that is mineralized serves as the growth substrate and energy source for the microorganism. Cometabolism is the degradation of organic compounds usually via nonspecific enzymatically mediated transformations. In contrast to mineralization, cometabolism does not result in the increase in cell biomass or energy. Consequently, the ability to cometabolize a compound is not benefit to the microorganism.

Halogenated compounds can be utilized as a growth substrate or cometabolized by anaerobic and aerobic microorganisms and consortia. Degradation of halogenated compounds can occur through the combined activities of fortuitously acting enzymes present in one or more microorganisms. Until recently, reductive dechlorination was generally considered a nonenergy-yielding detoxification mechanism. Hence, reductive dechlorination can be regarded as a form of 'anaerobic co-metabolism,' whether or not the compound is further degraded. Since reductive dechlorination is generally accepted to be a co-metabolic process, except in a few isolated cases, the microorganism, making enrichment and adaptation difficult derive benefit. Even highly chlorinated, poorly water soluble aromatic hydrocarbons that do not contain polar functional groups can also undergo reductive dehalogenation (Judith *et al*, 1991). Hexachlorobenzene (HCB) has generally been considered recalcitrant to microbial attack, particularly in the absence of oxygen.

However, HCB was shown to degrade to tri- and dichlorobenzenes by Fathepure *et al.* (1988). Typically, the chlorine atom in the middle of the three adjacent chlorines of chlorobenzenes (CBs) is easily eliminated. Two pathways of dechlorination were proposed: (1) a major pathway in which 1,3,5-trichlorobenzene (1,3,5-TCB) is formed via pentachloroenzene and 1,2,3,5-tetrachlorobenzenes (1,2,3,5-TTCB); and (2) a minor pathway in which dichlorobenzenes are formed via 1,2,4,5-TTCB and 1,2,4-TCBare formed via 1,2,4,5-TTCB and 1,2,4-TCB, see Figure 2.4.

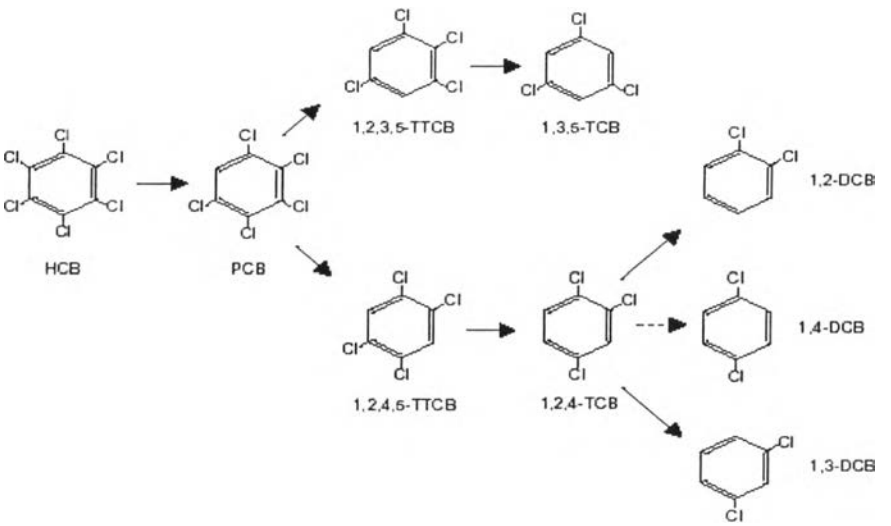

Figure 2.4 Proposed pathway for HCB dechlorination by an anaerobic microbial community (Fathepure *et al,* 1988).

2.4 Literature reviews

Many researches have been done regarding to HCB dechlorination. In one study, hexachlorobenzene (HCB) was dechlorinated to tri- and dichlorobenzenes in anaerobic sewage sludge. Dechlorination of HCB in fresh anaerobic sludge occurred with a lag time of approximately one week and biotransformation of 190 μM HCB (~50 ppm) was completed within 3 weeks. The major route of HCB dechlorination was HCB to pentachlorobenzene (PCB) to 1,2,3,5-tetrachlorobenzene (TeCB) to 1,3,5-trichlorobenzene (TCB). The minor route was HCB to PCB to 1,2,4,5-TeCB to 1,2,4-TCB to dichlorobenzenes (Fathepure et al, 1988).

There was the study that the mixture of chlorinated organic compounds could be degraded to water soluble metabolic intermediates and carbon dioxide by two-stage biofilm reactor (anaerobic biofilm column followed by aerobic column) (Fathepure and Vogel, 1991). HCB, PCE and chloroform can be dechlorinated on all tested primary carbon sources such as glucose, methanol and acetate, however, acetate provide the maximum extent of dechlorination in anaerobic biofilm column. This two-stage bioreactor system was recommended as the potential application for treating groundwater and industrial effluent composed of highly chlorinated aliphatic and aromatic hydrocarbons.

Nies and Vogel (1991)'s study showed that the source of the hydrogen atom added to the aromatic ring in reductive dechlorination of PCBs is the proton from water. This is consistent with a two-step mechanism, which involve the transformation of one electron to form an anion radical followed by the rapid expulsion of the halide to form a carbon radical. Another electron is then transferred the highly reactive radical to form a carbonion. It was also express that microbially mediated reductive dechlorination requires a source of electrons and protons. The reducing power is derived from the oxidation of the electron donors utilized by the microorganisms.

Anid *et al* (1992) found that reductive dechlorination by coenzymes can occur on aromatics, not only aliphatics as former belief, by demonstrate the ability of Vitamin B12 to mediate the reductive dechlorination of highly aromatic chlorinated compounds, pentachlorobiphenyl and HCB. However, more research is needed to assess the exact role of Vitamin B_{12}. Using available electron fortuitously by microbial mediated reaction, without earning any significant energetic benefit might occur. Coenzymes were derivative of Vitamin B_{12} and synthesized by microorganisms for use in normal metabolic functions. An increase in substrate level would lead to increase the total amount of coenzymes and a number of electrons available for reductive dechlorination.

Anaerobic metabolism of chlorinated benzenes was found in soil slurry microcosms under methanogenic conditions (Ramanand *et al*, 1993). The lag period for HCB metabolism was about two months and reached low levels of HCB concentration at the end of 142 days and their transformation resulted in transient accumulation of dechlorination products. Methane was produced in tested bottles, confirming the methanogenic condition under which the inoculation was carried out. Metabolism of HCB to chlorinated benzenes has not been observed in one single biological system and anaerobic microbial communities with dehalogenation activities were known to have high degrees of substrate specificity.

Jacobus and Connie (1994) studied the HCB dechlorination activity on lactate addition in the absence of sediment by microorganism derived from sediment

slurry from a shallow freshwater lake. The first amount of HCB disappearance to nearly zero amount was during 17 days; the subsequent addition of HCB disappeared rapidly. Fermentation of lactate in the medium apparently resulted in a sufficient H_2 production to serve as the source of reducing equivalent.

Chang *et al* (1997) found that the optimal dechlorination conditions from HCB to pentachlorobenzene (PCB) to 1,2,3,5-tetraclorobenzene (TeCB) to 1,3,5-trichlorobenzene (TCB) were 29-37°C and pH 6.1-6.9. Adding Ferrous Chloride and Manganese dioxide as an electron acceptor delayed the dechlorination. Adding lactate and pyruvate as electron donors enhance dechlorination. Adding acetate may not serve as electron donor of HCB dechlorination. Methane-producing bacteria was suggested to involved in the dechlorination of CBs because no dechlorination was observed when adding methanogenesis enzyme inhibitor.

Contaminant and organic matter bioavailability and their effect on the reductive dechlorination of sediment-bound chlorobenzenes were determined in another study. Hexachlorobenzene and other chlorinated benzene congeners were predominant chlorinated compounds encountered. Both the recalcitrant nature of the sediment organic matter and the strong partitioning of the chlorinated compounds were responsible for the low rate and extent of contaminant transformation. When frequent additions of degradable organic carbon source and ground sediment were used, an increased rate and extent of reductive dechlorination of the sediment-bound were observed from 2.2×10^{-3} day^{-1} to 2.1×10^{-2} day^{-1} (Prytula and Pavlostathis, 1996a).

Prytula and Pavlostathis (1996b) used five methods for studying the extraction of sediment-bound chlorinated organic compounds. Most of the problems associated with the extraction methods were related to high water content and the inherent heterogeneity of the sediment samples. Drying and grinding of the sample greatly improved both extraction efficiency and reproducibility. Analyses of wet sieve and fractionated sediment samples indicated that the contaminants are not evenly distributed throughout the sediment organic fraction but rather predominate in the larger particle sediment fraction.

Chang *et al* (1998) studied the potential dechlorination of HCB in medium by acclimated mixed culture under three reducing conditions. It was found that HCB dechlorination rates were 0.18 mg/l/d under methanogenic conditions and 0.14 mg/l/d under sulfate reducing conditions. Under denitrifying condition, HCB dechlorination was almost completely inhibited.

Rosenbroch *et al* (1997) investigated the potential for reductive dechlorination of hexachlorobenzene in different organic content, natural soils under anaerobic conditions. It was suggested that the indigenous anaerobic population was too low and growth was not improved by water saturation only because of the low inherent organic carbon content. A second effect of soil organic carbon is its role as an electron donor for reductive dechlorination.

Susarla *et al* (1997) examined the transformations of different classes of organic compounds in estuarine anaerobic sediment under sulfate-reducing condition by specific to determine the rate of transformation and half-life periods. Twenty-four halogenated benzenes were examined, including HCB. The rate constant for the reaction of HCB dechlorination followed a pseudo-first order reaction was 0.0256 d^{-1} with 27.1 days half-life. Dechlorination intermediate of HCB generally resulted in the accumulation of TCB.

Another study examined dechlorination of HCB in municipal sewage sludge by an anaerobic mixed culture. Optimal conditions was found at of pH 7.0 and 30°C. Biotransformation occurred along a pathway of HCB to pentachlorobenzene (PCB) to 1,2,3,4-tetrachlorobenzene (TeCB), 1,2,3,5-TeCB to 1,2,4-trichlorobenzene (TCB), 1,2,3-TCB, 1,3,5-TCB and 1,2-dichlorobenzene (DCB), 1,4-DCB. Dechlorination can be observed within substrate concentration of 2-50 mg/l, but slow down significantly at 50 mg/l or higher (Yuan *et al*, 1999).

The dechlorination occurs under anaerobic conditions and results in less chlorinated aerobically degradable products (Vogel, 1991). However, HCB is not directly dechlorinated by a single microbial activity but that various microbial systems are responsible for each step of dechlorination from HCB to TCB (Chen et al, 2000a).

The study of reductive dechlorination of hexachlorobenzene has found that the dechlorination was happened in a pH range of 5.96-7.70, and a temperature range of 29°C- 37°C. Mixed cultures from the sediment of Ho-Tsin River after a period of acclimation could dechlorinated HCB effectively. HCB-acclimated culture completely dechlorinated 4 times of re-introduced HCB all in one day and further nutrient addition is not necessary. After serial transfer, the lag phase of HCB dechlorination was shortened (Chen *et al*, 2002).

HCB was degraded to 1,2,5-trichlorobenzene (1,3,5-TCB) and 1,3-dichlorobenzene (1,3-DCB) in both a sludge medium and a synthetic medium with inoculated microorganisms. Chlorobenzene congeners showed that dechlorination by unacclimated microorganisms under anaerobic mixed cultures were more likely to occur under when larger amounts of energy were released when the transformation from the parent congener to daughter products were observed (Chen *et al*, 2000b).

Pavlostathis and Prytula (2000) studied the reductive dechlorination kinetics of all chlorinated benzenes. Using mixed culture derived from contaminated sediment. HCB was used as the primary contaminant. Pseudo first-order desorbed the transformation rate of HCB, obtained rate constants at 0.282±0.011 and half-life time of 2.5 days, with high value of correlation coefficients (r^2) 0.988. In addition, the ratio of the rate constant value of HCB dechlorination showed that almost directly proportional to the diluted initial biomass concentration. Besides, methanogens may not have been directly responsible for the observed reductive dechlorination of HCB and other chlorinated benzene as the sequential reductive dechlorination still took place but no methane production in methanogen-inhibitor amended culture series. Chlorinated benzene biotransformations by enrichment culture were considered to be cometabolic reductive dechlorination because of relatively very small energy as compared to those in pure cultures.

Adrian and Gorisch (2002) isolated microbial strain that couples reductive dechlorination of chlorinated benzene with energy conservations in additional anaerobic bacteria, thriving by dehalogenation of chlorinated benzenes or

chlorobiphenylic compounds. HCB has a result that highly persistent was tightly bound to sediment particles and therefore not bioavailable. Only grinding of the sediment and reinoculation led to complete HCB dechlorination.

From the literature reviews, there is no information about the sludge quantity in contaminated sediment that gives the most effective dechlorination. However, we can anticipate that the more sludge contained in the sediment, the more dechlorination efficiency. But it would be very useful for economic and technical reasons if the amount of biomass, which should be added to the contaminated soil to remove HCB in a certain amount of time effectively, is known. The various types of carbon source and using unacclimated sludge as inoculation has never been studied for reductive dechlorination of biomass in Thailand.

As various c-source that serve as electron donors stimulate different parts of anaerobic population (Perkins *et al*, 1994), they should be studied in order to see their role for dechlorinating activity. The type of c-source that provides the best result from this study would be obtained and valuable for further works either research or application. In addition, inoculation with unacclimated homogenized granular sludge is an important information because most of researches had been done by using acclimated sludge. In the other aspect, which this study focuses on, acclimation of sludge seed with HCB is not quite practical for real application because of high expense for reactor construction, operation, maintenance and transportation. Moreover, once that unacclimated sludge is used to remediate contaminated site which we could left it for a certain time, this unacclimated sludge will be able to develop or become acclimated by exposing to pollutant at a point of time.