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ABSTRACT

Mixed Culture Biodegradation of Pentachlorophenol,
Hexachlorobenzene, and Tetrachloromethane
under Anaerobic Conditions

by

Jesse Zahn

Three chlorinated compounds, namely, Pentachlorophenol (PCP), Hexachlorobenzene (HCB), and Carbon tetrachloride (CCl_4) were biodegraded under anaerobic conditions using a mixed culture obtained from an anaerobic sewage sludge digester in Linden, NJ.

Three sets of cultures capable of degrading each of the target compounds were established. Primary Enrichment Cultures (PECs) from the anaerobic sludge were exposed to each one of the target compounds. The cultures were able to degrade each target compound as follows: HCB, 0 to 1.2 ppm; PCP from 0 to 3-4 ppm; CCl_4 , 0.5 to 15.5 ppm.

One culture from each PEC group (CCl_4 , HCB, PCP) was transferred into fresh sterilized sludge with a mixture of all three compounds at a concentration of approximately 10 ppm each. Each culture produced some degradation: HCB 0.27 to 0.5 ppm; PCP, 1.7 to 6.3 ppm; and CCl_4 , 2.4 to 5 ppm. The maximum time period for degradation in both experiments was approximately three months.

This is the first time in the literature that these three chlorinated compounds have been anaerobically biodegraded at the same time.

MIXED CULTURE BIODEGRADATION OF PENTACHLOROPHENOL,
HEXACHLOROBENZENE, AND TETRACHLOROMETHANE
UNDER ANAEROBIC CONDITIONS

by
Jesse Zahn

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APPROVAL PAGE

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CHAPTER 1

INTRODUCTION

1.1 History of Hazardous Waste

The seeds of today's hazardous waste problem were planted early in the eighteenth century with the manufacture of large amounts of fuel from the discovery of the coal conversion process. Coal conversion is the production of a gaseous, liquid, or low-sulfur, low ash fuel from the original coal. The first municipal coal gas system was utilized to light Pall Mall in London in 1807. The coal-gas industry quickly spread to the United States in 1816, to reach an optimal 11,000 coal gasifiers operating in the 1920s. In 1947, 57% of U.S. manufactured gas was from the coal gasification process. During World War II, Germany developed a major synthetic petroleum industry based on coal which reached a peak capacity of 100,000 barrels per day in 1944 (Manahan, 1991).

This process was one of the first large scale processes that created hazardous waste in significant amounts, specifically waste liquids composed of aromatics and polycyclic aromatic hydrocarbons from incomplete combustion (Miller, 1988). A lack of understanding and mismanagement of the waste produced has resulted in several hundreds of these old plants being designated as hazardous waste sites because of the unhealthy residues that remain, threatening human and environmental health.

Apart from fuels, the War industry of the early 20th century also created the demand for vast amounts of new materials including munitions and poisonous gases. This resulted in generating thousands of tons of hazardous wastes.

The production of hazardous wastes continues today because the American consumer demands throw-away products that are often made from hazardous raw material feed stocks and/or produce hazardous waste as by-products.

The mismanagement of large amounts of hazardous wastes at "Love Canal" in Niagara Falls, NY and in HopeWell, Virginia, brought about the promulgation and passage of the Resource Conservation and Recovery Act (RCRA) of 1976 (Wentz 1989). It was specifically promulgated and enacted to deal with the generation, treatment, storage, and disposal of hazardous wastes, to prevent further hazardous waste disasters. In RCRA, Congress defines the term hazardous waste "as a waste, or combination of wastes, which because of its quantity, concentration, or physical, chemical, or infectious characteristics may (1) cause, or significantly contribute to, an increase in mortality or an increase in serious irreversible or incapacitating reversible illness or (2) pose a substantial present or potential hazard to human health or the environment when improperly treated, stored, transported, or disposed of" (Wentz, 1989).

Between the years 1950 and 1975, an estimated 6 billion tons of hazardous waste were deposited on or under the

land's surface throughout the United States. In addition, there are, by EPA estimates, at least 26,000 sites where hazardous materials were dumped before RCRA was promulgated and enacted. Annually, approximately 265 million metric tons of hazardous waste are produced in the US. (Miller, 1988), approximately one ton per person per year. This figure excludes those substances not regulated by RCRA: radioactive waste, sewage sludge, and household toxic and hazardous substances.

Regulatory agencies have just begun the monumental task of accounting for the production of hazardous waste in various industries. They have located and investigated the bulk of the unclaimed hazardous waste "dump" sites. However, the problem still remains of how to treat and dispose of the hazardous waste still produced today and remediate older disposal sites to the extent that risk to human health is minimal from any exposure to the waste or waste site.

Therefore, it is the intent of this thesis to determine the **biological treatability** of three common chlorinated chemicals that are ubiquitous in the environment because of their widespread use and persistence: Hexachlorobenzene (HCB), Carbon Tetrachloride (CCl_4) [or Tetrachloromethane], and Pentachlorophenol (PCP). While the three compounds are highly chlorinated, the similarity ends there, because they come from three distinctly different chemical groups.

Sections 1.3, 1.4, and 1.5 discuss the chemical class each compound belongs to and its associated characteristics.

1.2 Hazardous Waste Treatment Methods

After site assessments have been conducted, and the waste has been identified and quantified, the site remediation process can begin. There are several ways to remove hazardous waste once it has been deposited, purposefully or unintentionally, in the environment.

Currently there are four primary methods that are available for the treatment of hazardous wastes; physical, chemical, biological and incineration processes (Wentz, 1989).

Physical processes typically do not destroy hazardous wastes. Through concentration or phase changes, physical treatment produces a waste that is more easily and safely disposed of. Carbon adsorption, stripping, and sedimentation are some examples of physical treatment. A waste stream treated by such a process generates a hazardous waste, since the original contaminants have not been destroyed or detoxified, just concentrated. Low cost and well understood principles of operation are the primary advantages of physical treatment processes.

Chemical treatment may render hazardous waste harmless by destroying the constituents or changing them chemically. Neutralization, precipitation reactions, and wet air oxidation are three examples of chemical treatment. There

are several disadvantages to chemical treatment processes.

(1) The volume of the treated waste is usually much increased, requiring more disposal volume for the newly created hazardous sludge; (2) The hazardous waste that is being treated must be of known, and homogenous composition; (3) Complete destruction of waste is often not accomplished; (4) The chemicals used for the treatment process are often hazardous and pose a risk to workers and environmental health if mismanaged.

The advantages of chemical treatment includes: wide range of treatment applicability, low cost, and well understood processes of treatment.

Thermal treatment processes use high temperature as the mechanism of destruction for hazardous wastes. The advantages of incineration are significant: (1) Complete destruction or removal of hazardous constituents; (2) Low volume waste-ash produced to dispose; (3) Mobile treatment. A "traveling" incinerator (Wentz, 1989) can be moved to the hazardous waste site, instead of transporting the hazardous waste to the incinerator and increasing the risk of exposure to the workers and the environment. Additionally, start-up costs are reduced since the incinerator unit only has to be constructed once.

The disadvantages of thermal treatment are also significant: (1) Highly skilled operators are needed; (2) Precise operation parameters, test burns, and Destruction Removal Efficiencies (DREs) need to be complied with; (3)

Construction of new facilities is often hampered by State and Federal processes, and citizen group actions; (4) Only defined waste streams can be used, and high volumes of these waste streams need to be available for continuous operation of the facility; (5) High cost.

If an incinerator can be built operated, the advantages of permanent waste destruction are far superior to other methods of "treatment" that do not change the human and environmental toxicity of the initial hazardous waste.

Biological treatment utilizes microorganisms to biodegrade waste contaminants into less toxic or completely non-toxic products. It may be applied through conventional approaches or by *in-situ* treatment.

Conventional approaches for biological degradation (including aerobic and anaerobic processes) are used in sewage treatment plants, such as Publicly Owned Treatment Works (POTWs), and in small and large scale bioreactors.

Sewage treatment plants use sequential aerobic and/or anaerobic treatment to purify water by decreasing the concentration of suspended solids, oxygen demanding wastes, and nitrogen and phosphorus compounds. Destruction of many pathogens, but not all, also occurs during this process, possibly as a result of high temperatures experienced during the activated sludge process (Miller, 1988). Most removal of hazardous waste in this process occurs through the concentration of metals in the activated sludge and through

the biological degradation of the organic content of the waste that occurs during the process.

The advantages to this system are many. If the environmental conditions are adjusted correctly, and there is no shock-loading, then large volumes of waste can be treated with moderate to complete degradation of many xenobiotics (Verschuieren, 1983).

The principal disadvantage to sewage treatment plants with respect to hazardous wastes is that even when operating optimally, the treatment plants are only effective for a small number of hazardous compounds and in low concentrations.

Small and large scale bioreactors are the real "workhorses" of the biological remediation field. The process often consists of inoculating a simulated natural environment with specially acclimated microorganisms are able to utilize one or more xenobiotic substrates. The special microorganisms may be isolated from an environment that is contaminated with the hazardous compound of interest. For example, chlorophenol degrading organisms are often encountered near paper mill effluents because chlorophenols are used at most paper mills as slimicides in the pulp process (Allard et al., 1991; Sahm et al., 1986). The microorganism may also be bio-engineered from two or more bacterial strains (Bhatnagar and Fathepure, 1991).

Several environmental parameters must be controlled in the reactor to achieve maximum degradation rates. pH,

temperature, residence time, concentration, alkalinity, oxygen concentration, and media composition are a few.

The control of environmental conditions is the primary advantage to using a bioreactor in the degradation of hazardous waste. Speed of degradation is also another advantage offered by bioreactors operating with low to moderate waste stream flows. Bioreactors are especially suited for low level contaminant concentrations often found in drinking or ground water supplies. Many microorganisms grow very well with low concentrations of substrate. (Prescott *et al.*, 1990). Physical and chemical processes often are not able to separate and treat hazardous constituents at low levels, and the cost associated rises quickly to remove the last few ppm of a xenobiotic compound from a given waste stream by physical and chemical means. The ability of the bioreactor to remove hazardous constituents at low concentrations, at lower cost than most physical methods, is another advantage of this tool.

In-Situ treatment is the newest method by which environmental, organic and pesticide, contaminants can be treated (Bhatnagar and Fathepure, 1991). It is an on-site process that uses indigenous or native microorganisms to degrade contaminants. The process involves identifying the contaminants, their concentration, and distribution on site. The local biological population and environmental conditions are then evaluated to determine what nutrients may be added to enhance their growth in order to increase their

detoxification rate. Often times a "fertilizer" containing nitrogen or phosphorus may be sprayed on the site.

Alternatively, air can be pumped into the ground if it has been determined the aerobic population is the principal detoxifier and is limited by oxygen.

There are several advantages to in-situ treatment: (1) The in-place waste is not concentrated or mobilized for treatment, thus minimizing further risk to human and environmental health; (2) The cost of treatment is often very low compared to physical, chemical, and thermal technologies; (3) Removal of low level contamination will not cost proportionately more than treating higher concentrations, as is often the case with other treatment technologies (Manahan, 1991).

There are also several significant disadvantages to In-situ biotreatment: (1) Only organic compounds, some pesticides, esters, and amides can be treated since In-situ treatment is a biological process; (2) The hostile environment of a chemical waste landfill, including the presence of enzyme-inhibiting heavy metal ions, is deleterious to many biological processes, thus limiting, if not eliminating their productivity; (3) If a loss of contaminant is measured, it must be demonstrated that the loss resulted from microbial metabolism (Madsen et al., 1991), and not from physical/chemical processes.

In-situ biological treatment is a tool presently best used in conjunction with other methods in well-defined sites

with organic contamination. The enhanced petroleum degradation demonstrated at Prince William Sound, Alaska after the Exxon Valdez spill in some locations showed in-situ bioremediation's potential, and limitations. The magic "bioremediation bullet" does not exist yet. Microorganisms can perform well in some well defined systems, but most hazardous waste sites are not well defined, nor hospitable to bioremediation.

1.3 Chloroaromatics

Hexachlorobenzene can be classified as a chlorinated aromatic hydrocarbon. Aromatic hydrocarbons are a class of common chemicals found throughout the biosphere derived from natural and anthropogenic sources. Lignin and its derivatives, cell walls from algae and algal spores, and *p*-isopropyltoluene harvested from plants are all natural sources of aromatic hydrocarbons (Young, 1984; Healy, Jr. and Young, 1979). Indeed, Hager (1981) showed that some haloorganic compounds are natural products of living cells, though occurring only in traces. In general, naturally occurring haloaromatics (benzene ring with a halogen directly attached, in place of hydrogen) have potent biological activity, including the iodo aromatic hormone thyroxine found in mammals and the antibiotics griseofulvin and aureomycin produced by actinomycetes (Sahm *et al.*, 1986).

Over 70% of anthropogenic aromatic hydrocarbons are derived from petroleum, with the remainder derived from coal. When bituminous coal is heated to 100-1300°F in the absence of air, a light oil fraction is separated from the tar. The latter supplies the polycyclic aromatic hydrocarbons, naphthalenes, and anthracenes, while the light oil supplies benzene, toluene, xylenes, and solvent naphthas. Preparation of aromatic hydrocarbons from crude oil requires distillation, solvent extraction, and crystallization processes. These compounds are used as starting materials for the synthesis of plastics, paints, pesticides, resins, and dyes. They are also used as solvents for paints, dyes, resins, rubber, and plastics, and in automotive and aviation fuels (Young, 1984).

The deleterious effect of halogenated aromatic compounds upon the biosphere has been well publicized. 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), dichlorodiphenyltrichloroethane (DDT), polychlorinated and polybrominated biphenyls (PCBs & PBBs) are just some of the more infamous representatives of this class of chemical compounds. Entry into, and persistence in, the environment of compounds such as DDT is expected since they were applied in large amounts to crops and are resistant to degradation, both biotic and abiotic. Additionally, the widespread contamination of the environment (including the remote Antarctic) is facilitated by DDT's properties. Because it is highly soluble in fatty tissues, it accumulates in the

lipids of plants and animals. It undergoes partial degradation, largely from DDT to DDE, and has a half-life of approximately 20 years. DDT has a vapor pressure high enough to ensure direct losses from plants. It can be adsorbed to particles or remain as a vapor, and in either state, be transported by normal atmospheric circulation then return to land and sea on rainwater (Smith, 1986).

Kayden (1979) reports skin lesions, abnormal liver function, neurological effects such as numbness and vision impairment, suppression of the immune system, and interference with reproductive hormones associated with these halogenated hydrocarbons. Since many aromatics exhibit a high degree of nonpolarity, they partition and accumulate (biomagnification) quite readily into the fatty tissues of higher mammals at each successive trophic level, with top level carnivores (including humans) at risk from these concentrated compounds. Halogenated aromatic carcinogenic and teratogenic effects are discussed by Barr (1979) and Halling (1979).

On a global scale, reduced photosynthetic activity and loss in species diversity in phytoplankton populations (Wurster, 1968; SCEP, 1970) are direct results of halogenated aromatic hydrocarbons. Biomagnification through the food chain of the aromatic hydrocarbons has damaged reproductive capacity of bird species and caused their numbers to directly decline (Cooke, 1973; Robinson, 1979). Seal and sea lion populations behaved similarly (Young,

1984). The ban placed on DDT in the early 1970s by the United States and most Western nations has caused its levels to decrease in certain animal populations and mammals, from 7.88 ppm in 1970 to 4.99 ppm in 1974 (Schneider, 1979; Smith, 1986).

1.4 Chlorophenols

Pentachlorophenol, the second of the three target compounds studied here, comes from the class of compounds called the chlorophenols. They are the chlorinated aryl analogs of alcohols and have different properties from those of the aliphatic and olefinic alcohols. The addition of nitro and especially halogen groups strongly changes the toxicity of the specific phenol under consideration (Manahan, 1991).

Phenols, which are colorless, are produced on a scale of tens of thousands of pounds annually. Over 80 million pounds of pentachlorophenol were produced in 1977, making it the most used pesticide in the United States (Tiedje *et al.*, 1987). Rappe and workers (1979) quote over 150,000 tons annual world production of phenol.

The phenol family is used as a herbicide in rice and sugar cane fields, fungicides, and in general biocides, usually for the paper making industry, and are often found in the vicinity of bleachery discharges (Allard *et al.*, 1991).

Phenol, the simplest of its family, was the first antiseptic used on wounds and in surgery (Manahan, 1991).

Phenolics are tuberculocidal, effective in the presence of organic material and remain active on surfaces long after application. Phenol is still an effective antiseptic today, used in mouthwash and lip ointment products at concentrations of two percent or less. The principal use of phenol in manufacturing is for synthesis of phenol-formaldehyde polymers (Morrison and Boyd, 1987).

1.5 Chlorinated Aliphatics

As important in the biosphere as halogenated aromatics are halogenated aliphatic compounds (HACs). They are prevalent groundwater contaminants (Bouwer and Wright, 1988; Rahn, 1986; Speitel and Closmann, 1989) and are significant components of hazardous wastes, landfill leachates, permitted industrial and municipal discharges, urban and agricultural runoff and leaky underground pipes and storage tanks. Some sediments below industrial sites contain chlorinated hydrocarbons in excess of 1000 mg/liter. Small volumes of these compounds can contaminate entire drinking water aquifers. A recent random EPA sampling of 466 subsurface drinking water sources documented that 22% of the sources contained detectable levels of some volatile organic Hacs (Fathepure and Vogel, 1991). The EPA reports serious contamination of drinking-water wells by toxic organics in almost every state. A 1981 study of 670 wells representative of ground water in New Jersey found that 111 of them contained elevated levels of toxic organic

chemicals. In 1980, Rockaway Township, NJ discovered all three of its public wells were contaminated with tetrachlorethylene (TCE) and other chemicals (Rahn, 1986).

Most HACs released from industrial, commercial, and agricultural sources are brominated or chlorinated alkanes and alkenes that contain between one and three carbon atoms. Chlorinated ethanes and ethenes are in common use as cleaning solvents in dry-cleaning operations and semiconductor manufacture. Some brominated compounds such as 1,2-dibromoethane (EDB) and 1,2-dibromo-3-chloropropane (DBCP) are used as pesticides. The six most common volatile organic chemicals are tetrachloroethylene (PCE), trichloroethylene (TCE), 1,1,1-trichloroethane (TCA), cis-1,2-dichloroethylene (cis-1,2-DCE), 1,2-dichloroethane (1,2-DCA), and 1,1-dichloroethylene (1,1-DCE) (Bhatnagar and Fathepure, 1991).

The apparent hazard to human health from HACs has prompted investigations concerning their fate in the human body, ground water, and in treatment facilities (Vogel *et al.*, 1987; Bouwer and Wright, 1988).

CHAPTER 2

BIOLOGICAL DEGRADATION PROCESSES

2.1 Aerobic Biological Degradation Processes

Although this thesis is primarily concerned with anaerobic biodegradability, a brief overview of aerobic processes is presented here for completeness.

An aerobe is described as an "...organism that grow[s] in the presence of atmospheric oxygen." Aerobic respiration may be defined as, "A metabolic process in which organic molecules are oxidized with oxygen as the final electron acceptor" (Prescott *et al.*, 1990). Obligate aerobes and facultative anaerobes must protect themselves against the harmful effects of free oxygen, and usually do with the enzymes superoxide dismutase and/or catalase which catalyze the destruction of the superoxide radical and hydrogen peroxide, insuring that their own cell membranes and enzymes are not destroyed by oxygen.

Molecular oxygen is used for two purposes by microorganisms: (i) as a terminal electron acceptor where electrons are released during oxidation of organic substrates and energy is generated, and (ii) as a substrate for a biochemical reactions.

Eukaryotes such as fungi and mammals use monooxygenases to introduce a single atom of molecular O₂ into aromatic or aliphatic hydrocarbons. The resulting epoxide intermediate undergoes hydration with water to form a trans-1,2-

dihydroxy-1,2-dihydro intermediate, which is converted to a trans-dihydroxy compound (Gibson, 1976).

The single most important rate-limiting step in aerobic haloorganic biodegradation is the removal of the halogen from the organic compound. There are two ways in which this can be accomplished: (a) elimination of halogen during the initial stages of degradation via a reductive, hydrolytic, or oxygenolytic removal mechanism, (b) generation of nonaromatic structures which spontaneously lose halide by hydrolysis or hydrogen halide by beta-elimination.

Concerning *halogenated aliphatics*, bacteria that grow on these hydrocarbons usually introduce molecular O₂ into the organic molecule by oxygenases. Both types of oxygenases, mono and di, have been implicated in the oxidation of halogenated hydrocarbons (Bhatnagar and Fathepure, 1991).

A second oxidation mechanism occurs in some bacteria that can utilize these chemicals as a **sole source** of carbon and energy (Leisinger and Brunner, 1986; McCarty, 1988; Hartmans et al., 1985). Many species of *Pseudomonas* and *Hypomicrobium* are able to metabolize chlorinated alkanes as their primary substrates. Using glutathione-dependent dehalogenase, chlorine removal from dichloromethane is possible. Degradation of 1,2-dichloroethane proceeds in two ways. Soil bacterium strain DE2 transform 1,2-dichloroethanol, which is further converted to 2-chloroacetaldehyde and 2-chloroacetic acid, whereas

Xanthobacter autotrophicus degrade 1,2-dichloroethane to 1,2-chloroethanol by a hydrolytic dehalogenase (Janssen *et al.*, 1985).

Typically, aerobic processes play an important role in the catabolism of lesser chlorinated xenobiotics. An anaerobe may dechlorinate two or three halogens from the initially highly halogenated xenobiotic, creating an accumulation of the partially dehalogenated substrate. Then if the environment becomes aerobic, aerobes may complete dehalogenation and metabolize the substrate. If the system remains anaerobic, further degradation will not continue.

Some researchers have found that a sequential anaerobic-aerobic reactor system is the most effective in treating a highly halogenated xenobiotic compound (Dienemann *et al.*, 1990; Fathepure and Vogel, 1991; Kafekewitz *et al.*, 1992; Armenante *et al.*, 1992) for complete catabolism. Under either consideration, aerobic processes are as important as anaerobic catabolism, and the researcher must understand the sensitive link between the two for successful utilization of microorganisms for biodegradation to laboratory and especially environmental systems where the boundary between aerobic/anaerobic changes spatially and temporally.

2.2 Anaerobic Biological Degradation Processes

Anaerobic respiration is described by Prescott *et al.*, (1990) "...as an energy-yielding process in which the electron transport chain acceptor is an inorganic molecule other than oxygen." Some anaerobic electron acceptors are nitrate, sulfate, and carbon dioxide. NO_3^- accepts electrons to become reduced to NO_2^- , N_2O and finally to nitrogen gas, N_2 . Sulfate is reduced to hydrogen sulfide gas, H_2S , and carbon dioxide is reduced to methane, CH_4 .

Anaerobes are divided into three subclasses:

facultative anaerobes which do not require O_2 for growth, but grow better in its presence, **strict or obligate anaerobes** which will not tolerate free oxygen and die upon exposure to even small quantities of O_2 , and **aerotolerant anaerobes** which grow equally well in the presence or absence of O_2 .

Environmentally significant anoxic conditions are likely to exist in waterlogged soils, stagnant water, municipal landfills, sewage treatment digesters, and the alimentary tract of all animals. Occasionally in groundwater, and in the sediments of oceans and other natural water bodies.

Tarvin and Buswell provided the first decisive chemical evidence, in 1934, that the aromatic nucleus of common aromatic compounds was completely decomposed when incubated anaerobically with sewage sludge (Evans & Fuchs 1988). The

compounds reduced were natural oxygenated aromatics such as benzoate, phenylacetate, phenyl propionate and others.

Anaerobic transformation of non-oxygenated aromatic hydrocarbons was first demonstrated in 1980 by Ward and workers. Only seven years ago, Vogel and Grbic-Galic (1986) were able to provide evidence for the initial oxidative mechanism of toluene and benzene. In the short time elapsed since then, research has delved deeply into the study of halogenated aliphatic and aromatic biodegradation. The following is a brief overview partially from Bhatnagar and Fathepure (1991) of anaerobic degradative pathways recently elucidated and still evolving. Only examples of pathways pertinent to this thesis will be discussed, i.e., halogenated aliphatic and aromatic biological degradative processes.

The specific halogen studied in this thesis was chlorine, but many researchers have used bromine and iodine, as models to chlorine mechanisms, and in many experiments have found the degradation to be more expedient and complete (Edward and Wright, 1988; Vogel *et al.*, 1987; Wolf, 1976; Bini *et al.*, 1975; Watanabe, 1986), therefore some discussion of these halogens will be included. Literature coverage of pentachlorophenol, carbon tetrachloride, and hexachlorobenzene will be discussed in Section 4.0.

Anaerobic processes become crucial with respect to contaminants released into the environment by man. Recent research has shown that anaerobic microorganisms are able to

degrade some compounds also degraded by aerobes, as well as many important others that cannot be metabolized by aerobes. Two anaerobic processes used by the microbes are **reductive dechlorination** of highly chlorinated aliphatic and aromatic hydrocarbons and reduction of the **aromatic ring to an alicyclic ring** structure, and finally **ring fission**.

Reductive dehalogenation is the key process by which halogenated organics are degraded. This is an anaerobic process by which one or more halogens, located on a ring or carbon skeleton, is replaced by a hydrogen and an HX acid (X representing Cl, I, or Br) is formed as product of the substitution. The newly reduced, less halogenated compound is now easier to attack for further partial or complete degradation by aerobic microorganisms. Without this first key dehalogenation step, degradation of the compound would not proceed.

Reductive dehalogenation performed by mixed cultures was first reported by Sulfita and co-workers (1982). Using halogenated benzoates with methanogenic enrichment cultures, the benzoates were partially or completely degraded to methane, CO₂, and hydrochloric acid. Several researchers studying anaerobic dehalogenation of various halogenated benzoates and phenols derivatives suggest that dehalogenation is an oxygen sensitive process (Boyd and Shelton, 1984; Boyd et al., 1983; Horowitz et al., 1983; Sulfita et al., 1983).

The complex interaction between multiple organisms and the task each one performs in the complete degradation of a chlorinated compound was demonstrated clearly by Shelton and Tiedje (1984). They characterized a mixed microbial community in sludge that performed the dechlorination of 3-chlorobenzoate. The chlorobenzoates are popular test compounds for biological degradation studies because they are a toxic constituent of hazardous wastes, an intermediary product of several pollutants, (PCBs, chlorophenols) and components of some herbicides. Nine different organisms were isolated by Shelton and Tiedje out of which only five were thought to have major roles in the carbon and electron flow: (a) The dechlorinating organism, DCB-1, that was responsible for removing the chlorine(s) and producing benzoate, (b) The benzoate degrading strain, BZ-2, that metabolized the ring, and produced H₂ and acetate, (c) The hydrogen utilizers, *Methanospirillum* and *Methanobacterium*, and *Methanothrix*, which use acetate to produce CO₂ and CH₄.

Recent studies on pentachlorophenol (PCP) degradation have uncovered two different dechlorination pathways. Mikesell and Boyd (1986) used fresh sludge in batch culture studies when they demonstrated PCP was sequentially converted to 3,4,5-trichlorophenol, 3,5-dichlorophenol, 3-chlorophenol which was finally metabolized to CH₄ and CO₂. Thus, they concluded the *ortho* positions of PCP were dechlorinated.

Bhatnagar and co-workers (1989) developed granular anaerobic mixed cultures from specific enrichments of PCP which dechlorinated PCP in an upflow anaerobic sludge blanket reactor via 2,4,6-trichlorophenol and 4-chlorophenol. They concluded a *meta* dechlorination pathway as the initial dechlorination step.

Regarding the chlorinated benzenes, dechlorination also occurs via two routes. Hexachlorobenzene incubated anaerobically with sewage sludge for three weeks accumulated 1,3,5-trichlorobenzene which remained unchanged. The isomer 1,2,4-trichlorobenzene formed via a minor route, and further dechlorinated to all isomers of dichlorobenzene (Fathepure et al., 1988).

The initial step in the degradation of **halogenated aliphatics** is also reductive dehalogenation. Unlike halogenated aromatic compounds, halogenated aliphatics can lose one or two halides during the reductive process. Vogel (1987) provides evidence for a dihalogen (adjacent) removal with the concurrent production of a double bond. Bhatnagar and Fathepure (1991) suggest that chloroform and carbon tetrachloride in sediment samples probably go through an initial reductive dehalogenation step before leading to the final product, CO₂.

A final topic to be covered under this section is the subject of **cometabolism**. This is especially important, because this type of metabolism is most likely a factor in the degradation of xenobiotics by mixed cultures, including

this thesis work. Bhatnagar and Fathepure (1991) describe cometabolism in the following way: "...many microorganisms growing on one substrate may be able to transform or degrade a different co-substrate in a reaction or sequence of reactions that is not directly associated with the organism's energy generation, carbon assimilation, and biosynthesis or growth." Cometabolism was formerly termed cooxidation, but the concept also includes reactions not necessarily involved in breakdown of the substrate.

Cometabolism is suggested as a possible mechanism by Goulding and workers (1988). They used an ethanol solution for stock solutions of the lower chlorobenzenes and pentachlorophenol from which they obtained 100% degradation for 19 out of 24 compounds by an aerobic mixed culture. They state, "All substrates were provided in ethanol solution (0.1-1%) so the possibility of co-oxidation of aromatic compounds cannot be excluded." High concentrations of alcohol produces a sterilizing action, but low concentrations, less than 1%, are easily degraded, so that the ethanol may have acted as a desirable substrate for the PCP degraders.

Matsumura (1982) discusses the concept of cometabolism in relation to several microbiologically mediated catabolic processes for pesticide degradation. He further defines the indistinct "cometabolism" to *incidental* metabolism, stating that "...the pesticides themselves, including any part of the pesticide molecules, do not serve as the energy and

carbon source for the microorganisms. Therefore, addition of pesticides does not affect their growth, which is always controlled by other nutrients...It may be also generalized that incidental metabolism is the more prevalent form of microbial metabolism, when the amount of pesticides is low in comparison with other carbon sources. Catabolic metabolism could occur when the amount of pesticide is high, coupled with the favorable chemical structure of the insecticide that allows it to be microbially degradable and utilizable as a carbon source."

Therefore, degradation may not indicate that an organism possesses or has specifically evolved a separate catabolic pathway for a certain xenobiotic, especially if the xenobiotic concentration is low, as is often the situation in many research experiments. The only method to determine if the microorganism is unequivocally using a certain xenobiotic as a primary substrate for energy and biosynthesis, is to utilize a defined media with the xenobiotic as the only carbon source.

2.3 Mixed Culture Biological Degradation Processes

As will be discussed more extensively below, the approach used in this study to biologically degrade carbon tetrachloride, pentachlorophenol, and hexachlorobenzene was to initially use a mixed culture from anaerobic sewage sludge that would acclimate to each of the target compounds independently, then combine the three target compounds and

use each of the acclimated cultures to attempt to degrade a mixture of the three target compounds at once in the same system. The following is a discussion of mixed cultures and the advantages they offer to degradation of hazardous waste.

The initial culture used from the anaerobic sewage sludge is called a *mixed culture* [The following is partially readapted from Zeikus and Johnson, 1991]. Mixed cultures are those in which the inoculum always consists of two or more organisms. Pure cultures consist of only one strain of microorganism growing in isolation; excluded are inocula in which two or more strains of the same species are used. Mixed cultures may consist of known species, or they may be composed of mixtures of unknown species. The mixed culture may consist of just bacteria, or a mixture of organisms such as fungi, bacteria and yeasts.

In nature, it is much more common to have a mixed culture than a pure one, but a few special situations occur where a single microorganism may be growing separately, alone, as in the interior of stems, roots, leaves, and seeds. These places are normally sterile. When an infection does occur in these areas, the infecting organism may grow in pure culture in the host. In a location of extreme environmental conditions such as a hot springs or in a fen (alkaline environment), a single microorganism may be responsible for the fermentation of the substrate.

There are many advantages that mixed cultures offer over pure culture: (i) increased overall growth rates; (ii)

creation of a very stable microorganism association; (iii) better utilization of substrates, i.e. destruction of toxic substrates; (iv) potential for bringing about multi-step transformations that would be impossible for a single microorganism; (v) with proper control of temperature, mass of substrate, length of fermentation, and kind of substrate, it is not difficult matter to carry out repeated successful fermentations; (vi) reduced potential for phage infections since mixed cultures have a wider genetic base of resistance to phage. This keeps the culture from losing any of the strains it possesses and thus keeps it operating optimally; (vii) Mixed culture fermentations enable the utilization of cheap and impure substrates. Trace contaminants or impurities will not affect a mixed culture as much as a pure culture. A pure culture may be destroyed by an abiotic or biotic impurity, while a mixed culture would more likely remain unaffected; (viii) Some organisms in the mixed culture may provide necessary nutrients for optimal growth. Growth factors, vitamins, and other substances may be provided by organisms in the mixed culture for other organisms (symbiotic associations); (ix) Mixed cultures are more stable and recover more rapidly when growth conditions are disturbed i.e. in anaerobic digesters of sewage treatment plants. Changes in pH, temperature, xenobiotic concentration etc., will affect a mixed culture much less than a pure culture; (xi) Foaming is usually not a problem with mixed cultures, hence no antifoaming agents have to be

added to the system. This becomes especially significant in large scale digester and reactor operation.

The disadvantages offered by a mixed culture system are few, but significant: (i) The scientific study of the mixed culture is much more difficult than in most pure strain (monoculture) studies. Manipulation of any one parameter in the system will affect each strain in the culture differently, some adversely, some beneficially, and some with no apparent effect; (ii) Defining the product and the microorganisms involved becomes more difficult for patent and regulatory procedures.

Zeikus and Johnson (1991) conclude by stating that the performance of a pure culture in the degradation of a toxic organic xenobiotic compound is very poor when compared to that of an adapted mixed culture. They suggest that individual microbes do not have the genetic information to code for all the enzymes required for biodegradation because they have not been exposed to xenobiotics long enough to expect the evolution of a separate complete catabolic pathway. With skillful manipulation of microorganisms, there may be the opportunity to "direct the evolution" of organisms and their enzymes for specific xenobiotic substrates that are currently being degraded in a nebulous mixed culture system.

For the highly chlorinated more recalcitrant compounds like hexachlorobenzene, used in this study, a mixed culture from anaerobic sewage sludge has shown to be the only method

able to effect degradation. In fact, mixed cultures harvested from anaerobic digesters are often the microorganisms of choice for biodegradation of recalcitrant chlorinated xenobiotics in the laboratory (Sulfita *et al.*, 1984; Fathepure *et al.*, 1988; Topping, 1987; Reineke and Knackmuss, 1984; Fathepure and Vogel, 1991; Vargas and Ahlert, 1987).

CHAPTER 3

OBJECTIVE OF THIS STUDY

The objectives of this thesis were twofold. The first objective was to establish three distinct enrichment cultures from anaerobic digester sewage sludge by exposing the primary culture to the three compounds of interest in this work, i.e., pentachlorophenol, carbon tetrachloride, hexachlorobenzene. It was expected that each of the resulting three mixed cultures would have specific degradative abilities against the compound to which the culture was exposed.

The second objective was to determine if the three now distinct mixed cultures would be able to degrade a mixture of all three of the xenobiotics at once, and if so, in what amounts.

Biological degradation was determined by loss of parent compound and concomitant appearance of daughter products.

CHAPTER 4

LITERATURE REVIEW FOR THE SUBSTRATES STUDIED IN THIS WORK

4.1 Hexachlorobenzene

Hexachlorobenzene (HCB) is the most chlorinated benzene family, with six chlorines. HCB is a by-product of tetrachloroethylene manufacture. HCB is used in manufacture of pentachlorophenol, as a wood preservative, fungicide, seed treatment (for control of wheat bunt), in the production of aromatic fluorocarbons, in organic synthesis, for the impregnation of paper, in technical pentachlorophenol up to 13%, in herbicide DCPA up to 10-14%, and in pesticide PCNB between 1 and 6%. HCB may be found in the following industries: aluminum manufacture (fluxing agent), vinyl chloride monomer production, synthetic rubber production, and in the wood preservative industry, as well as several others.

Residues in peanut butter are reported at an average of 7.4 ppb (range 0.97-38.0 ppb) (Verschueren, 1983). Rabbit minimum lethal single oral dose was 2600 mg/kg body weight. Long term HCB effects are primarily concerned with increased liver weight, renal lesions, and decreased reproduction in the rat. The NO Effect Level (NOEL) is 20 ppm. Feeding female minks at doses as low as 1 ppm during gestation and lactation resulted in increased mortality of kits (USEPA (1985a; Verschueren, 1983).

HCB is not manufactured as a commercial product in the United States. Up until 1979, an estimated 2 to 5 million pounds were produced each year during the synthesis of several chlorinated chemicals (USEPA (1985a)). Rogers (*et al.*, 1989) examined twelve U.K. sewage sludges for the presence of chlorobenzenes. He reports a range of <0.01 to 40.2 mg/kg (dry weight) with dichlorobenzenes representing the majority, and HCB samples at 0.11 mg/kg. In one Federal survey of drinking water supplies from 104 surface water and 12 ground water supplies, no supply contained levels above the detection limit of 0.1 ug/L, (USEPA, 1985a) probably due to HCB's strong nonpolar behavior keeping it from partitioning into the aqueous phase.

The largest exposure of HCB to a human population occurred when seed wheat treated with HCB (to control wheat bunt) in Turkey was ground for bread-making instead of planting, and ingested by the local population. The primary medical diagnosis was HCB-induced porphyria turcica (PCT). Symptoms included disturbed porphyrin metabolism, cutaneous lesions, and hyperpigmentation. Researchers estimated 0.05 to 0.2g/day of HCB ingestion, with a 95% mortality rate in children under one year of age. Follow-up studies with patients 20 to 25 years after the onset of porphyria showed that a few patients (10%) still had active porphyria, >50% exhibited hyperpigmentation, and 83% had scarring (USEPA, 1985a).

Nair and Pillai (1989) measured hexachlorobenzene residues in Dehli and Faridabad, India in 1987. Samples were taken of the soil, earthworms, water, fish, lake water, buffalo milk, human milk, human fat, and pigeon breast muscle. They found HCB residues in 60 out of the 78 samples analyzed with a range of 0.0 to 2102 ng/g. Soil samples averaged 24 ng/g, lower than that reported in the United States by Carey (1986). Earthworm samples were also low, as compared to values reported in Germany. Fish values were also positive for HCB, with an average of 122 ng/g, but lower than values reported from the Netherlands. Buffalo milk samples showed no HCB residues, and human fat samples were also quite low, with a mean of 12.19 ng/g, much lower than values reported from Poland and Italy. The human milk samples from Delhi were high, with a mean of 449 ng/g, but still lower than reported values from West Germany.

Nair and Pillai concluded that HCB residue levels in India are low compared to many developed countries because HCB has never been used as a fungicide in India.

As far as research directed at anaerobic degradation of HCB, there are two significant papers, discussed in the following. Fathepure and workers (1988) have shown reductive dechlorination of hexachlorobenzene to tri- and dichlorobenzenes in anaerobic sewage sludge. They used fresh anaerobic sludge from a primary digester in Jackson, Michigan, which receives approximately 40% industrial and 60% residential wastewater. HCB was dissolved in diethyl

ether and added to a 160mL empty serum bottle. 50mLs of sludge were added to the bottle, after the ether was removed by drying, under a continuous nitrogen stream. Bottles were sealed with 20-mm Teflon lined rubber septa and aluminum crimp caps.

The initial concentration of hexachlorobenzene, 1,3,5 - trichlorobenzene, and 1,2,3,5-tetrachlorobenzene used was 50ppm. Dechlorination of HCB occurred with a lag time of approximately one week and was complete by three weeks. 85% of 1,2,3,5-tetrachlorobenzene was converted to 1,3,5-trichlorobenzene at a rate of $11\mu\text{mol liter}^{-1}\text{day}^{-1}$ with complete conversion occurring at eight weeks. 1,3,5-trichlorobenzene sludge amendments showed no dechlorination. This demonstration of HCB dechlorination is significant because the poorly water soluble halogenated aromatic hydrocarbons that do not contain polar functional groups like -COOH or -OH have not been shown to be amendable to dechlorination, and they are a significant class of environmental pollutants.

Fathepure (1988) suggests that since most of the HCB accumulated as 1,3,5-trichlorobenzene, *meta*-substituted benzene rings are structurally more stable.

The second significant experimental work involving HCB degradation was conducted by Fathepure and Vogel (1991). Using a two stage anaerobic-aerobic biofilm reactor, reductive dechlorination of HCB at 75 **ppb** was 98.7% effective, with the concurrent appearance of 1,2,3-

trichlorobenzene and 1,2-dichlorobenzene. Fathepure and Vogel (1991) also tested the capabilities of their mixed culture with different primary carbon sources, discovering that acetate yielded the highest dechlorination rates, superior to glucose and methanol. Chloroform and tetrachloroethylene were also successfully degraded by this system.

This research was particularly significant because of the method used to introduce the HCB standard to the column. Fathepure and Vogel used isooctane to dissolve HCB and administer to the column. The solvent used in this thesis was hexadecane, which is similar to isooctane in that they are both relatively long chain non-polar hydrocarbons. Other researchers have used diethyl ether (Fathepure *et al.*, 1991).

Gantzer and Wackett (1991) used hexachlorobenzene as a model compound "to test the susceptibility of chlorinated aromatics to reductive dechlorination by purified bacterial redox cofactors and proteins." They showed that vitamin B₁₂ yielded faster HCB degradation than hematin and coenzyme F₄₃₀. Concentrations were not reported, but the authors did note that the experiments were carried out at 65 degrees Celsius in order to produce the dechlorination reaction.

A chlorobenzene paper by Oliver and Karen (1982) that is cited in some of the chlorobenzene literature is presented below. Its significance related to microbial degradation of chlorobenzenes is dubious at best. I wish to

present the paper, as many other researchers have done, but additionally discuss a very pertinent comment by Robert Bailey (1983), who criticized the authors for suggesting that all CBs are resistant to microbial oxidation. Bailey's comment is often not cited by the same authors who use the Oliver and Karen results, thus not providing a fair presentation and evaluation of the data.

Oliver and Karen (1982) suggest that the whole chlorobenzene class is persistent because of, "The concentration profile of CB's in lake sediments...". They measured chlorobenzene concentrations in sediments, water and fish from lakes Superior, Huron, Erie, and Ontario. The highest levels of HCB were measured in Lake Ontario. HCB concentrations ranged from 13-127 ppb (no reported avg).

The predominance of HCB relative to the other CB's in fish is remarkable, according to Oliver and Karen. HCB comprises <0.5% of the total CBs in water samples, between 2% and 20% of the total CBs in sediments and between 53 and 76% of the total CBs in fish. Although they acknowledge that HCB has a high octanol/water partition as compared to the other CBs ($\log P_{\text{Oct}} 6.18$; Verschueren, 1983), they still suggest that levels of HCB are highest in fish because of metabolism of the lower CBs by the fish, and an inability of HCB to be metabolized. The elevated HCB levels in fact could be a product of simple biological amplification by the fatty fish tissue since the HCB is very non-polar, while the lesser chlorinated benzenes are more polar and are likely to

be excreted by the fish as easily as they are ingested. Additionally, the temporal distribution of specific CBs into the Great Lakes may not have been constant, as Bailey suggested.

Bailey (1983) commented on Oliver and Karen's measurement of CBs in the Great Lakes, refuting their argument that all the CBs are persistent and also commented on the distribution of the different CB congeners in the sediment core data that they reported. Bailey states that the ratio of the lower CBs to penta and hexachlorobenzene increases dramatically as sample depth increases, suggesting a slow dehalogenation process of HCB in the anaerobic sediment or a much different release pattern [from industry sources] in the past. However, there is too much supposition associated with the interpretations of the data. Karen and Oliver's data is only useful for revealing the distribution of CBs in the Great Lakes, that is all. As Bailey stated in his commentary, "The survey of chlorobenzenes (CBs) in the Great Lakes by Oliver and Nicol provides a valuable picture of their present distribution. Study of additional sediment cores might reveal more precisely the rate of change in distribution and those conditions that cause this change."

4.2 Pentachlorophenol

Pentachlorophenol, introduced into the United States during the 1930's (Pignatello *et al.*, 1983), is a wide

spectrum biocide, disinfectant, and ingredient in antifouling paints. Approximately 80% of the world production of 25 million kg in 1978 was estimated to be used by the wood preserving industry. Because of its efficiency and solubility in organic and inorganic solvents, PCP and its sodium salt are extremely useful and sold under more than twenty trade names (Valo *et al.*, 1985).

Technical grade PCP contains chlorodibenzo-p-dioxins, chlorodibenzofurans, and octachlorodibenzo-p-dioxin. Sewage treatment removal varied from 4.4% to 76% in an Oregon plant. Seepage from a landfill at 30-500m from the source yielded <1-2ppb. Peanut butter samples range from 12-64ppb with an average of 28ppb. PCP in human semen samples was concentrated in the sperm cells by a factor of 9 (Verschuieren, 1983). PCP is toxic to algae at 0.001 mg/L, with an LC₅₀ for large size goldfish of 0.250 ppm at 24 hours and 0.190 ppm at 96 hours (Verschuieren, 1983).

PCP is a fat soluble chemical known to accumulate in water organisms directly through the skin or through ingestion. PCP has also been shown to be stable in benthic sediments in various studies throughout the world. Soil adjacent to a Finnish saw mill was contaminated up to 500 mg/kg (dry wt.) to a depth of several meters (Valo *et al.*, 1985).

The literature is replete with biological degradation of PCP by anaerobic and aerobic organisms and in a variety of environments. Ide *et al.*, (1972) reported PCP

decomposition in paddy soil. Murthy and workers (1979) demonstrated PCP degradation in aerobic and anaerobic soils. A discussion of some anaerobic research follows.

PCP was anaerobically degraded by Woods and co-workers (1989) using a continuous upflow anaerobic sludge blanket reactor in the presence of high concentrations of readily biodegradable organic compounds. The original inoculum was from a municipal sludge digester at the West Point Waste Water Treatment Facility in Seattle, Washington. The organisms were acclimated to a dilute caustic extraction effluent for two years, then grown in the column for three months prior to the addition of the chlorinated substrate to the column. A PCP concentration of 100 ppb was completely degraded into 2,3,4,5-tetrachlorophenol and 3,4,5-trichlorophenol within 80 hours. 2,3,4,5-trichlorophenol was removed at 95 to 97% efficiency during the last six days of the experiment. The authors thus conclude that the degradative pathway thus favors reductive dechlorination of chlorines in the *ortho* position, regardless of *meta* position chlorines.

Mikesell and Boyd (1986) used a mixed culture from a Jackson, Michigan sewage treatment plant which had been acclimated to monochlorophenol degradation for two years to degrade PCP. They established three different experiments with regards to various sludges: (i) individual MCP-acclimated sludges, (ii) mixture of equal volumes of the three MCP-acclimated sludges, and (iii) sludge acclimated

simultaneously to the three MCP isomers. PCP of concentration 11 ppm was added to the various sludge mixtures via an ethanol stock solution.

PCP degradation was evident in each of the individual MCP-acclimated sludges, with the fastest breakdown in the 2-CP sludge at three days. The 3-CP sludge was the slowest degrader, with seven days' degradation providing approximately 64% removal of the parent compound. 3-CP and 4-CP degradation was not much longer at 5 days for complete removal of parent compound. Mikesell and Boyd state that these data taken into consideration with previous work suggest that chlorine members *ortho* to the phenolic OH group are removed more rapidly than chlorine in the *meta* and *para* positions.

Combining the results from **each** of the MCP-acclimated cultures, all positions of the aromatic ring were able to be dechlorinated. Subsequently, the three MCP-acclimated were combined to determine if they were able to dechlorinate PCP. PCP additions of 11ppm to the tri-culture system were successfully degraded within 3 to 6 days with the concomitant appearance of daughter products.

This research indicated that the complete reductive dechlorination of PCP is dependent upon at least two, if not three or more organisms.

Mikesell & Boyd (1985) demonstrated degradation of seven aromatic compounds in sludge, including PCP, and made a comparison regarding the degradation capability of three

different sludges from Michigan using the seven aromatics. Adrian, Jackson, and Mason Michigan sludges were used. The experiments were conducted in anaerobic 160 mL serum bottles, with ethanol stock solutions. Non-acclimated 75 mL sludge samples were pipetted into the serum bottles under anaerobic conditions.

The Jackson sludge was the most effective, degrading 12.5 ppm of PCP in 14 days, with the Adrian sludge second at 14.6 days, and the Mason sludge at 28.5 days. The Jackson sludge also degraded the six other chlorinated compounds in a significantly shorter period of time. The two *ortho* chlorine atoms were removed rapidly from PCP to give 3,4,5-trichlorophenol, which is consistent with the rapid *ortho* dechlorination observed in the 2,4-D and 2,4,5-trichlorophenol in this experiment, and others (Boyd *et al.*, 1983; Boyd and Shelton, 1984).

Of particular interest, 2,4,6-trichlorophenol was degraded to a recalcitrant 4-chlorophenol. The anaerobic degradation of 2,4,6-trichlorophenol had not been demonstrated previous to Mikesell and Boyd (1985).

Bryant and co-workers (1991) investigated the potential of various freshwater sediments, adapted and non-adapted to two dichlorophenols, to reductively dechlorinate PCP. Sediments from a Georgia pond, the East River in New York, and a lake close to the Black Sea of the Soviet Union were used in this experiment. Non-adapted and adapted 2,4- and 3,4-dichlorophenol microbial communities were amended with 5

to 10 ppm of compound and PCP amended communities had up to 20 ppm added to them.

The first set of results dealt with non-adapted communities. In the first experiment with the Georgia sediment, there was no PCP degradation in the first 40 days. In a second experiment, 29 of the initial 65 ug/mL PCP remained after 144 days. In the East River sediment, all 20 ppm of PCP was present at 19 days, but could not be detected at 33 days. The Black Sea sediment showed a considerable loss of PCP (20 ppm), after a 13 day lag period. A second addition of PCP (37 ppm) was reduced to less than 50% after 45 days of incubation. No further loss of PCP was detected.

A mixture of the three sediments was prepared and adapted to 2,4- or 3,4-dichlorophenol. The progress of PCP removal by these adapted sediment communities did not follow an extended lag phase, as observed for the non-adapted communities above. More than 75% of the PCP was dechlorinated by the 2,4-DCP-adapted communities within 5 days. In the 3,4-DCP-adapted community, PCP was decreased to less than 1 ppm by day 5.

Although the chlorophenol-adapted communities were more successful in the degradation of PCP, the authors could not explain the decrease in the rate of degradation by the acclimated communities after the third addition of PCP at 20 ppm. Product inhibition was ruled out by the authors, leaving no alternative explanation.

In addition to demonstrating the superior dechlorination ability of the chlorophenol-adapted sediment communities over non-adapted chlorophenol sediment communities, this research also indicated that sediments may contain distinctly different anaerobic organisms than those found in an anaerobic sewage sludge because of the observed dechlorination preferences as compared to those discovered by Mikesell and Boyd (1985 and 1986) using anaerobic sewage sludge organisms.

4.3 Carbon tetrachloride

The specific HAC under study in this thesis, carbon tetrachloride, is very significant because of its contribution to the destruction of stratospheric ozone. Title VI under the Clean Air Act Amendments of 1990 lists carbon tetrachloride under Class I substances which are believed to be most damaging to the ozone layer, in addition to CFCs, halons, methyl chloroform and all isomers of these substances excluding 1,1,2-trichloroethane. Pursuant to section 604 of the act, production and consumption of all Class I substances must be terminated by January 1, 2000 (Arbuckle, 1991), including carbon tetrachloride, which has a long half life. Mabey (1978) gives an environmental half life for carbon tetrachloride at **7,000 years**, based on abiotic hydrolysis or dehydrohalogenation at 20°C.

A solid waste is characterized as a hazardous waste if a representative sample contains 0.5 ppm or more carbon tetrachloride. The EPA Hazardous Waste number for carbon

tetrachloride is D019/D001 and U211 designating it as a listed Toxic waste (Librizzi, 1991).

Carbon tetrachloride is used in fire extinguishers, dry cleaning, manufacturing of refrigerants, aerosols and propellants, veterinary medicine, metal degreasing, as a fumigant, and for the process of chlorinating organic compounds. The toxicity threshold for *Pseudomonas putida* is reported at 30ppm, guppy lethal concentration (LC₅₀) is 67ppm, and severe toxic effects by absorption in man is reported at 20ppm (Verschueren, 1983).

Household use of carbon tetrachloride was prohibited in 1970 by the FDA because it was found that it was a systemic poison that affected the nervous system when inhaled, and the gastrointestinal tract, liver, and kidneys when ingested. The biochemical mechanism of carbon tetrachloride toxicity involves reactive radical species including CCl₃• that react with biomolecules such as proteins and DNA. Most damage from these reactions occurs in the liver as lipid peroxidation, which destroys liver cells (Manahan, 1991).

Animal toxicology studies of carbon tetrachloride demonstrated that it is readily absorbed in fatty tissues and metabolized to chloroform. Bini and co-workers (1975) found that only fifteen minutes after oral administration of CCl₄ to rats, CCl₄ and chloroform (CHCl₃) were found in the liver tissues. Butler (1961) observed that involved in the *in vivo* conversion of carbon tetrachloride to chloroform, shown in dogs and rats, is the acquisition of two electrons.

He suggested that the acquisition of the two electrons, as well as other unproven but plausible reactions involving the free radical postulated to be formed by homolytic fission of the carbon-halogen bond, underlies the hepatotoxic effects of carbon tetrachloride. Fowler (1969), studying rabbits, found some evidence supporting the hypothesis of free radical formation following carbon tetrachloride administration. He also discovered that the compound was distributed to the fat, liver, kidney, and muscle within 48 hours.

Chloroform, the daughter product of CCl_4 metabolism of serious concern, also results from the chlorination of drinking water in a reaction involving dissolved organics in the water. A known carcinogen, chloroform has been limited to a maximum contaminant level (MCL) of 100 ug/L (Manahan, 1991; Miller, 1988; Vogel et al., 1987; Motosugi and Koda, 1983) by the EPA.

There is limited information concerning the biodegradation of carbon tetrachloride in the literature. Bower and Wright (1988) conducted transformation experiments of several halogenated aliphatics including carbon tetrachloride and chloroform. They used continuous-flow columns containing glass beads seeded with mixed bacterial cultures to simulate anoxic groundwater conditions. Acetate was used as the primary substrate, with halogenated aliphatics providing the secondary carbon source.

Three parallel columns were run under three different conditions: denitrification respiration, sulfate respiration and methanogenesis. Under methanogenic conditions, substrate concentrations of 250 ppm were used. The columns were operated for eighteen months at a feed rate of 20 mL/day. Primary digested sewage sludge of unreported location was used to seed the anoxic column for methanogenesis. Short term radioactive studies were performed to confirm that removal was by a biological mechanism.

Under methanogenic conditions, more than 99% carbon tetrachloride was transformed in a 9-12 week period. Chloroform in the sulfate reducing column showed an increase in concentration, while carbon tetrachloride was degraded by more than 99% in less than two weeks. The denitrifying biofilm column yielded a greater than 99% degradation of carbon tetrachloride, with an increase in the amount of chloroform produced. The authors suggest that the increased chloroform concentration originated from the degradation of carbon tetrachloride. Acetate proved to be the most successful substrate used in this study.

Fathepure and Vogel (1991) also report acetate to be the best substrate for xenobiotic degradation, compared to methanol and glucose, for chloroform. An upflow anaerobic and aerobic biofilm reactor was used with 3 mm diameter glass beads. Inocula came from a primary anaerobic digester at Jackson, Michigan. Prior to seeding the column, the

organisms were batch grown on PREM containing glucose as the only carbon source. The aerobic seed bacteria were from East Lansing, Michigan. After the bacterial population had attained steady state, HCB, tetrachloroethylene, and chloroform (0.5 to 1.0 mg/L) were added to the anaerobic column influent. Radioactive substrates were dissolved in isooctane to determine the ^{14}C distribution in different metabolic fractions. Chloroform degradation was at 7% (initial 45, 157 dpm/2mL) for the anaerobic portion of the reactor. Sequential anaerobic/aerobic reactor degradation yielded 83% conversion for chloroform.

Isooctane stock solutions were used in this work for hexachlorobenzene, tetrachloroethylene, and chloroform stock solutions, a high molecular weight organic non-polar solvent similar to the solvent used in this thesis, hexadecane, for carbon tetrachloride and hexachlorobenzene stock solutions.

A summary of abiotic and biotic carbon tetrachloride degradative mechanisms is given by Vogel (1987) and co-workers. The general mechanisms they propose for HAC degradation are: (i) Substitution by solvolysis and hydrolysis, and conjugation and other nucleophilic reactions, (ii) dehydrohalogenation, (iii) oxidation by alpha hydroxylation, halosyl oxidation, epoxidation, biohalogenation, (iv) and reduction by hydrogenolysis, dihalo-elimination and coupling. The reader is directed to Vogel (1987) for further elucidation upon these important

abiotic and biotic mechanisms for chlorinated aliphatic removal mechanisms.

Another important mechanism involved in the transformation of CCl_4 is the use of **cytochrome P450** by some organisms. Vogel reports that many microorganisms possess cytochrome P450, an important cytochrome involved in halogenated compound transformation. *Pseudomonas putida's* enzyme closely resembles the microsomal enzyme, and whole cells can use it to mediate the reduction of carbon tetrachloride and bromotrichloromethane to chloroform.

Wolf (1976) and workers demonstrated a P450- CCl_4 complex and suggested that CCl_4 only, and not chloroform, is the initiator of lipid peroxidation and therefore responsible for hepatotoxic effects.

Methanogens do not have the cytochrome systems that are present in aerobic organisms. However, methanogens do have nickel containing enzymes or cofactors such as F430. Several of the microbial reductants have much greater reducing potentials than the mammalian enzymes, Vogel suggests that this may lead to a broader reductive dehalogenation ability.

Klecka and Gonsior (1984) demonstrated that reduced iron was able to degrade carbon tetrachloride and chloroform to lower chlorinated homologues, lending support to the nickel enzyme reductive research discussed briefly above. A naturally-occurring iron porphyrin, hematin (ferriprotoporphyrin hydroxide), was added to 0.99ppm of a

sterile carbon tetrachloride solution. Within 48 hours, the level of CCl_4 remaining in the system was below detection (below 0.00032ppm). There was a commensurate increase in the concentration of chloroform, but no other degradation products.

Klecka also suggest that the cell free dehalogenation involving iron porphyrins uses a similar if not the same mechanism that enzymes as well as free iron porphyrins present in living organisms that also catalyze such reactions utilize. Ahr and workers (1982) have shown cytochrome P-450 to catalyze the reductive dehalogenation of carbon tetrachloride under anaerobic conditions.

Gantzer and Wackett (1991) demonstrated the reductive dechlorination of CCl_4 with vitamin B_{12} and coenzyme F_{430} and suggest that "...elevating the biosynthesis of redox-active cofactors or the levels of critical reductants may lead to microbial strains enhanced in biodegradative capabilities."

Bouwer and McCarty (1983) suggest that carbon tetrachloride may undergo reduction to chloromethane and subsequent oxidation to carbon dioxide, as described in mammalian systems, and that more than one organism's participation in the process is likely.

Carbon tetrachloride degradation mechanisms involving abiotic and biotic processes are both very important pathways for transformation of the compound in a variety of mammalian and environmental systems.

CHAPTER 5

EXPERIMENTAL WORK

5.1 Materials, Inoculum and Equipment

5.1.1 Materials

The following is a list of the significant materials used in this research:

- Hexachlorobenzene, 99% purity. Aldrich Chemical Company, Milwaukee, Wisconsin.
- Pentachlorobenzene, 98% purity. Aldrich Chemical Company, Milwaukee, Wisconsin
- Hexadecane, 98% purity. Fischer Chemical Company, Fairlawn, NJ.
- Carbon tetrachloride, 99% purity. Fischer Chemical Company, Fairlawn, NJ.
- Acetone, 99.6% purity. Fischer Chemical Company, Fairlawn, NJ.
- Pentachlorophenol, 99% purity. Sigma Chemical Corp.
- Acetonitrile: 99.9% purity. B&J Brand.
- N₂ gas, dried and de-oxygenated: Liquid Carbonic Specialty Gases, Chicago, IL.

5.1.2 Inoculum

The sewage sludge inoculum for this research was obtained from an anaerobic digester at the Linden Sewage Authority, Linden, NJ.

5.1.3 Equipment

The following is a list of equipment used in this research:

- Anaerobic chamber: Coy Products, palladium catalyst, with 4% H₂ atmosphere, with automatic anaerobic airlock
- Incubator: Environmental Growth Chamber (Walk in), Chargin Falls, Ohio.
- Autoclave: Harvard/LTE Series 300 Lab Autoclave, #302/0662/00
- Centrifuge: ICE International Centrifuge - model PR-2, Needle Heights, MA.
- Serum bottles: Wheaton Glass company, Millville, NJ., 160 mL volume
- Stoppers: Teflon coated stoppers
- Crimped Seals: Bellco Biotechnology, Vineland, NJ., aluminum
- Decrimper: Supelco, Inc.
- HPLC: Waters Inc. Model #600E.
 - Column: Alltech C8 Econosphere Porapak
 - Eluent: 50/50 Acetonitrile/Water 5% Acetic acid
 - Detector: UV Waters Model #484, Waters Autosampler Model #715.
- GC: Hewlett Packard Series II 5890 Gas Chromatograph, with autosampler.
 - Column: Supelco Inc., Bellefonte, PA. SPBTM-608 fused silica capillary column (30mX0.25mmID, with 0.25um film thickness.
 - Integrator: Hewlett Packard HP3396A, 7673
 - Detector: ⁶³Ni electron-capture detector at 290°C.
 - Run Conditions:** Isothermal at 220°C, with injector at 240°C. Helium Carrier gas at a flow rate of approx. 0.64 mL/min. and 50 psi, make-up gas of N₂ at 34.53 mL/min. and 30 psi. Split ratio of 10.4 and anode purge of 3.175 mL/min.

5.2 Biological Methods

5.2.1 Sludge Acquisition, Treatment, and Sampling

On three occasions, approximately eight liters of anaerobic sewage sludge from the Linden Sewage Authority,

Linden, NJ were collected in a Nalgene container. This amount represented approximately half the container storage capacity.

The sludge was stored in a laboratory fume hood, with the screw top loosened, for approximately 2-3 days. 50 mL samples were taken from the spigot (located approximately 5 cm above the container bottom) of the Nalgene container and placed in 160 mL serum bottles. This process was not conducted in the absence of air.

5.2.2 Culture Establishment

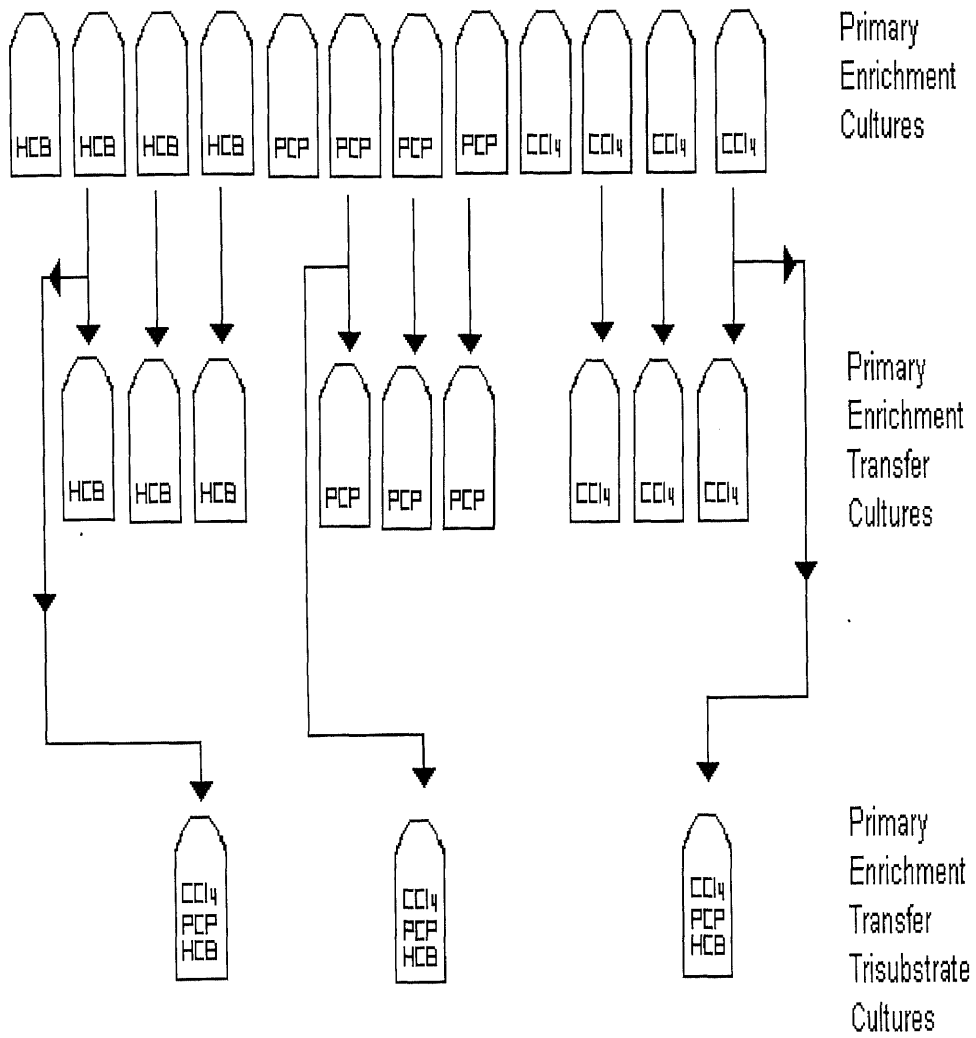
To biodegrade Pentachlorophenol, Hexachlorobenzene, and Carbon tetrachloride, three sets of cultures were established for each target compound. The three sets of cultures established were: Primary Enrichment Cultures (PECs), Primary Enrichment Transfer Cultures (PETCs), and Primary Enrichment Transfer Trisubstrate Cultures (PETTCs). As the names imply, the PETCs and PETTCs originate from the PECs. See Figure 1 for the experiment design.

The following discusses the establishment of the three sets of cultures for each target compound.

5.2.2.1 Hexachlorobenzene Cultures

For HCB, the Primary Enrichment Cultures were established by the following procedure.

Figure 1. Experiment Design



50 mL sludge samples were withdrawn from the Nalgene container and placed into the serum bottles following Section 5.2.1 procedure.

Next, a thirty milliliter layer of hexadecane was poured into the serum bottle. The hexadecane layer floated on top of the aqueous sludge. HCB was pipetted from a hexadecane stock solution concentration of 100 ppm into the serum bottles, on top of the hexadecane layer. The resulting concentration range of HCB used was from 0 ppm to 20.0 ppm as measured in the 30 mL hexadecane layer. Since hexachlorobenzene, one of the three target compounds studied in this thesis, is very nonpolar and hydrophobic (0.11 ppm solubility in pure water; Verschueren, 1983), it tends to partition to suspended and deposited sediment, reducing greatly its bioavailability (Ekelund et al., 1987). Consequently, a system had to be developed in such a way that: (1) HCB was made available to the organisms in the aqueous 50 mL sewage sludge; (2) a high extraction efficiency for accurate quantification of HCB disappearance from the bottle (the major mechanism by which biodegradation was measured) could be obtained; and (3) the organisms would be protected against toxic concentrations of the chlorinated target compounds. Therefore, a 30 mL hexadecane layer was established on top of the 50 mL sludge sample in the serum bottle, by simply pouring thirty milliliters of hexadecane into the bottle.

Because of its lower density, the hexadecane floated on top of the aqueous sludge sample, and formed a distinct water-immiscible organic phase. HCB partitioned primarily into the hexadecane layer which acted as a reservoir, permitting some of the target compound to cross into the aqueous layer, establishing an equilibrium. As the HCB was consumed by the organisms in the aqueous phase, more HCB was transferred from the hexadecane layer in to the aqueous layer to maintain equilibrium. Thus, the organisms were not exposed to high concentrations of HCB that could be in principle toxic or inhibitory to the non-acclimated sludge organisms.

Because an oxidative mechanism is employed in its catabolization, hexadecane is not metabolized under anaerobic conditions by bacteria. Therefore, since the serum bottles were kept under anaerobic conditions, hexadecane was not metabolized.

After the addition of HCB to the hexadecane layer, Teflon-covered gray butyl stoppers were placed on top of the bottles, but they were not yet crimp-sealed. The bottles were flushed with dry, de-oxygenated nitrogen for three minutes, following modified methods of Macy *et al.*, (1972) and Miller and Wolin (1974). The bottles were then crimped-sealed. Photosensitive HCB bottles were wrapped in aluminum foil. All serum bottles were incubated in the dark at 30 degrees Celsius, under stationary conditions. Twenty-two PEC cultures were established.

For sampling purposes, 1 mL samples were removed from the bottles at various times throughout the experiment with pure nitrogen-filled syringes (to prevent the introduction of air into the now anaerobic bottles) through the Teflon stoppers.

The second set of HCB cultures created were the Primary Enrichment Transfer Cultures (PETCs). The purpose of these cultures was to determine if the PEC degradation results could be reproduced. The Primary Enrichment Transfer Cultures were created by opening (in the anaerobic glovebox) the Primary Enrichment Culture bottle selected for transfer and withdrawing by sterile pipette 8 mL from the aqueous layer. 4 mL of these 8 mL were transferred to a control bottle and the remaining 4 mL to the "live" PETC bottle. A "live" bottle was a bottle that contained 50 mL of sterile sludge, 30 mL of hexadecane, and which had been inoculated and incubated. The live bottle was expected to perform the biological degradation of the target compound. The bottles were wrapped in aluminum foil and incubated in the dark at 30 degrees Celsius, under stationary conditions. Three PETCs were established.

The third and last set of cultures created were the Primary Enrichment Transfer Trisubstrate Cultures (PETTCs). The purpose of these cultures was to determine if a mixture of the three target compounds could be degraded at the same time. The cultures were established similarly to the PETCs. A bottle with 50 mL sterile sludge and 30 mL hexadecane was

inoculated in the anaerobic glove box. The source of inoculum was a PEC with hexachlorobenzene as the target substrate. The PETTCs differ from the two previous cultures in one important way; the PETTC bottle contained a mixture of PCP, HCB, and CCl_4 at a concentration of approximately 10 ppm for each compound (the concentration of CCl_4 and HCB is based on the 30 mL hexadecane layer). One "live" PETTC bottle was established that used an HCB PEC inoculum.

The method by which an HCB Primary Enrichment Culture was selected for transfer to make PETCs and PETTC was simple. The PEC chosen was based upon the amount of target compound it degraded. Primary Enrichment Cultures demonstrating the most target compound biodegradation were selected as inocula for the creation of Primary Enrichment Transfer and Primary Enrichment Trisubstrate Transfer Cultures.

5.2.2.2 Carbon Tetrachloride Cultures

The CCl_4 Primary Enrichment, Primary Enrichment Transfer, and Primary Enrichment Trisubstrate Transfer cultures were established following the same procedures discussed above in Section 5.2.2.1 for HCB.

Since CCl_4 , like HCB, is very nonpolar, the hexadecane system was also used for all the CCl_4 cultures. The system is the same as described in 5.2.2.1 for HCB.

The range of CCl_4 concentrations used in the experiments was 0 to 21.0 ppm (as measured in the hexadecane layer).

The methodology used to choose PECs for transfer in the CCl_4 set of cultures was the same as outlined above for HCB; that is Primary Enrichment Cultures demonstrating the most target compound biodegradation were selected as inocula for the creation of Primary Enrichment Transfer and Primary Enrichment Trisubstrate Transfer Cultures.

All CCl_4 bottles were incubated in the dark, stationary, and without aluminum foil.

5.2.2.3 Pentachlorophenol Cultures

The creation of Pentachlorophenol cultures varied from the procedures used for CCl_4 and HCB. 50 mL sludge samples were withdrawn from the Nalgene container and placed in the serum bottles as previously described in Section 5.2.2.1.

At low concentration, PCP is soluble in the aqueous phase. Consequently hexadecane was not used for cultures that had PCP as the target compound.

The creation of Primary Enrichment Cultures followed the same procedures used for HCB and CCl_4 PECs. 50 mL of sludge was placed in the bottle and PCP was added to the serum bottle via a 50% ethanol solution. The ethanol solubilized PCP at concentrations greater than 25 ppm PCP.

The PCP was pipetted into the bottles at the desired concentration. The concentration range of PCP used was from

0 ppm to .4 ppm. Teflon-covered gray butyl stoppers were placed on top of the bottles, but they were not yet crimp-sealed. The bottles were flushed with dry, de-oxygenated nitrogen for three minutes, following modified methods of Macy *et al.*, (1972) and Miller and Wolin (1974). The bottles were then crimped-sealed. For sampling purposes, 1.0 mL samples were removed from the bottles at various times throughout the experiment with pure nitrogen-filled syringes (to prevent the introduction of air into the now anaerobic bottles) through the Teflon stoppers. All serum bottles were incubated in the dark at 30 degrees Celsius, under stationary conditions.

The second set of PCP cultures created were the Primary Enrichment Transfer Cultures (PETCs). The purpose of these cultures was to determine if the PEC degradation results could be reproduced. The Primary Enrichment Transfer Cultures were created by opening (in the anaerobic glovebox) the Primary Enrichment Culture bottle selected for transfer and withdrawing by sterile pipette 8 mL from the aqueous layer. 4 mL of these 8 mL were transferred to a control bottle and the remaining 4 mL to the "live" PETC bottle. A "live" bottle was a bottle that contained 50 mL of sterile sludge and had been inoculated and incubated. The live bottle is expected to perform the biological degradation of the target compound. The bottles were incubated in the dark at 30 degrees Celsius, under stationary conditions. Three PETC were established.

The third set of cultures created were the Primary Enrichment Transfer Trisubstrate Cultures (PETTCs). The purpose of these cultures was to determine if a mixture of the three target compounds could be degraded at the same time. The cultures were established similarly to the PETCs. A bottle with 50 mL sterile sludge was inoculated in the anaerobic glove box. The source of inoculum was a PEC with PCP as the target substrate. The PETTCs differed from the two previous cultures in one important way; the PETTC bottle contained a mixture of PCP, HCB, and CCl_4 at a concentration of approximately 10 ppm for each compound. One "live" PETTC bottle was established that used a PCP PEC inoculation.

The selection process to determine which PEC was to be used for transfer inoculations was different for serum bottles containing PCP. Because of the poor detection limit of PCP at low concentrations, there was not a high degree of confidence in the data generated for PCP degradation. Extraction of PCP from control bottles containing less than 2 ppm of compound was not consistently reproducible. Therefore, the process of choosing the best three degradation bottles for transfer [creating Primary Enrichment Transfer Cultures] was different for PCP samples, compared to HCB and CCl_4 bottles. Bottles that were under the highest pressure and were darkest in color were selected for transfer. [Autoclaved bottles which were microbiologically inactive, usually exhibited a yellowish-

clear color. "Live" bottles were black and appeared organic/mucky].

A relative determination of the pressure in the bottles with respect to that in other bottles was obtained by observation during sampling. When samples were withdrawn by syringe, the magnitude of the relative pressures could be established by comparing the rate, if any, of the syringe plunger "blow back" caused by gas accumulation inside the air tight bottle once the syringe was inserted through the Teflon stopper. The PCP bottles that exerted the highest pressure were selected for transfer.

5.3 Analytical Methods

The following is a discussion detailing the methodology for sampling and measuring the three target compounds.

5.3.1 Hexachlorobenzene Sampling and Measurement

HCB is extremely insoluble in water. Therefore, as mentioned before, HCB standards were made in the non-polar solvent hexadecane, creating a two phase system. I have discovered from repeated sampling and extraction of the aqueous and organic layers of HCB serum bottles in concentrations ranging from 1 to 60 ppm, that over 99.0% of HCB resides in the organic layer, once equilibrium is achieved. Measurement of the aqueous layer of bottles yields concentrations of 0.1 ppm or less of HCB.

Consequently, the amount of HCB consumed by the organisms could be determined accurately by withdrawing samples from the organic layer (hexadecane layer) and measuring the concentration of HCB. In addition, the samples could be directly injected into the gas chromatograph or stored in an air tight sample bottle for autosampling soon thereafter.

All HCB concentrations for bottles containing HCB as the target compound are reported in parts per million with respect to the 30 mL volume of the organic layer, and not the combined liquid volume of 80 mL [50 mL sludge + 30 mL organic layer]. Because HCB remained in the organic layer, all concentration values reported in Tables 6.1 through 6.3 are the concentrations in the organic layer.

Measurement of HCB by the gas chromatograph was excellent, with measurement reproducibility within +/- 0.1 ppm throughout the concentration range of 1 - 45 ppm.

To test the recovery efficiency of HCB, a 50 mL sample of sterile sewage with 30 mL hexadecane was spiked with 5.0 ppm HCB. HCB recovery from the system (organic + aqueous layer) was 95.6%.

Serum bottles selected for transfer were tested qualitatively for the presence of methane. Head space samples were withdrawn from the bottles using a Hamilton gas-tight syringe and injected into a gas chromatograph with a FID detector.

5.3.2 Carbon Tetrachloride Sampling and Measurement

The sampling methodology used for HCB was also used for sampling CCl_4 . CCl_4 is nonpolar and preferentially resides in the hexadecane layer. The solubility of CCl_4 in pure water is 800 ppm (Label on CCl_4 Bottle, Fischer Chemical Co.), but decreases dramatically with the presence of dissolved salts in the sewage sludge (personal communication, G. San Agustin). Samples taken from the aqueous layer of the sludge, under equilibrium conditions, and extracted into pure hexadecane indicated a CCl_4 concentration of less than 0.1 ppm. Consequently, the amount of CCl_4 consumed by the organisms could be determined accurately by withdrawing samples from the organic layer (hexadecane layer) and measuring the concentration of CCl_4 . The samples could also be stored similarly to HCB samples for injection by autosampler soon thereafter.

All concentrations for bottles containing CCl_4 as the target compound are reported in parts per million with respect to the 30 mL volume of the organic layer, and not the combined liquid volume of 80 mL [50 mL sludge + 30 mL organic layer]. Because CCl_4 remained in the organic layer, all concentration values reported in Tables 6.1 through 6.3 are the concentrations in the organic layer.

Measurement of CCl_4 by the gas chromatograph was excellent, with measurements reproducible within +/- 0.1 ppm throughout the concentration range of 1 - 25 ppm.

To test the recovery efficiency of CCl_4 , a 50 mL sample of sterile sewage with 30 mL hexadecane was spiked with 15.0 ppm CCl_4 . CCl_4 recovery from the system (organic + aqueous layer) was 98.3%.

Serum bottles selected for transfer were tested qualitatively for the presence of methane. Head space samples were withdrawn from the bottles using a Hamilton gas-tight syringe and injected into a gas chromatograph with a FID detector.

5.3.3 Pentachlorophenol Sampling and Measurement

Serum bottles containing PCP as the target compound did not have the hexadecane layer. Therefore, nitrogen-filled syringe withdrawals were extracted using a 1:1 sample dilution with acetonitrile. They were then vortexed for approximately one minute, shaken for thirty seconds, and centrifuged at 4500 RPMs for 20 minutes on the ICE centrifuge. The sample was filtered through a Millipore 0.45um filter and run isocratically on HPLC.

As mentioned previously, the measurement of PCP concentrations below 2-3 ppm was not accurate. Measurements of concentrations 5 ppm to 60 ppm PCP were reproducible +/- 0.5 ppm.

To test the recovery efficiency, a 50 mL sample of sterile sewage was spiked with 76.0 ppm PCP. PCP recovery from the system (aqueous layer) was 98.6%.

Serum bottles selected for transfer were tested qualitatively for the presence of methane. Head space samples were withdrawn from the bottles using a Hamilton gas-tight syringe and injected into a gas chromatograph with a FID detector.

5.3.4 Experiment Controls

There were two different sets of controls used in this work: Biological and Target Compound Interactive.

The purpose of the Biological Controls was to ascertain the amount, if any, of target compound lost through abiotic processes. Biological Controls were used with all three culture groups: PECs, PETCs, and PETTCs.

Biological control bottles were identical, in preparation, to the live bottles, except that they were autoclaved twenty minutes a day for three days. Biological control bottles did not show significant change in target compound concentration in any phase of the experiment.

The Target Compound Interactive control bottles were used in the PETTCs. The Primary Enrichment Trisubstrate Transfer Cultures contained a mixture of the three target compounds. The purpose of this control was to ensure that there was not an interaction between the compounds that affected their extraction from PETTC serum bottles. Each of the three PETTCs established were sterile with the same

mixture of the three target compounds at the same concentration as "live" cultures. Sampling and analysis revealed that extraction efficiency from the aqueous and organic layer was not affected.

CHAPTER 6

RESULTS AND DISCUSSION

The following is a discussion of results. The presentation is subdivided to treat each target compound separately.

6.1 Results of Experiments with Hexachlorobenzene

Table 6.1a indicates the initial and final HCB concentration in the Primary Enrichment Cultures after the specified number of days, the percent change of HCB, if methane was detected, and if the bottle was used for Primary Enrichment Transfer Cultures. The % Change column was calculated using the equation: $[(\text{Final Conc. of target compound} - \text{Initial Conc. of target compound}) / \text{Initial Conc. of target compound}] * 100$. A negative % Change signifies that more of the compound was extracted than was initially placed in the bottle. All results are reported in actual final concentrations, adjusted for recoveries where applicable. A positive value indicates loss of HCB.

"0/No Pk" in the Final HCB concentration column signifies that the final concentration of HCB was approximately 0 ppm, and that there was no peak on the chromatogram at the usual retention time of HCB. "0/Pk" signifies that the concentration of HCB was very close to 0 ppm, and that the integrator recorded some HCB, but not enough to generate a peak distinct from baseline.

The "Methane" column indicates if any methane was present in the serum bottle. A "Yes" simply represents a spot qualitative check for the presence of methane. The test was only performed on Primary Enrichment Cultures with the highest degradation yields.

All initial and final concentrations are calculated on a weight per weight basis.

All tables are arranged by increasing concentration of initial target compound used in the culture.

"Average degradation rate" for Tables 6.1c, 6.2c, and 6.3c was calculated by averaging the degradation rates of the three target substrates combined.

Table 6.1a. HCB Results for Primary Enrichment Cultures *

Final Time (Days)	Bottle #	Initial HCB Conc. (ppm)	Final HCB Conc. (ppm)	%Change	Methane Generation	Transfer Culture
64	7	0	0/Pk	0		
48	12	1.03	0.87	15.5	Yes	Yes
48	13	1.03	0.84	18.4	Yes	Yes
41	14	2.05	1.81	11.7	Yes	
41	15	2.05	1.83	10.7		
63	6	2.05	2.23	-8.8		
64	5	2.56	2.36	7.8		
64	4	2.56	2.36	7.8		
2	10	3.08	3.03	1.6		
62	11	3.08	2.87	6.8		
51	9	5.0	4.98	0.4		
51	8	5.0	4.38	12.4	Yes	
64	1	5.13	5.17	-0.8		
64	2	5.13	5.14	-0.4		
64	3	5.13	5.22	-1.8		
39	16	10.26	10.39	-1.3		
39	17	10.26	10.08	1.8	Yes	Yes
28	48	20.0	19.45	2.8		
146	50	20.0	18.80	5.3		
28	49	20.0	19.11	4.5		

*Concentration reported as g HCB/10⁶ g hexadecane

Table 6.1a indicates degradation results for PECs containing HCB as the target compound.

The maximum degradation obtained in approximately 7 weeks was 18.4% by Bottle #13, representing approximately a decrease of 0.20 ppm HCB out of the total 1.03 ppm.

The amount degraded in the HCB PECs was more than that reported to be degraded by Fathepure and Vogel (1991). They demonstrated degradation of 75 ppb HCB using a continuous flow anaerobic reactor.

The 0.20 ppm HCB degradation was much less than that demonstrated by Fathepure and co-workers (1988) using Jackson, Michigan anaerobic digester sludge. They obtained degradation of approximately 50 ppm in 21 days by the serum bottle technique used in this work.

Table 6.1b. HCB Results for Primary Enrichment Transfers

Final Time (Days)	Bottle #	Initial HCB Conc. (ppm)	Final HCB Conc. (ppm)	%Change
50	17	10.3	9.99	3.0
50	13	10.3	10.0	2.9
50	12	10.3	10.1	1.9

Table 6.1b indicates the degradation results of Primary Enrichment HCB-exposed cultures that were transferred to create Primary Enrichment Transfer Cultures. The bottle number indicates the PEC bottle that was used as inoculum.

The Bottles #17, #12, and #13 showed less than half a ppm of HCB degradation. This amount of degradation is less than that obtained from Primary Enrichment Cultures, (Table 6.1a). This result may be explained by the fact that the PETCs had less time for degradation than the PECs.

Theoretically, it can be predicted that the PETC degradation would exceed PEC degradation since the PETCs should be more acclimated to HCB.

Table 6.1c. HCB Inoculation: Primary Enrichment Transfer Trisubstrate Cultures (Bottle JZ8)

	Initial Conc. (ppm)	Final Conc. (ppm)	%Change*	Days
HCB	10.27	9.84	4.2	48
PCP	10.00	3.7	63.0	50
CCl ₄	12.68	10.26	19.1	48

*Average change of 28.8%

Table 6.1c shows the results of the experiments obtained with Primary Enrichment Transfer Trisubstrate Cultures (PETTCs). Each serum bottle contained a mixture of the three target compounds, and was inoculated with a different PEC. The initial and final target compound concentrations are given in the table, as well as percent change and days elapsed from inoculation to sample date.

Hence, the Primary Enrichment Transfer Trisubstrate Cultures are only different from each other because of the origin of inoculation [See Figure 1].

Bottle JZ8 was inoculated with a PEC that contained HCB as the target compound.

Bottle JZ8 produced the highest average degradation rate at 28.8%. The inoculum was from a PEC (Bottle #12), which degraded 0.2 ppm HCB in the PEC experiment, and 0.2 ppm in the PETC experiment.

In the PETTC (Table 6.1c), this culture degraded 0.43 ppm HCB. This amount is an improvement over the amount of HCB degraded in the PECs and PETCs by two times.

This culture produced remarkable PCP degradation at 6.3 ppm and also produced the least CCl_4 degradation amount of the three PETTCs.

6.2 Results of Experiments With Carbon Tetrachloride

Table 6.2a indicates degradation results for PECs containing CCl₄ as target compound.

Table 6.2a CCl₄ Results for Primary Enrichment Cultures

Final Time (Days)	Bottle #	Initial CCl ₄ Conc. (ppm)	Final CCl ₄ Conc. (ppm)	%Change	Methane Generation	Transfer Culture
0	Blank	0	0/No Pk	0		
69	35	0	0/Pk	0		
69	41	0	0/Pk	0	Yes	
28	42	2.1	0.21	90.0		
28	43	2.1	0.20	90.5	Yes	Yes
34	39	2.1	0.21	90.1	Yes	
34	40	2.1	0.21	90.0		
68	36	10.0	1.90	81.0	Yes	Yes
68	38	10.0	2.69	73.1	Yes	Yes
33	46	12.68	5.53	56.4		
33	47	12.68	12.09	4.7		
33	45	21.0	5.42	74.2		
33	44	21.0	12.82	39.0		

The carbon tetrachloride bottles demonstrated much better overall degradation for most of the cultures than HCB and PCP PECs.

Bottle #46 showed a degradation of approximately 7 ppm CCl_4 (56.4% conversion) in 33 days.

This amount is significantly higher than removals obtained by Bouwer and Wright (1988) of 7 to 17 ppb. Using hematin, Klecka and Gonsior (1984) transformed 1 ppm CCl_4 to chloroform. Bouwer and McCarty (1983) obtained degradation of 145 ppb of CCl_4 in 54 days using methanogenic bacteria in a serum bottle system with a methanogenic population that was obtained from a waste activated sludge-fed laboratory digester.

Consequently, the degradation of 7 ppm CCl_4 in this thesis is significantly higher than that previously reported in the literature.

Table 6.2b. CCl_4 Results for Primary Enrichment Transfers

Time (Days)	Bottle #	Initial CCl_4 Conc. (ppm)	Final CCl_4 Conc. (ppm)	%Change
56	43	12.68	9.25	27.1
56	38	12.68	9.12	28.1
56	36	8.81	8.81	30.5

The results for the Primary Enrichment Transfer Culture experiments with CCl_4 are reported in Table 6.2b. The results are consistent with the data obtained from the Primary Enrichment Cultures in which degradation of several ppm of CCl_4 was observed.

Table 6.2c. CCl₄ Inoculation: Primary Enrichment
Transfer Trisubstrate Cultures (Bottle JZ6)

	Initial Conc. (ppm)	Final Conc. (ppm)	%Change*	Days
HCB	10.27	9.84	4.2	48
PCP	10.00	6.78	32.2	50
CCl ₄	12.68	10.22	19.4	48

*Average change of 18.6%

The bottle inoculated with the CCl₄-degrading PECs yielded the lowest overall average degradation rates. This would seem to make sense since the culture came from a bottle that contained an aliphatic compound, while HCB and PCP are aromatics and may not be metabolized by the same mechanisms.

6.3 Results of Experiments with Pentachlorophenol

Table 6.3a indicates the initial and final concentrations for PECs containing PCP as the target compound.

Table 6.3a. PCP Results for Primary Enrichment Cultures

Final Time (Days)	Bottle #	Initial PCP Conc. (ppm)	Final PCP Conc. (ppm)	%Change	Methane Generation	Transfer Culture
89	32	0	0	0		
110	27	0	0	0	Yes	
111	24	1.04	0/Pk	100		
105	28	1.04	0/Pk	100		
89	30	1.04	0/Pk	100	Yes	Yes
110	25	1.04	0/No Pk	100	Yes	Yes
105	29	1.04	0/Pk	100	Yes	Yes
87	33	1.04	0/Pk	100		
87	34	1.04	1	3.8		
89	31	1.04	0/Pk	100		
111	18	1.28	0/No Pk	100		
111	19	1.28	0/No Pk	100		
110	23	5.2	0/No Pk	100		
110	22	5.2	0/No Pk	100		
111	21	6.4	5.6	12.5		
111	20	6.4	6.24	3		

As discussed previously, the reported % changes for PCP are not reliable for concentrations below 3 ppm. The detection of PCP below approximately 3 ppm generates a poor signal, not discernible from the baseline.

While there was some degradation in this set of PECs, it was limited to low concentrations of 1 to 2 ppm PCP.

Table 6.3b PCP Results for Primary Enrichment Transfers

Final Time (Days)	Bottle #	Initial PCP Conc. (ppm)	Final PCP Conc. (ppm)	%Change
60	25	13.5	11.55	14.8
70	30	10.0	NA	-
67	29	10.0	3.0	70.0

NA - Not available

The results of the Primary Enrichment Transfer Culture experiments in bottles containing PCP indicate a degradation of 2 to 7 ppm PCP in Bottles #25 and #29, respectively. This is much higher than what was achieved previously in the PECs. Extraction difficulties were encountered with Bottle #30. Consequently the results for this culture are not reported.

Table 6.3c. PCP Inoculation: Primary Enrichment
Transfer Trisubstrate Cultures (Bottle JZ7)

	Initial Conc. (ppm)	Final Conc. (ppm)	%Change*	Days
HCB	10.27	10.0	2.6	48
PCP	10.0	8.3	17.0	50
CCl ₄	12.68	7.92	37.5	48

*Average change of 19.0%

It is interesting to note that bottle JZ7, inoculated with a PCP acclimatized culture (Bottle #27), had the highest CCl₄ degradation, almost double that of the other two bottles. However, the PCP degradation was the lowest. This indicates that PCP was not the most desirable target compound for this culture.

CHAPTER 7

CONCLUSIONS

Both objectives of this thesis i.e., 1) to establish Primary Enrichment Cultures that would degrade PCP, HCB, and CCl₄, and 2) to use the Primary Enrichment Cultures to degrade a mixture of PCP, CCl₄, and HCB were successfully achieved. The maximum degradation time period reported in this work was approximately four months.

In the Primary Enrichment Cultures, degradation of the target compounds ranged as follows: HCB, 0 to 1.2 ppm; PCP, undetectable to 3-4 ppm; CCl₄, 0.5 to 15.5 ppm.

Primary Enrichment Transfer Trisubstrate Culture experiments also demonstrated significant degradation ability. Degradation obtained for the target compounds ranged as follows: HCB, 0.27 to 0.5 ppm; PCP, 1.7 to 6.3 ppm; and CCl₄ 2.4 to 5 ppm.

LITERATURE CITED

- Ahr, H.J., L.J. King, W. Nastainczyk, and V. Ullrich. 1982. "P-450 in the reductive dehalogenation of carbon tetrachloride." *Biochem. Pharm.* 31: 2855.
- Allard, A., P. Hynning, C. Lindgren, M. Remberger, and A. Neilson. 1991. "Dechlorination of chlorocatechols by stable enrichment cultures of anaerobic bacteria." *Appl. Environ. Microbiol.* 57: 77-84.
- Bailey, R. 1983. Comment on "Chlorobenzenes in sediments, water, and selected fish from Lake Superior, Huron, Erie, and Ontario." *Environ. Sci. Technol.* 17: 504-505.
- Bhatnagar, L., and B. Fathepure. 1991. "Mixed Cultures in detoxification of hazardous waste." Chapter 11, in Zeikus, G., Johnson, E. (eds.), *Mixed Cultures in Biotechnology*, New York: McGraw Hill, Inc.
- Bini, G., G. Vecchi, G. Vivoli, V. Vannini, and C. Cessi. 1975. "Detection of early metabolites in rat liver after administration." *Pharm. Res. Commun.* 7: 143-149.
- Bouwer, E.J., and J.P. Wright. 1988. "Transformation of trace halogenated aliphatics in anoxic biofilm columns." *J. Contam. Hydrol.* 2: 155-169.
- Bouwer, E., and P. McCarty. 1983. "Transformation of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions." *Appl. Environ. Microbiol.* 45:1286-1294.
- Boyd, S., and D. Shelton. 1984. "Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge." *Appl. Environ. Microbiol.* 47: 272-277.
- Boyd, S., D. Shelton, D. Berry, and J. Tiedje. 1983. "Anaerobic biodegradation of phenolic compounds in digested sludge." *Appl. Environ. Microbiol.* 46: 50-54.
- Bryant, F., D. Hale, and J. Rogers. 1991. "Regiospecific dechlorination of pentachlorophenol by dichlorophenol-adapted microorganisms in freshwater, anaerobic sediment slurries." *Appl. Environ. Microbiol.* 57: 2293-2301.
- Butler, T. 1961. "Reduction of carbon tetrachloride *In Vivo* and reduction of carbon tetrachloride and chloroform *In Vitro* by tissues and tissue constituents." *J. Pharmac. exp. Ther.* 134: 311-319.

Closmann, F.B. 1989. "The cometabolism of chlorinated one-carbon and two-carbon compounds in unsaturated soil systems by methylotrophic organisms." A thesis presented to the University of Texas, at Austin, Texas in partial fulfillment of the requirements for the degree of Master of Science.

Dienemann, E., D. Kosson, and R. Ahlert. 1990. "Evaluation of serial anaerobic/aerobic packed bed bioreactors for treatment of a superfund leachate." *J. Hazardous Mater.* 23: 21-42.

DiGeronimo, M., M. Nikaido, and M. Alexander. 1979. "Utilization of chlorobenzoates by microbial populations in sewage." *Appl. Environ. Microbiol.* 37: 619-625.

Ekelund, R., A. Granmo, M. Berggren, L. Renberg, and C. Wahlberg. 1987. "Influence of suspended solids on bioavailability of hexachlorobenzene and Lindane to the deposit-feeding marine bivalve, *Abra nitida* (Müller)." *Bull. Environ. Contam. Toxicol.* 38: 500-508.

Evans, W., and G. Fuchs. 1988. "Anaerobic degradation of aromatic compounds." *Ann. Rev. Microbiol.* 42: 289-317.

Fathepure, B., J. Tiedje, and S. Boyd. 1988. "Reductive dechlorination of hexachlorobenzene to tri- and dichlorobenzenes in anaerobic sewage sludge." *Appl. Environ. Microbiol.* 54: 327-330.

Fathepure, B., and T. Vogel. 1991. "Complete degradation of polychlorinated hydrocarbons by a two-stage biofilm reactor." *Appl. Environ. Microbiol.* 57: 3418-3422.

Fowler, J. 1969. "Carbon tetrachloride metabolism in the rabbit." 1969. *Br. J. Pharmac.* 37: 733-737.

Gantzer, C., and L. Wackett. 1991. "Reductive dechlorination catalyzed by bacterial transition-metal coenzymes." *Environ. Sci. Technol.* 25: 715-722.

Genther, B., W. Price II, and P. Pritchard. 1989. "Anaerobic degradation of chloroaromatic compounds in aquatic sediments under a variety of enrichment conditions." *Appl. Environ. Microbiol.* 55: 1466-1471.

Gibson, D., and V. Subramanian. 1984. "Microbial degradation of aromatic hydrocarbons." Page 181 in D.T. Gibson (ed.) *Microbial Degradation of Organic Compounds*. New York: Marcel Dekker.

Goulding, C., C. Gillen, and E. Bolton. 1988. "Biodegradation of substituted benzenes." *J. Appl. Bacteriol.* 65: 1-5.

- Handy, M. 1988. "Effect of hexachlorobenzene on growth and survival of various microorganisms." *Bull. Environ. Contam. Toxicol.* 41: 936-942.
- Hartmans, J., J. de Nont, J. Tramper, and K. Luyben. 1985. "Bacterial degradation of vinyl chloride." *Biotechnol. Lett.* 7: 383.
- Ide, A., Y. Niki, F. Sakamoto, I. Watanabe, and H. Watanabe. 1972. "Decomposition of pentachlorophenol in paddy soil." *Agric. Biol. Chem.* 36:1937-1944.
- Janssen, D., A. Scheper, Dijkhuizen, and B. Witholt. 1985. "Degradation of halogenated aliphatic compounds by *xanthobacter autotrophicus* DJ10." *Appl. Environ. Microbiol.* 49: 673.
- Klecka, G., and S. Gonsior. 1984. "Reductive dechlorination of chlorinated methanes and ethanes by reduced iron (II) porphyrins." *Chemosphere.* 13: 391-402.
- Leisinger, T., and W. Brunner. 1986. "Poorly degradable substances." Page 475 in J.J. Rehm and G. Reed (eds.) *Biotechnology*, vol. 8, New York: VCH Publishers.
- Librizzi, William. 1991. *Law in Environmental Engineering*, EVSC-631, a Graduate course offered at the New Jersey Institute of Technology, lecture notes distributed in class.
- Mabey, W., and T. Mill. 1978. *J. Phys. Chem. Ref. Data.* 7: 383-415.
- Macy, J., J. Snellen, and R. Hungate. 1972. "Use of syringe methods for anaerobiosis." *Amer. J. Clinical Nutrition.* 25: 1318-1323.
- Madsen, E., J. Sinclair, and W. Ghiorse. 1991. "In-situ Biodegradation: Microbiological patterns in a contaminated aquifer." *Science.* 252: 830-834.
- Manahan, Stanley. 1991. *Environmental Chemistry*. Michigan:Lewis Publishers, Inc.
- Martinson, M., J. Steiert, D. Saber, W. Mohn, and R. Crawford. 1985. in *Biodeterioration Six: Proceedings of the Sixth International Symposium* (O'Rear, E. E., ed.) Commonwealth Agricultural Bureau in press.
- Matsumura, F. 1982. "Degradation of pesticides in the environment by microorganisms and sunlight." *Biodegradation of Pesticides*. Plenum Press, New York, 67-87.
- McCarty, P. 1988. "Bioengineering issues related to in-situ remediation of contaminated soils and groundwater." Page 143

in G.S. Omenn (ed.) *Environmental Biotechnology*. New York:Plenum Press.

Miller, T. 1988. *Living in the Environment*. California:Wadsworth, Inc.

Miller, T., and M. Wolin. 1974. "A serum bottle modification of the Hungate technique for Cultivating Anaerobes." *Appl. Microbiol.* 27: 985-987.

Mikesell, M., and S. Boyd. 1985. "Reductive dechlorination of the Pesticides 2,4-D, 2,4,5-T, and Pentachlorophenol in Anaerobic sludges." *J. Environ. Qual.* 14: 337-340.

Mikesell, M., and S. Boyd. 1986. "Complete reductive dechlorination and mineralization of pentachlorophenol by anaerobic microorganisms." *Appl. Environ. Microbiol.* 52: 861-865.

Morrison, R., and R. Boyd. 1987. *Organic Chemistry*. Massachusetts: Allyn and Bacon, Inc.

Motosugi, K., and K. Koda. 1983. "Microbial degradation of synthetic organochlorine compounds." *Experientia.* 39: 1214-1220.

Murthy, N.B., D. Kaufman, and G. Fries. 1979. "Degradation of pentachlorophenol (PCP) in aerobic and anaerobic soil." *J. Environ. Sci. Health.* B14:1-14.

Nair, A., and M. Pillar. 1989. "Monitoring of hexachlorobenzene residues in Delhi and Faidabad, India." *Bull. Environ.. Contam. Toxicol.* 42: 682-686.

Nastainczyk, W., H.J. Ahr, W. Nastainczyk, and V. Ullrich. 1982. *Biochem. Pharm.* 31: 381.

Oliver, B., and Nicol Karen. 1982. "Chlorobenzenes in sediments, water, and selected fish from lakes Superior, Huron, Erie, and Ontario." *Environ. Sci. Technol.* 16: 532-536.

Pignatello, J., M. Martinson, J. Steiert, R. Carlson, and R. Crawford. 1983. "Biodegradation and photolysis of pentachlorophenol in artificial freshwater streams." *Appl. Environ. Microbiol.* 46: 1024-1031.

Prescott, L., J. Harley, and D. Klein. 1990. *Microbiology*. Iowa: Wm. C. Brown Publishers.

Rahn, Perry. 1979. *Engineering Geology*. New York: Elsevier Science Publishing Co., Inc.

Reineke, W. 1984. "Microbial degradation of halogenated aromatic compounds." Page 319 in D.T. Gibson (ed.) *Microbial Degradation of Organic Compounds*. New York: Marcel Dekker.

Reineke, W., and H. Knackmuss. 1984. "Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene-degrading bacterium." *Appl. Environ. Microbiol.* 47: 395-402.

Reineke, W., and H. Knackmuss. 1988. "Microbial degradation of haloaromatics." *Ann Rev. Microbiol.* 42: 263.

Rogers, H., J. Campbell, B. Crathorne, and A. Dobbs. 1989. "The occurrence of chlorobenzenes and permethrins in twelve U.K. sewage sludges." *Wat. Res.* 23: 913-921.

Shelton, D., and J. Tiedje. 1984. "Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid." *Appl. Environ. Microbiol.* 48: 840-848.

Speitel, Jr., and F.B. Closmann. 1989. "Chlorinated solvent biodegradation by methanotrophs in unsaturated soils." *J. Environ. Eng.* 117: 541-559.

Sulfita, J., J. Stout, and J. Tiedje. 1984. "Dechlorination or (2,4,5-Trichlorophenoxy) acetic acid by anaerobic microorganisms." *J. Agric. Food Chem.* 32: 218-221.

Sulfita, J., J. Robinson, and J. Tiedje. 1983. "Kinetics of microbial dehalogenation of haloaromatic substrates in methanogenic environments." *Appl. Environ. Microbiol.* 45: 1466-1473.

Topping, T. 1985. "The biodegradability of *para*-dichlorobenzene and its behaviour in model activated sludge plants." *Wat. Res.* 21: 295-300.

USEPA (1985a). "Hexachlorobenzene." *Reviews of Environ. Contam. Toxicol.* 106(1988): 143-154.

Valo, R., J. Apajalahti, and M. Salkinofa-Salomen. 1985. "Studies on the physiology of microbial degradation of pentachlorophenol." *Appl. Microbiol. Biotechnol.* 21: 313-319.

Vargas, C., and R. Ahlert. 1987. "Anaerobic degradation of chlorinated solvents." *J. Water Pollut. Control Fed.* 59: 964-968.

Verschueren, Karel. 1983. *Handbook of Environmental Data on Organic Chemicals* 2ed. New York: Van Nostrand Reinhold Company, Inc.

- Vogel, T., C. Criddle, and P. McCarty. 1987. "Transformations of halogenated aliphatic compounds." *Environ. Sci. Technol.* 21(8): 722-736.
- Watanabe, I., T. Kashimoto, and R. Tatsukawa. 1986. "Hexabromobenzene and its debrominated compounds in river and estuary sediments in Japan." *Bull. Environ. Contam. Toxicol.* 36: 778-784.
- Wentz, C. 1989. *Hazardous Waste Management*. New York: McGraw-Hill Inc.
- Wolf, C., D. Mansuy, W. Nastainczyk, G. Deutschmann, and V. Ullrich. 1976. "The Reduction of polyhalogenated methanes by liver microsomal cytochrome P450." *Molecular Pharmacology* 13: 698-705.
- Woods, S., J. Ferguson, M. Benjamin. 1989. "Characterization of chlorophenol and chloromethoxybenzene biodegradation during anaerobic treatment." *Environ. Sci. Technol.* 23: 62-68.