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ABSTRACT

Attempts to Demonstrate Reductive
Dechlorination of Chlorobenzenes
by Anaerobic Microorganisms

by

Melissa S. Gross

Chlorobenzenes are a source of environmental pollution throughout the world. Investigations of the potential biodegradability of these compounds have been conducted employing different media under various conditions. In this study, the biodegradability of hexachlorobenzene (HCB), pentachlorobenzene (QCB), and 1,2,3,4-tetrachlorobenzene (1,2,3,4-TetCB) at a concentration of 70 uM (20 ppm), 80 uM (20 ppm), and 93 uM (20 ppm), respectively, was investigated. The source of inoculum was fresh anaerobic digester sewage sludge. Compound and sludge were combined in a serum bottle incubation system under anaerobic conditions and allowed to incubate at 30^{o}C for several weeks. An extraction method utilizing hexane and acetone was developed. Analysis after 8 weeks for HCB and QCB, and 10 weeks for 1,2,3,4-TetCB revealed no biodegradation of the compounds. In addition, it was also discovered via extraction of the butyl rubber stoppers in a mixture of hexane and acetone that the compounds absorb into the stoppers used to close the serum bottles.

ATTEMPTS TO DEMONSTRATE REDUCTIVE DECHLORINATION OF CHLOROBENZENES BY ANAEROBIC MICROORGANISMS

by

Melissa S. Gross

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Dechlorination of Chlorobenzenes

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1. INTRODUCTION

1.1 Preface

Environmental pollution is a major challenge facing the world today. An even bigger dilemma arises in deciding how to remedy this problem. At present, almost everyone is familiar with such environmental disasters as Love Canal, the Valley of the Drums, and the toxic releases of methyl isocyanate gas from a Union Carbide plant in Bhopal, India, which killed approximately 3700 persons (Lepkowski, 1990). As a result, public and government concern and protest is demanding that the scientific community uncover the solution.

In her book, *Silent Spring*, Rachel Carson (1962) exposed to the public the detrimental affects suffered by the environment due to pesticides. This marked the beginning of the true environmental awareness age. Ever since, the public and scientific communities have been on a crusade to stop such toxic pollutants from entering the biosphere and causing damage. However, the work is not nearly done in this fight to eliminate environmental pollution of all kinds. This is apparent from a report recently released (Thayer, 1991) which shows approximately 1200 sites on EPA's National Priorities List (NPL) which are targeted for cleanup under the Comprehensive Environmental Response, Compensation & Liability Act (Superfund), and as many as 4000 sites which may need remediation under the

corrective action side of the Resource Conservation & Recovery Act (RCRA). In addition, it is anticipated that the number of sites requiring investigation and cleanup under RCRA will far exceed those on the Superfund NPL. Tens of thousands more sites, sites such as oil or chemical spills and underground storage tanks, are expected to require treatment.

Many chemicals make their way into the biosphere. In 1985, EPA conducted a National Screening Survey of Hazardous Waste Treatment, Storage, Disposal, and Recycling (TSD) Facilities and found that there are 2959 facilities, regulated under RCRA, which managed a total of 247 million tons of wastes, and 322 million tons of hazardous waste which was handled by units exempt from RCRA reporting requirements (Wentz, 1989). Of the variety of chemicals being released, the persistence and toxic nature of one group of chemicals known as the chlorinated aromatic compounds are demanding the need for immediate attention. Within this group, chlorobenzenes are now being investigated by the scientific community.

Large amounts of chlorobenzenes are being released into the environment because of their use as solvents, fumigants, and intermediates in the production of pesticides and dyes (Haigler *et al.*, 1988). In the United States, they are produced in amounts in excess of 500 metric tons annually (Onuska and Terry, 1985). Hexachlorobenzene (HCB), pentachlorobenzene (QCB), and 1,2,3,4-tetrachlorobenzene

(1,2,3,4-TetCB) all have similar uses (Rogers *et al.*, 1989). HCB is used in the manufacture of pentachlorophenol and as a grain fungicide in paper impregnation preparations, styrene and nitroso rubber production, and in ordnance items. QCB has no significant industrial uses. However, it is found as an impurity in the pesticide pentachloronitrobenzene. The primary use of 1,2,3,4-TetCB is in the synthesis of the herbicide 2,4,5-T. It has limited industrial use but is also used in fire retardant compositions and in transformer coils. Trichlorobenzenes (TCB) are used as coolants in electrical equipment and in glass tempering while dichlorobenzenes (DCB) are used as fuel additives, coolants, and engine cleaners.

Chlorobenzenes are being detected worldwide in various environmental media (Balley, 1983; Hites, 1988; Rogers *et al.*, 1989; Masunaga *et al.*, 1991; Masunaga *et al.*, 1991). In the U.K., twelve different sewage sludges of varying treatments were analyzed for eleven chlorobenzenes. These included 1,3-, 1,4-, 1,2-DCB, 1,3,5-, 1,2,4-, 1,2,3-TCB, 1,2,4,5-, 1,2,3,5-, 1,2,3,4-TetCB, QCB, and HCB. They occurred at concentrations of the order of <0.01 - 40.2 mg/kg (dry wt) with the disubstituted compounds being the most abundant. There was a general reduction in concentration with increased chlorine substitution and pentachlorobenzene was not detected in any of the samples analyzed (Rogers *et al.*, 1989). Chlorobenzenes have also been found in numerous areas of the United States including

a hazardous waste dump, operated by the Hooker Chemical Company from approximately 1953 - 1975, located in Niagara Falls, N.Y., and the sediments, water, and selected fishes of lakes Superior, Huron, Erie, and Ontario (Balley, 1983; Hites, 1988). At the Hooker Chemical Company site, 55,000 tons of halogenated wastes were buried in the ground. Analysis of the site revealed aromatic compounds containing chlorine and fluorine (Hites, 1988). At the Great Lakes area, chlorobenzene biodegradability in several layers of sediments ranging from youngest to oldest were studied. In the older layers, the ratio of dichlorobenzenes (DCB) and trichlorobenzenes (TCB) to HCB and QCB increases greatly. This is consistent with a slow dehalogenation process in the anaerobic sediment (Balley, 1983). In addition, chlorobenzene studies have also been conducted at Ise Bay which is located in the middle of Honshu, Japan's main island (Masunaga, 1991). These various studies determined the behavior and distribution of chlorobenzenes in various environmental media. Investigations revealed that the chlorobenzenes were adsorbed by suspended particulates in the surface water and settled to the bottom swiftly through less polluted seawater in the lower water layer with little desorption (Masunaga, 1991). It was also discovered that the distribution patterns could be explained by the following effects: the distance from the source, horizontal transport caused by the river inflows, and the sedimentation caused by downward water flow (Masunaga, 1991).

As a result of the abundance of chlorobenzenes throughout the environmental media of the world, there has been an increased awareness of their potential danger. According to Hazardous Substance Fact Sheets developed by the New Jersey Department of Health, HCB is a carcinogen and repeated exposure may damage the liver, immune system, thyroid, kidneys, and nervous system. Lower chlorinated benzenes may damage the lungs, liver, kidneys, and blood cells. In addition, they may damage the developing fetus. Therefore, the elimination of these compounds from the environment is critical and necessitates the use of some form of removal. There are several processes to remove chlorinated benzenes from the biosphere. These include chemical fixation, incineration, and landfilling. However, an innovative technology known as bioremediation may prove to be the solution.

1.2 Bioremediation

Bioremediation employs microorganisms to detoxify or destroy wastes. It is an area of great interest to industry and researchers due to the restrictions and expense of other methods. However, bioremediation is only a small part of the very large and diverse hazardous-waste treatment and remediation market. Nonetheless, it is among the fastest growing sectors in environmental management. In 1991, the market for the bioremediation of hazardous wastes is about $60 million and based on an average annual growth rate of

about 25%, the market is expected to grow to about $150 million by the year 1995 (Thayer, 1991).

Bioremediation can and has been applied in one of two approaches. The first promotes the growth of existing microbes in situ by the addition of specific nutrients based on the principle that during the time the indigenous microbial populations have been exposed to the pollutants, a sub-population will have developed the ability to utilize and hence degrade the compounds. The rate of removal of the pollutants will be stimulated by adding other nutrients to promote growth of these organisms. This approach was successful on the beaches along Prince William Sound and the Gulf of Alaska to clean up the oil spilled from the Exxon Valdez. Nutrients added to the polluted beaches stimulated the native microbes which acted on the oil-creating metabolites (Hardman, 1991).

The second approach involves taking samples from a polluted site to the laboratory and inoculating them into selected nutrient conditions that promotes the enrichment and selection of microbes present in the samples which are competent at degrading the target compounds. Once isolated, the mixture of organisms can be returned to the site and added back with growth-promoting nutrients, once again stimulating the rate of removal of the pollutants (Hardman, 1991).

Three broad areas comprise bioremediation methods. These include land treatment, bioreactors, and in situ

treatment. Other approaches being explored are biofilters for treating gaseous streams, adding solids, sludges, or liquid wastes into surface soils, and composting of the waste which is similar to land treatment. One difference between composting and land treatment is the addition of bulking agents during composting (Thayer, 1991).

Land treatment has been employed by the oil-refining industry for treating contaminants generated during oil recovery. However, the land treatment approach now faces restrictions under RCRA land disposal regulations (Thayer, 1991). Also, even though land treatment is considered the least expensive method of biological treatment, it requires large amounts of land to be utilized solely for treatment for several months or more.

Bioreactors have been proven to be a quicker and more efficient means of degrading hazardous wastes by providing on-site treatment. Nevertheless, this approach is not without any disadvantages. Expenses can be high due to maintenance, operating, and capital costs. In addition, added hazards may be placed upon the workers because of wastes being pumped or excavated or from soils being handled.

In situ bioremediation avoids this potential hazard of worker contamination by eliminating the problems associated with handling the hazardous wastes for treatment. Depending on the conditions at the site, this method can prove to be very successful or very unsuccessful. Unlike bioreactors,

the control of environmental conditions can be quite difficult and more complex. Therefore, unexpected and unpredicted outcomes could result which explains why this is still considered an innovative technology. One study of in situ biodegradation, focusing on the microbiological patterns in a contaminated aquifer, points out that proof of in situ biodegradation must show that the mass of pollutant compounds has decreased and that microorganisms are the causative agent (Madsen *et al.*, 1991). Obtaining this information may be a difficult task in a field setting because mass balances may be prevented by the open complexity of the site. In addition, other abiotic attenuating processes such as dilution, volatilization, migration, and/or sorption may occur simultaneously with biodegradation.

In determining whether to employ bioremediation, several parameters must be addressed. These include its ability to meet time, cost, and regulatory constraints for clean up (Thayer, 1991). Computer modeling, pilot-scale studies, and extensive laboratory investigations may have to be conducted. Only when questions as to its potential, disadvantages and limitations, and applicability are answered, will bioremediation be considered as a proven technology.

2. MICROBIAL PROCESSES

2.1 Introduction

The fate of chlorobenzenes in environmental media has been investigated. However, the significance of biodegradation is still not precise. Studies have been conducted to determine biodegradation of chlorobenzenes, as well as other chlorinated aromatic compounds such as chlorophenols and polychlorinated biphenyls, under a variety of experimental conditions. Many of these investigations take into consideration both biotic and abiotic reactions. One area receiving great attention is the ability of microorganisms to degrade toxic compounds under anaerobic conditions.

Microbial activity is almost always responsible for the mineralization, or complete biodegradation of an organic molecule. As these microorganisms convert the organic substrate to inorganic products, some of the carbon in the substrate is utilized and converted into cell constituents. Simultaneously, energy is released and the microbial population increases in number and biomass due to the prescence of the utilizable carbon source. As a result, detoxification is a common outcome of mineralization.

There is great interest in the microbial degradation of aromatic compounds. This interest centers on (i) the study of the metabolic intermediates involved in the degradation of different aromatic substrates, (ii) the

elucidation of the enzymatic mechanisms of hydroxylation and ring fission, and (iii) the relatively recent investigations into the control of the enzymes taking part in the metabolism of aromatic compounds (Gibson, 1968).

2.2 Mineralization via Aerobic Bacteria

The mineralization of many aromatic compounds under aerobic conditions has been extensively studied. Information on the aerobic biodegradation of halogenated benzenes was first reported by Shelat and Patel (1973), who found that bromobenzene should be utilized by a *Bacillus polymyxa*. Since that time, much research has taken place in an attempt to elucidate the pathways of microbial biodegradation under aerobic conditions. For example, chlorophenols and polychlorobiphenyls have been investigated (Miller *et al.*, 1988; Sugimoto *et al.*, 1988; Gibson and Suflita, 1986). Polyhalogenated aromatic hydrocarbons (e.g., PCB's and HCB) have been reported (Sugimoto *et al.*, 1988) to be rapidly degraded by superoxide ion (in dimethylformamide) to carbonate and halide ions. This was found to be accomplished by the in situ electrolytic reduction of dissolved oxygen to generate superoxide ion which reacts with polyhalo aromatics by nucleophilic substitiution. Gibson and Suflita (1986) compared the reductive biodegradation of a variety of haloaromatic substrates within a shallow anoxic aquifer to that of freshwater sediment and sewage sludge. The metabolic

capacity existing in the media compared were found to be similar in that three of four chlorobenzoates, five of seven chlorophenols, and one of two chlorophenoxyacetate herbicides were reductively dehalogenated. One study even examined the affect of brief UV (300-nm) photolysis of 2,4-dichlorophenol and 2,4,5-trichlorophenol (Miller *et al.*, 1988). It was found that this greatly facilitates the removal of the two chlorophenols from sewage through accelerated mineralization and binding of polar products. It also adds that the addition of 0.1 M hydrogen peroxide accelerated the photolysis.

Reports of the aerobic biodegradation of chlorobenzenes have been established and will be discussed in greater detail in the following chapter. However, a brief overview will be given at this time. Marr and Stone (1960) were interested in studying the first steps of benzene oxidation and determining at what points its oxidation products might coincide with the known reactions of the so-called aromatic pathway. This study was conducted in response to a well-defined pathway of the bacterial degradation of the aromatic nucleus proposed by Stanier (1950) and several workers, including Murphy and Stone (1955) and Rogoff and Wender (1957, 1959). Since this intial research, specific studies have been reported. For example, several *Pseudomonas* species have been found to degrade chlorobenzenes (Gibson *et al.*, 1968; Marinucci and Bartha, 1979; Spain and Nishino, 1987; Haigler *et al.*, 1988; Pettigrew *et al.*, 1991; van der

Meer *et al.*, 1991; Sander *et al.*, 1991; Spain *et al.*, 1990). *Pseudomonas putida* can oxidize chloro, bromo, iodo, and fluorobenzenes to their respective 3-halogenated catechol derivatives using toluene as the sole source of carbon. Degradation of 1,2-DCB and 1,4-DCB in activated sludge by a *Pseudomonas* strain has also been documented (Spain and Nishino, 1987; Haigler *et al.*, 1988) while specific strains have also been isolated (Pettigrew *et al.*, 1991; van der Meer *et al.*, 1991; Sander *et al.*, 1991; Spain *et al.*, 1990). These report on *Pseudomonas* sp. strain JS6 which is able to grow on a wide range of chloro- and methylaromatic substrates, strain P51 which is able to use 1,2-DCB, 1,4-DCB, and 1,2,4-TCB as sole carbon and energy sources, and strain PS12 and PS14 which grew on monochlorobenzene, all three isomeric DCBs, 1,2,4-TCB, and 1,2,4,5-TetCB, respectively. Degradation of chlorobenzenes has also been found to be accomplished by *Alcaligenes* species (de Bont *et al.*, 1986; Schraa *et al.*, 1986). 1,3-DCB has been used as the sole carbon and energy source for an *Alcaligenes* sp. which was isolated from a mixture of soil and water samples while 1,4-DCB was degraded by *Alcaligenes* sp. strain A175.

2.3 Mineralization via Anaerobic Bacteria

On the other hand, anaerobic biodegradation of aromatic compounds has been less studied, especially chlorobenzenes. However, studies have been done on polychlorinated biphenyls and chlorophenols, and other aromatic compounds under

anaerobic conditions. Take for instance the enormous contamination of the Hudson River in New York State due to the prescence of polychlorinated biphenyls (Rhee *et al.*, 1989; Quensen III *et al.*, 1988). These investigations into the anaerobic sediments of the river revealed organisms capable of dechlorinating PCB's. The most rapid dechlorination was observed at the highest concentration (700 ppm Aroclor) and it was also determined that dechlorination occurred primarily from the *meta* and *para* positions. In the case of chlorophenols, extensive research has been conducted under anaerobic conditions (Woods *et al.*, 1989; Mikesell and Boyd, 1986; Mikesell and Boyd, 1988; Bryant *et al.*, 1991; Mikesell and Boyd, 1985; Boyd *et al.*, 1983; Boyd and Shelton, 1984). Unacclimated bacteria have been shown to preferentially remove chlorines from the position adjacent to the hydroxyl group for chlorophenols while with acclimation, *meta* chlorines were also removed. Pentachlorophenol has been found to be anaerobically dechlorinated via acclimation to monochlorophenol degradation and produced di-, tri-, and tetrachlorophenols. Other investigations (Harwood and Gibson, 1988; Fitzsimons *et al.*, 1990; Healy, Jr. and Young, 1979) include the study of the photosynthetic bacterium known as *Rhodopseudomonas palustris* which can use diverse aromatic compounds for growth under both anaerobic and aerobic conditions, the anaerobic biodegradation of simple aromatic lignin derivatives to methane and carbon dioxide, and the study of

bleach plant effluents which discovered that the rate of dechlorination of high molecular mass material proceeded too slowly to be of any practical use in effluent treatment.

Literature concerning chlorobenzene biodegradation under anaerobic conditions is limited and will be discussed further in the following chapter. As a result of this limited amount of research, chlorobenzene biodegradation will be extended to other poorly water soluble aromatic compounds which constitute significant environmental pollutants. However, HCB has proven to undergo reductive dechlorination employing anaerobic sewage sludge under anaerobic conditions (Fathepure *et al.*, 1988). As can be seen, Boyd, Tiedje, and Mikesell have become important participants in the studies of aromatic compounds under these conditions. Also, from a study at the University of Georgia (Hamdy, 1988), HCB contaminated wastes supported the growth of *Serratia liquefaciens* and *Pseudomonas aeruginosa*.

2.4 Microbial Processes of Mineralization

As previously stated, mineralization, or complete biodegradation of an organic molecule usually leads to detoxification which is of great interest to scientists and engineers who deal with bioremediation. Of more interest is how these microorganisms are capable of doing this and how the scientist and engineer can perhaps alter a few conditions to enhance this act. Therefore, investigators are endlessly studying microbial processes.

Aerobic degradation of aromatic chlorine compounds does not generally proceed directly but rather is accomplished in a sequence of reactions (Neilson, 1990). This sequence is as follows:

(a) hydroxylation or dioxygenation of the ring to form catechols;

(b) cleavage of the aromatic ring;

(c) elimination of chloride from the aliphatic intermediates;

(d) incorporation of the products into metabolic pathways.

Chlorocatechols are key metabolites in almost all degradative reactions of chlorinated aromatic compounds because they are produced by hydroxylation of chlorophenols and by dioxygenation of chlorinated aromatic hydrocarbons (Neilson, 1990). Further, an alternative pathway proposes that chlorophenols are intermediates in the degradation of chlorobenzenes (Rochkind and Blackburn, 1986).

Ring cleavage may occur either between the oxygen atom (*ortho, endo,* or 1:2 cleavage) or between the C2 hydroxyl and the adjacent carbon atom (*meta, exo,* or 2:3 cleavage) (Neilson, 1990). Of the two mechanisms, which takes place may be critical to the organism. The metabolites from the *exo* cleavage may be toxic and inhibit further degradation of the original substrate. Also, if the compound contains rings with varying degrees of chlorination, it is generally

accepted that the ring with fewer chlorine substituents is attacked first (Neilson, 1990).

Aerobic degradation of aromatic compounds via substitution of chlorine before ring cleavage occurs has been less commonly reported with the aromatic chloro compounds (Neilson, 1990). Direct replacement of chlorine substituents with hydroxyl groups has, however, been observed in 3-chlorobenzoate, 4-chlorobenzoate, and 4-chlorophenylacetate.

In contrast to aerobic degradation, the first step in anaerobic degradation appears to be the removal of the halogen, i.e., reductive dechlorination, resulting immediately in the formation of a less toxic, more biodegradable compound (Sharak Genthner *et al.*, 1989). Reductive dechlorination removes chlorine and replaces it with a hydrogen; a well known case is the conversion of DDT to TDE (=DDD) (Matsumura, 1982). However, due to the experimental difficulties in isolating pure cultures of anaerobic bacteria as compared to aerobic studies which generally use pure cultures, scientists and engineers must rely upon metabolically stable consortia. Nonetheless, two important facts have emerged from these anaerobic studies:

(a) Only certain congeners seem to be produced from, for example, pentachlorophenol, HCB, and chlorocatechols which may plausibly be related to the established dechlorination of lower chlorinated congeners and possibly the higher

chlorinated congeners.

(b) The more highly chlorinated congeners appear generally to be more readily dechlorinated which is consistent with the hypothesis that dechlorination is an energy-producing reaction coupled to metabolism of the substrate (Neilson, 1990).

3. LITERATURE REVIEW OF CHLOROBENZENES

3.1 Biodegradation under Aerobic Conditions

As was stated earlier, literature on chlorobenzene biodegradation is limited. However, studies have been completed. Whereas 1,3,5-TCB and other highly halogenated benzene isomeres still seem resistant to aerobic biodegradation (Sander *et al.*, 1991; Oldenhuis *et al.*, 1989), the reductive dehalogenation of tri- and dichlorobenzenes to monochlorobenzene and even that of hexachlorobenzene, resulting in a mixture of tri- and dichlorobenzenes, has been demonstrated to occur under rather unspecific anaerobic conditions (Sander *et al.*, 1991; Fathepure *et al.*, 1988).

In 1960, Marr and Stone wanted to study the first steps of aerobic benzene oxidation. They were able to isolate *Mycobacterium rhodochrous* and *Pseudomonas aeruginosa* from soil by an enrichment culture technique. With cells of *P. aeruginosa* grown on benzene, oxygen uptake accounted for the oxidation of nearly 90% of the benzene to carbon dioxide and water. The oxidation rate for *M. rhodochrous* was somewhat lower. These organisms also oxidized catechol rapidly. However, when grown in a nutrient broth medium, both organisms failed to show significant activity on benzene. What resulted from this study was that a metabolic pathway for the breakdown of benzene via 3,5-cyclohexadiene-1,2-diol and catechol was proposed and that the first steps

in oxidation of benzene and other aromatic compounds are apparently unique and can be carried out only by a limited number of strains.

Following this study, another group of researchers studied the metabolism of toluene and benzene by *Pseudomonas putida* (Gibson *et al.*, 1968). This paper describes the isolation and identification of the products formed from chloro-, bromo-, iodo-, and fluorobenzenes and also *p*-chlorotoluene when these substrates are incubated with cells of *P. putida*, grown with toluene as the sole source of carbon. They discovered that oxidation of chlorobenzene, bromobenzene, and iodobenzene occurred at approximately equal rates while fluorobenzene was oxidized at half the rate and *o*-, *m*-, and *p*-chlorotoluenes were metabolized at a comparatively reduced rate. Chromatographs of the reaction products formed from each of the halogenated aromatic substrates revealed the presence of a catechol derivative via a dioxygenase mechanism. Ring cleavage of these catechols can then be carried out, resulting in at least partial mineralization of the ring carbon. To deactivate the effects of the halogens on the ring, direct ring dechlorination may occur but is not common.

More recent studies of the aerobic biodegradation of chlorobenzenes have now been reported and the isolation of many different strains is now being completed. Spain, Nishino, Haigler, and Pettigrew have conducted research on the degradation of chlorobenzenes utilizing *Pseudomonas* sp.

A *Pseudomonas* sp. capable of growth on 1,2-DCB (*o*-DCB) and 1,4-DCB (*p*-DCB) or chlorobenzene as a sole source of carbon and energy was isolated by selective enrichment from activated sludge (Spain and Nishino, 1987; Haigler *et al.*, 1988). This is further strengthened by a study conducted by Topping (1987) who found that the overall removal of *p*-DCB during the aeration stage of activated sludge sewage treatment will be high (>95%), even if adverse conditions prevail. He also states that under normal operating conditions, the major proportion of *p*-DCB reaching biological treatment (i.e. >76%) is likely to be removed by biodegradation. Biodegradation of *o*-DCB and *p*-DCB was detected in a mixed culture after 14 months and 10 months, respectively. Initial steps in the pathway for *o*-DCB degradation were found to be the conversion to 3,4-dichloro-*cis*-1,2-dihydroxycyclohexa-3,5-diene (*o*-DCB dihydrodiol). This suggests that *o*-DCB is initially converted by a dioxygenase to a dihydrodiol, which is converted to 3,4-dichlorocatechol by an NAD^+-dependent dehydrogenase. Ring cleavage of 3,4-dichlorocatechol is by a catechol 1,2-oxygenase to form 2,3-dichloro-*cis*,*cis*-muconate. Chloride is then eliminated during subsequent lactonization of the 2,3-dichloro-*cis*,*cis*-muconate, followed by hydrolysis to form 5-chloromaleylacetic acid (Haigler *et al.*, 1988). Initial steps in the pathway for *p*-DCB degradation were determined to be the conversion by a dioxygenase to 3,6-dichloro-*cis*-1,2-dihydroxycyclohexa-3,5-diene, which was

converted to 3,6-dichlorocatechol by an NAD^+-dependent dehydrogenase. Ring cleavage of 3,6-dichlorocatechol was by a 1,2-oxygenase to form 2,5-dichloro-*cis,cis*-muconate (Spain and Nishino, 1987).

In two other studies (Pettigrew *et al.*, 1991; Spain, 1990), these investigators suggest that *Pseudomonas* sp. strain JS6 has the ability to degrade a variety of chloro- and methylaromatic compounds by means of a modified *ortho* ring fission pathway. In this specific study, they investigate chlorobenzene and toluene by JS6. Methyl-substituted aromatic substrates are generally degraded via *meta* ring fission catalyzed by catechol 2,3-dioxygenase, whereas chloro-substituted aromatic substrates are degraded via modified *ortho* ring fission catalyzed by catechol 1,2-dioxygenase. This incompatibility of pathways has been demonstrated in that the catechol 2,3-dioxygenase of the *meta* cleavage pathway is inactivated by chlorocatechols (Pettigrew *et al.*, 1991). However, they show that JS6 can grow on toluene and chlorobenzene simultaneously. The initial conversion of toluene and chlorobenzene to the corresponding substituted catechols is mediated by dioxygenase and dihydrodiol dehydrogenase enzymes which is similar to the well-characterized pathway for degradation of toluene by *P. putida* as stated above.

Another *Pseudomonas* sp. strain, strain P51, is able to use 1,2-DCB, 1,4-DCB, and 1,2,4-TCB as sole carbon and energy sources (van der Meer *et al.*, 1991). This report

describes the cloning and genetic characterization of two gene clusters encoding the degradation of 1,2-DCB, 1,4-DCB, and 1,2,4-TCB.

Alcaligenes sp. has also been identified in the aerobic biodegradation of 1,3-DCB and 1,4-DCB (de Bont *et al.*, 1986; Schraa *et al.*, 1986). As compared to the study above with the *Pseudomonas* sp., 1,3-DCB was metabolized via 3,5-dichloro-*cis*-1,2-dihydroxycyclohexa-3,5-diene to 3,5-dichlorocatechol. This was then cleaved yielding 2,4-dichloromuconate. In the degradation of 1,4-DCB, *cis*-1,2-dihydroxycyclohexa-3,5-diene oxidoreductase and 1,2-pyrocatechase, but not 2,3-pyrocatechase, were found in cell extracts, while 3,6-dichlorocatechol and (2,5-dichloro)muconic acid could be detected as intermediates.

The biodegradation of 1,2,3- and 1,2,4-trichlorobenzenes in soil has also been investigated (Marinucci and Bartha, 1979). These compounds have been shown to be mineralized by microbial action but at the very slow rates of 0.35 and 1.00 nmol/day per 20 g of soil, respectively. Further, the total CO_2 evolution of soil samples declined markedly with increasing concentrations of 1,2,4-TCB. Marinucci and Bartha found that probably the most dramatic influence on degradation is the TCB concentration itself. They used 50 ug of TCB per g, as compared with 10 ug/g used by another researcher. At the latter concentration, mineralization was about twice as high as at 50 ug/g. In addition, the effect of inoculation with

pure bacterial cultures on the degradation of benzene, toluene, *o*-, *m*-, and *p*-xylene, chlorobenzne, *o*-dichlorobenzene, and 1,3,5-TCB in soil slurries was investigated (Oldenhuis *et al.*, 1989). The compounds for which organisms were added were rapidly degraded. However, without inoculation, degradation of benzene, toluene, *m*- and *p*-xylene and chlorobenzene was slow, while *o*-xylene and *o*-dichlorobenzene were only slightly degraded. Results showed that degradation was due to growth of the inoculated cells using the aromatic compounds as sources of carbon and energy. However, addition of activated sludge did not stimulate degradation.

In the area of water, studies to determine the diversity of degradative abilities of microbial communities from pristine aquifer solids samples (Swindoll *et al.*, 1988), and the microbial transformation of dimethyl- and dichlorobenzenes in laboratory aquifer columns simulating saturated-flow conditions typical for a river water/groundwater infiltration system (Kuhn *et al.*, 1985) have been conducted. All compounds were biodegraded under aerobic conditions. However, the dimethylbenzenes were biodegraded faster than the dichlorobenzenes. Evidence for this is presented which suggests that dimethylbenzenes are degraded by denitrifying bacteria under anaerobic conditions whereas dichlorobenzenes were not transformed under these conditions (Kuhn *et al.*, 1985).

3.2 Biodegradation under Anaerobic Conditions

On the other hand, investigations done over the past few years have indicated that anaerobic degradation of chlorinated aromatic compounds does occur, including the chlorobenzenes. However, this is only true for the higher chlorinated benzenes. There is evidence that HCB, TCB, and a variety of PCB congeners are subject to reductive dechlorination. In general, these reactions appear to take place at a much slower rate than those of the chlorobenzoates and chlorophenols. In addition, Battersby and Wilson (1989) found that the presence of chloro and nitro groups inhibited anaerobic gas production, while carboxyl and hydroxyl groups facilitated biodegradation. Nevertheless, reductive dechlorination may still prove to be a significant detoxification mechanism in contaminated anaerobic environments.

Studies on the reductive dechlorination of HCB have been completed employing anaerobic sewage sludge from a source that had previously shown the ability to degrade chlorophenols (Tiedje *et al.*, 1987; Fathepure *et al.*, 1988; Mikesell and Boyd, 1986; Mikesell and Boyd, 1985; Boyd *et al.*, 1983; Boyd and Shelton, 1984). These researchers are credited with much of the work done on these compounds under anaerobic conditions. Sludge was obtained from a wastewater treatment facility in Jackson, Mich, and used in the experiments. HCB was dechlorinated to tri- and dichlorobenzenes. This complete biotransformation of

approximately 50 ppm occurred within 3 weeks at a rate of 13.6 umol/(liter day). HCB was dechlorinated via two routes, both involving the sequential removal of chlorine from the aromatic ring. The major route was HCB - QCB - 1,2,3,5-TetCB - 1,3,5-TCB. More than 90% of the added HCB was recovered as 1,3,5-TCB and there was no further evidence of dechlorination past this point. Since most of the added HCB acccumulated as 1,3,5-TCB, which remained unchanged, this result is consistent with the general observation that *meta*-substituted benzene rings are structurally more stable. Although these products were not further metabolized by anaerobic populations, it is probable that they would be degraded by aerobic organisms or by facultative anaerobes that possess dechlorinating activity. The minor route was HCB - QCB - 1,2,4,5-TetCB - 1,2,4-TCB - DCBs (Fathepure *et al.*, 1988).

The above study also examined the biodegradation of 1,2,3,5-TetCB and found that there was a stoichiometric conversion to 1,3,5-TCB without a lag and with more than 85% of the initial 1,2,3,5-TetCB being converted to 1,3,5-TCB. After 3 weeks, the remaining TetCB was dechlorinated at reduced rates and required 8 weeks for complete conversion. No other products were detected, suggesting that the other TCB and DCB products of HCB did not originate from the 1,2,3,5-TetCB intermediate. In addition, experiments employing 1,3,5-TCB showed no dechlorination.

Sewage sludge from the same source, which had been acclimated to monochlorophenol degradation for more than 2 years, was shown to degrade pentachlorophenol (PCP) with the formation of di-, tri-, and tetrachlorophenols as products of biodegradation (Mikesell and Boyd, 1986). When the 2-chlorophenol- (2-CP), 3-CP-, and 4-CP-acclimated sludges were mixed in equal volumes, PCP was completely dechlorinated. Incubations with [^{14}C]PCP resulted in 66% of the added ^{14}C being mineralized to $^{14}CO_2$ and $^{14}CH_4$. In this case, degradation occurred most rapidly in the 2-CP-acclimated sludge in which the PCP was degraded within 3 days. In the 3- and 4-CP-acclimated sludges, PCP degradation was considerably slower, requiring 12 and 9 days, respectively. This suggests that Cl substituents *ortho* to the phenolic OH group are removed more rapidly than Cl in the *meta* and *para* positions.

Also investigated was the anaerobic biodegradation of chlorophenols in unacclimated sludge (Boyd and Shelton, 1984), and soil through induced anaerobiosis and bioaugmentation with anaerobic sewage sludge (Mikesell and Boyd, 1988). In the unacclimated sludge, each of the monochlorophenol isomers was degraded in the order of *ortho* > *meta* > *para* and for the DCP isomers, reductive dechlorination of the Cl group *ortho* to the phenolic OH was observed. In the case of the soil study, addition of biologically active anaerobic sewage sludge, previously shown to dechlorinate CP, to PCP contaminated soil resulted

in greatly enhanced rates of PCP degradation. The degradative pathway with PCP occurred as PCP - 2,3,4,5-TetCP - 3,4,5-TCP - 3,5-DCP - 3-CP. This addition of anaerobic sludge to anaerobic soil resulted in reducing the concentration from the initial 70 umol/kg to below the detection limit in 28 days.

Anaerobic degradation/dechlorination of high molecular mass material in bleach plant effluent was investigated in different systems (Fitzsimons *et al.*, 1990). A decrease in organically bound chlorine, measured as *adsorbable organic halogen* (AOX), was found with all molecular mass fractions. The rate and extent of dechlorination and degradation of soluble AOX decreased with increasing molecular mass. Part of the dechlorination appears to be dependent on the readily degradable carbon sources. They also reported that the rate of dechlorination of high molecular mass material proceeded too slowly to be of any practical use in effluent treatment.

Animals metabolize HCB by a different route. In a study conducted by Takazawa and Strobel (1986), HCB is converted to QCB by reductive dechlorination by *CYT P-450* in hepatic microsomes. However, this is an oxidative dechlorination of HCB by a *CYT P-450* monooxygenase system. In addition, Gantzer and Wackett (1991) have reported reductive dechlorination catalyzed by bacterial transition-metal coenzymes. Vitamin B_{12}(Co), coenzyme F_{430} (Ni), and hematin (Fe) catalyzed the reductive dechlorination of polychlorinated ethylenes and benzenes. For vitamin B_{12} and

coenzyme F_{430}, reductive dechlorination rates for different classes of perchlorinated compounds had the following order: carbon tetrachloride > tetrachloroethylene > HCB. For hematin, the order of reductive dechlorination rates was carbon tetrachloride > HCB > tetrachloroethylene. Within each class of compounds, rates of dechlorination decreased with decreasing chlorine content. QCB and PCP were each dechlorinated by vitamin B_{12} to yield two out of three possible isomeric TetCBs. Similar relative kinetics and dechlorination products have been observed in anaerobic cultures, suggesting a possible role of transition-metal coenzymes in the reductive dechlorination of polychlorinated compounds in natural and engineered environments.

4. OBJECTIVE

The objective of this study was to determine if reductive dechlorination of hexachlorobenzene, pentachlorobenzene, and 1,2,3,4-tetrachlorobenzene could occur under anaerobic conditions employing anaerobic digester sewage sludge. The anaerobic digester sewage sludge was obtained from the Livingston Water Pollution Control Facility located in Livingston, New Jersey. The concentrations of hexachlorobenzene, pentachlorobenzene, and 1,2,3,4-tetrachlorobenzene being investigated are 70 uM (20 ppm), 80 uM (20 ppm), and 93 uM (20 ppm), respectively. Evidence of reductive dechlorination will be based on the disappearance of hexachlorobenzene, pentachlorobenzene, and 1,2,3,4-tetrachlorobenzene and the appearance of dechlorinated products.

5. EXPERIMENTAL PROCEDURE

5.1 Introduction

Fresh anaerobic digested sewage sludge was obtained from the Livingston Water Pollution Control Facility located in Livingston, New Jersey. The facility receives primarily residential wastewater and no wastewater from industrial sources. However, if any industrial chemicals do enter the facility, they are always under the detection limit set for each pollutant.

After being transported to the laboratory, the containers of sludge were flushed with hydrogen gas (H_2) for approximately 1 hour by a degassing manifold. The bottles were then loosely stoppered and stored for 24 to 48 hours at room temperature to reduce excess methane evolution. The sludge contained no detectable levels of chlorobenzenes (Livingston Water Pollution Control Facility) (Table 5-1).

5.2 Materials and Equipment

This section lists and describes all materials and equipment utilized to conduct this experiment:

* glass serum incubation bottles (160 ml volume)- Wheaton Glass Company, Millville, New Jersey
* butyl rubber stoppers- Bellco Biotechnology, Vineland, New Jersey, catalog number 2048-11800
* aluminum seals (20 mm)- Bellco Biotechnology, Vineland, New Jersey, catalog number 2048-1120

Table 5-1 Anaerobic Digested Sewage Sludge Analysis

Parameter	Results (mg/kg)	
	Dry Wt.	Wet Wt.
Benzene	U	U
Carbon Tetrachloride	U	U
Chloroform	U	U
Methylene Chloride	1.681	0.0249
Tetrachloroethylene	U	U
Trichloroethylene	U	U
Vinyl Chloride	U	U
Aldrin	U	U
Chlordane	U	U
Dieldrin	U	U
DDT	U	U
Heptachlor	U	U
Lindane	U	U
PCB's	U	U
Toxaphene	U	U
Benzidine	0.290J	0.004J
Benzo (a) pyrene	0.164J	0.002J
Bis (2-ethylhexyl Phthalate	1.750	0.026
Hexachlorobenzene	U	U
Hexachlorobutadiene	U	U
N-nitrosodimethylamine	U	U

U- Indicates that the compound was analyzed for but not detected

J- Indicates that the compound meets the identification criteria but the result is less than the Method Detection Limit

- Printed with permission of the Livingston Water Pollution Control Facility

* chlorobenzenes- Aldrich Chemical Company, Inc. Milwaukee, Wis (used without further purification)

* anaerobic digested sewage sludge- Livingston Water Pollution Control Facility, Livingston, New Jersey

* resazurin- Eastman Kodak Company, Rochester, New York, catalog number 1137728

* H_2 and N_2 gases- Matheson Gas Products, Inc., East Rutherford, New Jersey

* incubator (30°C)- Environmental Growth Chamber, Chargin Falls, Ohio
* Harvard/LTE Series 300 Lab Autoclave- catalog number 302/0662/00, 121°C for 20 minutes
* Series 25 incubator shaker- New Brunswick Scientific Co., Inc., Edison, New Jersey, serial number 090822629, 30°C at 250 rpm
* hexane, acetone, toluene, and diethyl ether- Fischer Scientific, Springfield, New Jersey
* Corex glass centrifuge tubes (50 ml)- Corning Incorporated, Corning, New York
* ICE International Centrifuge- model PR-2, serial number 39546M, Needham Heights, Mass, 15°C, 5000 rpm
* solid state/ultrasonic FS-14- Fischer Scientific, serial number 170420
* sodium sulfate anhydrous (Na_2SO_4)- Fischer Scientific, Springfield, New Jersey
* Hewlett Packard Series II 5890 Gas Chromatograph- serial number 3115A35092, HP3396A Integrator, 7673 Controller, ^{63}Ni electron-capture detector at 290°C, injection temperature at 280°C, carrier gas of He at a flowrate of 0.64 ml/min and 50 psi, make-up gas of N_2 at a flowrate of 34.53 ml/min and 30 psi; Liquid Carbonic Speality Gas Corporation, Chicago, Illinois, split ratio of 10.4 and purge of 3.175 ml/min, SPB™-608 fused silica capillary column (30 m by 0.25 mm ID, 0.25 um film thickness; Supelco,

Inc., Bellefonte, PA), operated under an isothermal program at 180°C

5.3 Procedure

The evaluation of potential HCB, QCB, and 1,2,3,4-TetCB degradation in the fresh sludge under anaerobic conditions was conducted employing a serum bottle incubation system. 0.5 ml of a 2000 ppm toluene solution of HCB was added to the empty serum bottles. The toluene was then removed by flushing the bottle with deoxygenated nitrogen gas (N_2). 0.5 ml of a 2000 ppm diethyl ether solution of QCB and a 2000 ppm diethyl ether solution of 1,2,3,4-TetCB were also added to empty serum bottles. The diethyl ether was then removed by evaporation. All bottles contain 1 mg of compound based on the following:

$$\frac{2000 \text{ mg compound}}{1000 \text{ ml}} \times 0.5 \text{ ml} = 1 \text{ mg compound}$$

Fresh sludge (49 ml) was added to each serum bottle containing each compound while continuously sparging with H_2 utilizing the modified Hungate procedure (Miller and Wolin, 1974). While continuing to sparge the bottles with H_2, 1 ml of a 0.1% resazurin solution was added to each bottle to serve as an oxygen indicator. Therefore, the concentration of the bottles is the following:

$$\frac{1 \text{ mg compound}}{50 \text{ ml}} \times \frac{1000 \text{ ml}}{1 \text{ L}} = \frac{20 \text{ mg}}{\text{L}} = 20 \text{ ppm}$$

Finally, the bottles were flushed for an additional 5 minutes and sealed with butyl rubber stoppers and aluminum

crimp caps. To serve as sterile controls, a series of separate bottles containing HCB, QCB, and 1,2,3,4-TetCB and the fresh sludge was set up and autoclaved for 20 minutes on 3 successive days. Bottles of fresh sludge and bottles of sterilized sludge containing no compound were also set up to serve as controls. All bottles were then incubated stationary at 30^{o}C in the dark due to the photosensitivity of HCB. Active and control bottles were removed from incubation after 0, 3, 7, 14, 21, 28, 42, 56, 70, and 77 days and stored frozen until analyzed.

5.4 Analysis

An extraction procedure using a mixture of hexane and acetone was employed to recover HCB, QCB, and 1,2,3,4-TetCB from the samples. Toluene was considered based on a recent paper (Fathepure *et al.*, 1988), however, the recovery efficiences were not very high. This paper reports recovery efficiencies for HCB, 1,2,3,5-TetCB, and 1,3,5-TCB as 56, 71.5, and 70%, respectively. Therefore, in an attempt to increase the rate of efficiency, the mixture of hexane and acetone was used. Dr. Haggblom, from New York University Medical Center, suggested this mixture based on studies he has conducted on PCB's. He was using a 50:50 ratio of hexane and acetone to recover PCB's from sediments of the Hudson River and New Bedford Harbor and obtaining efficiencies greater than 90%. Other researchers have also used hexane/acetone and that of just hexane to recover

Table 5-2 Extraction Efficiencies of Preliminary Experiment

Concentration (ppm)	ppm Extracted	mg Extracted	% Recovery
20	8.5	0.85	85
	8.9	0.89	89
	9.2	0.92	92
	9.4	0.94	94
	8.8	0.88	88

chlorobenzenes (Nair and Pillai, 1989; Masunaga *et al.*, 1991; Masunaga *et al.*, 1991; Van Der Meer *et al.*, 1987; Bosma *et al.*, 1988; Schraa *et al.*, 1986). Several bottles containing 70 um (20 ppm) of HCB were analyzed by the following procedure to determine extraction efficiency (Table 5-2). In this preliminary experiment, the practice bottles testing extraction efficiency were set up 1 week prior to extracting HCB and were set up in plastic centrifuge tubes. The HCB was recovered, however, the solvents reacted with the plastic rendering the tubes unusable. In addition, due to the reaction with the plastic, the chromatograms became difficult to analyze due to the prescence of peaks resulting from the reaction. Therefore, plastic centrifuge tubes were not used in subsequent experiments.

Bottles used for this study were prepared for analysis by thawing and adding a 5 ml:5 ml hexane/acetone solution. The samples were then placed on the incubator shaker for 1 hour. After shaking, the sample volume was divided equally among 3 different glass centrifuge tubes (approximately 20 ml/centrifuge tube) in order to fit into the 50 ml glass

centrifuge tubes. The samples were centrifuged for 30 minutes. After centrifugation, the solvent layer was decanted and the remaining material including both water and solid material was rinsed with another 5 ml:5 ml mixture of hexane/acetone. Upon addition of hexane and acetone to the material, the samples were placed in an ultrasonic bath for 20 minutes in an effort to extract more compound. The samples were again centrifuged for 30 minutes and the solvent layer decanted. The remaining material was again rinsed with 10 ml of hexane only, ultrasonicated, and centrifuged. After combining all solvent layers, the volume was diluted to a known concentration of 100 ml for analysis. Samples containing 70 uM (20 ppm) HCB were diluted in hexane to a concentration of 10 ppm. QCB and 1,2,3,4-TetCB samples were also diluted in hexane to a concentration of 10 ppm. Based on the following equation, the amount of compound in mg can then be determined:

$$\text{mass}_{(mg)} = \text{concentration}_{(ppm)} \times \text{volume}_{(ml)}$$

Therefore, for example:

$$\text{mass}_{(mg)} = \frac{8.5 \text{ mg}}{1000 \text{ ml}} \times 100 \text{ ml} = 0.85 \text{ mg compound}$$

Using the amount recovered, the following can be obtained:

$$\frac{0.85 \text{ mg compound}}{50 \text{ ml total volume}} \times \frac{1000 \text{ ml}}{\text{L}} = \frac{17 \text{ mg}}{\text{L}} = 17 \text{ ppm}$$

Approximately 0.5 g Na_2SO_4 anhydrous was added to each solution to absorb any moisture and samples of each were injected into the GC under the conditions described in section 5.2.

6. RESULTS AND DISCUSSION

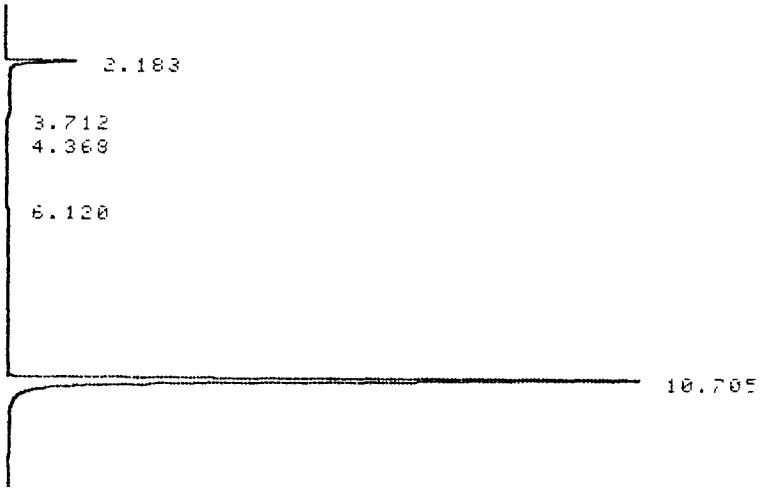
6.1 Outcome of Hexachlorobenzene Study

The bottles containing 70 uM (20 ppm) HCB showed no biodegradation of HCB when compared to the sterile control. There were no dechlorination products detectable (Table 6-1 and Figure 6-1). The bottles for day 0 through day 42, containing activated sludge plus compound and sterilized sludge plus compound, were prepared for analysis utilizing the procedure described in section 5.4. However, the bottles for day 14 were prepared with a slight modification to the original procedure. These bottles were shaken for a period of 2 hours on the incubator shaker instead of the original procedure time of 1 hour. The extraction was then completed following the original procedure. In addition, day 56 bottles were also prepared differently than the original procedure described in section 5.4. In this case, the bottles were thawed, and a 5 ml:5 ml hexane/acetone solution was added. The bottles were then shaken for 1 hour on the incubator shaker. Following this, the contents of the bottles were divided into 3 different glass centrifuge tubes and centrifuged. After decanting the solvent layer, the remaining portion was rinsed with a 5 ml:5 ml hexane/ acetone solution, ultasonicated, and centrifuged again. After decanting the solvent layer, the remaining portion was again rinsed with only 10 ml of hexane, ultrasonicated, centrifuged, and decanted. Thus far this is identical to

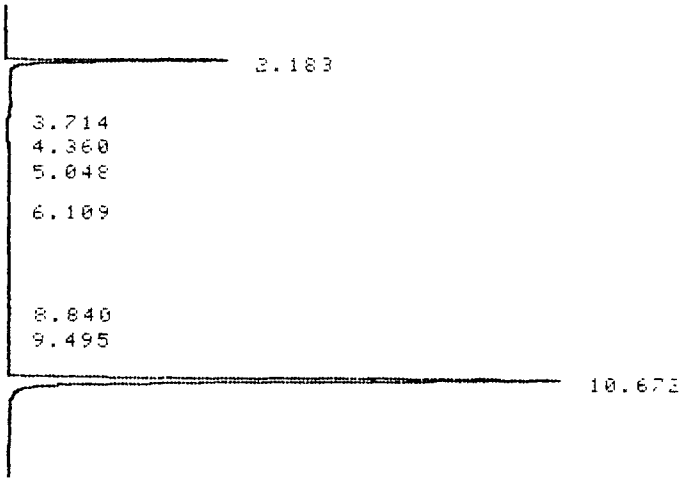
Table 6-1 Results of Hexachlorobenzene Analysis (20 ppm)

Time of Incubation (days)	Activated sludge plus compound (ppm)	mg	% Recovery
0	8.4	0.84	84
3	9.0	0.90	90
7	8.3	0.83	83
14	**8.4**	**0.84**	**84**
21	7.2	0.72	72
28	7.1	0.71	71
42	7.7	0.77	77
56	**7.0**	**0.70**	**70**
Time of Incubation (days)	Sterilized sludge plus compound (ppm)	mg	% Recovery
0	7.0	0.70	70
3	7.0	0.70	70
7	7.2	0.72	72
14	**4.2**	**0.42**	**42**
21	5.1	0.51	51
28	5.5	0.55	55
42	5.4	0.54	54
56	**8.1**	**0.81**	**81**

the original procedure. However, at this point an additional step was added. The water layer of the samples was removed and discarded and the remaining pellet of solid material was rinsed with a 5 ml:5 ml combination of hexane and acetone, ultrasonicated for another 20 mins, centrifuged for another 30 minutes, and the solvent layer was then decanted. This modification was based on the assumption that perhaps the water was in some way interfering or inhibiting the extraction of the compound since the higher chlorinated benzenes are insoluble in water. Finally, the solvent layers were combined and diluted to a volume of 100 ml. Therefore, the final concentration of each solution

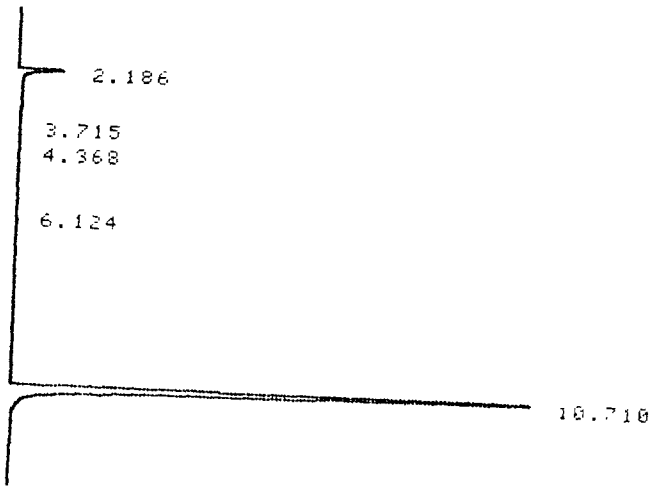


a. Activated sludge plus 20 ppm HCB at 0 day

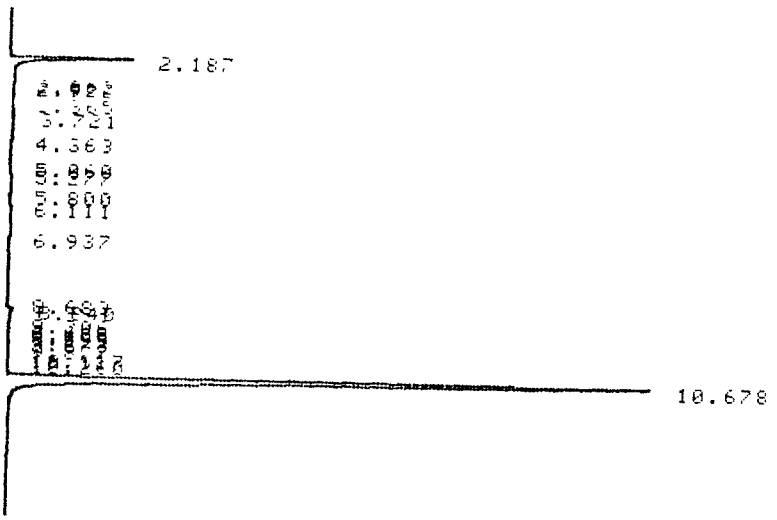


b. Activated sludge plus 20 ppm HCB at 56 days

Figure 6-1



c. Sterilized sludge plus 20 ppm HCB at 0 day



d. Sterilized sludge plus 20 ppm HCB at 56 days

Figure 6-1 (Continued)

should theoretically be 10 ppm based on the following equation:

$$\frac{20\ ug}{ml} \times 50\ ml = \frac{1000\ ug}{100\ ml} = \frac{10\ ug}{ml} = 10\ ppm.$$

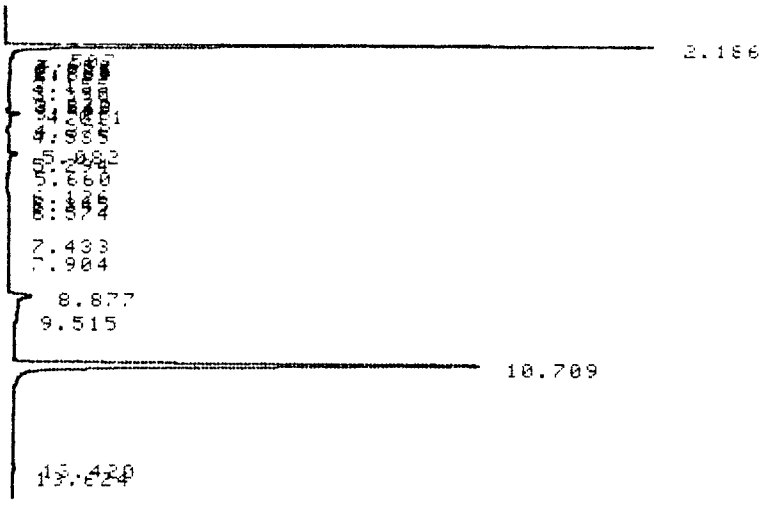
Acquiring the areas from the GC analysis chromatograms, the final concentration of HCB in each bottle was then obtained. Appendix A contains the standard calibration curve for HCB used in determining the concentrations of HCB in the bottles. Using the concentrations, the mass in mg was then determined following the equation in section 5.4.

It was believed that the difference in recoveries between the activated and sterilized sludge samples was a result of a chemical breakdown of HCB when the compound was autoclaved to serve as sterile controls. However, further investigation disproved this. As can been seen by Table 6-1, the extraction efficiencies of both the activated and sterilized sludge samples decrease as the days of incubation increase. It was thought that this was possibly due to HCB becoming more tightly bound to the sludge particles with increasing incubation time. This is the reasoning for the modifications to the original extraction procedure. The changes were made in an effort to increase extraction efficiencies. However, when the extraction efficiency of the day 56 sterilized sludge plus compound sample increased, other alternatives were explored. Nonetheless, the possibility of the HCB being more tightly bound to the sludge particles was not ignored.

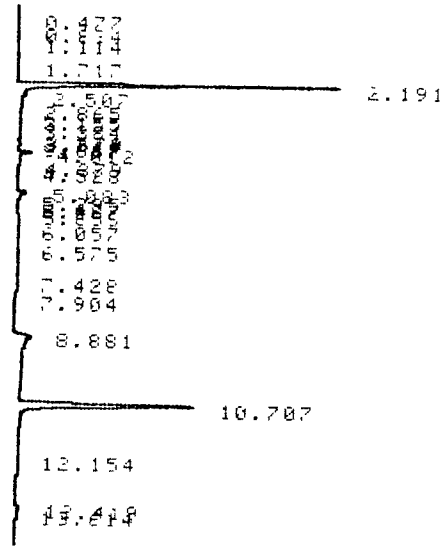
Table 6-2 Butyl Rubber Stopper Analysis (20 ppm HCB)

Activated sludge plus compound (ppm)	mg	Sterilized sludge plus compound (ppm)	mg
5.7	0.1425	2.1	0.0525

Since the HCB was decreasing in both the activated and sterilized sludge samples and there were no dechlorination products to suggest any type of biodegradation, the loss of HCB had to be explained. The next possiblity to be examined was to determine if the compound absorbed into the butyl rubber stoppers used to close the incubation bottles. In order to determine this, an extraction of the stoppers of the day 56 bottles and a blank control butyl rubber stopper was completed (Table 6-2 and Figure 6-2). This was completed by placing the stopper from each bottle and a new stopper to serve as the control into individual solutions of 10 ml:10 ml hexane and acetone and allowing them to extract overnight. After 24 hours, the solution was decanted and diluted to 25 ml and injected into the GC for analysis. It can be seen that HCB did absorb into the butyl rubber stopper based on the appearance of a definite peak and the absence of a peak in the control butyl rubber stopper in Figure 6-2. The next step was to add the amount that absorbed into the stopper with the amount extracted from the sludge to determine the total efficiency of extraction (Table 6-3). Upon completion of this step, the extraction efficiencies for both the activated and sterilized sludge samples at day 56 are almost identical. This factor in



a. Butyl rubber stopper of activated sludge plus 20 ppm HCB at 56 days



b. Butyl rubber stopper of sterilized sludge plus 20 ppm HCB at 56 days

Figure 6-2

Table 6-3 Total Extraction Efficiency of Hexachlorobenzene (Butyl Rubber Stopper and the Sludge Analysis)

	Activated sludge plus compound (mg)	Sterilized sludge plus compound (mg)
Stopper	0.1425	0.0525
Sludge	0.70	0.81
Total extracted	0.8425	0.8625
% Recovery	84.25	86.25

addition to the absence of dechlorination products reveals no biodegradation of HCB at a concentration of 70 uM (20 ppm) (Figure 6-3, 6-4).

6.2 Outcome of Pentachlorobenzene Study

The bottles containing 80 uM (20 ppm) QCB showed no biodegradation of QCB when compared to the sterile control. There were no dechlorination products detectable (Table 6-4 and Figure 6-5). These bottles, containing activated sludge plus compound and sterilized sludge plus compound, were prepared for analysis utilizing the extraction procedure used for the day 56 bottles of the HCB analysis. As stated earlier, this procedure is identical to that in section 5.4 except for the extra step of removing the water layer and rinsing the pellet with an additional combination of 5 ml:5 ml hexane and acetone. Again, the solvent layers were diluted to a known concentration of 10 ppm. After GC analysis of the samples, the concentration and amount in mg of QCB in the bottles was obtained. Appendix B contains the standard calibration curve for QCB used in determining

Analysis of HCB (Time vs Concentration)

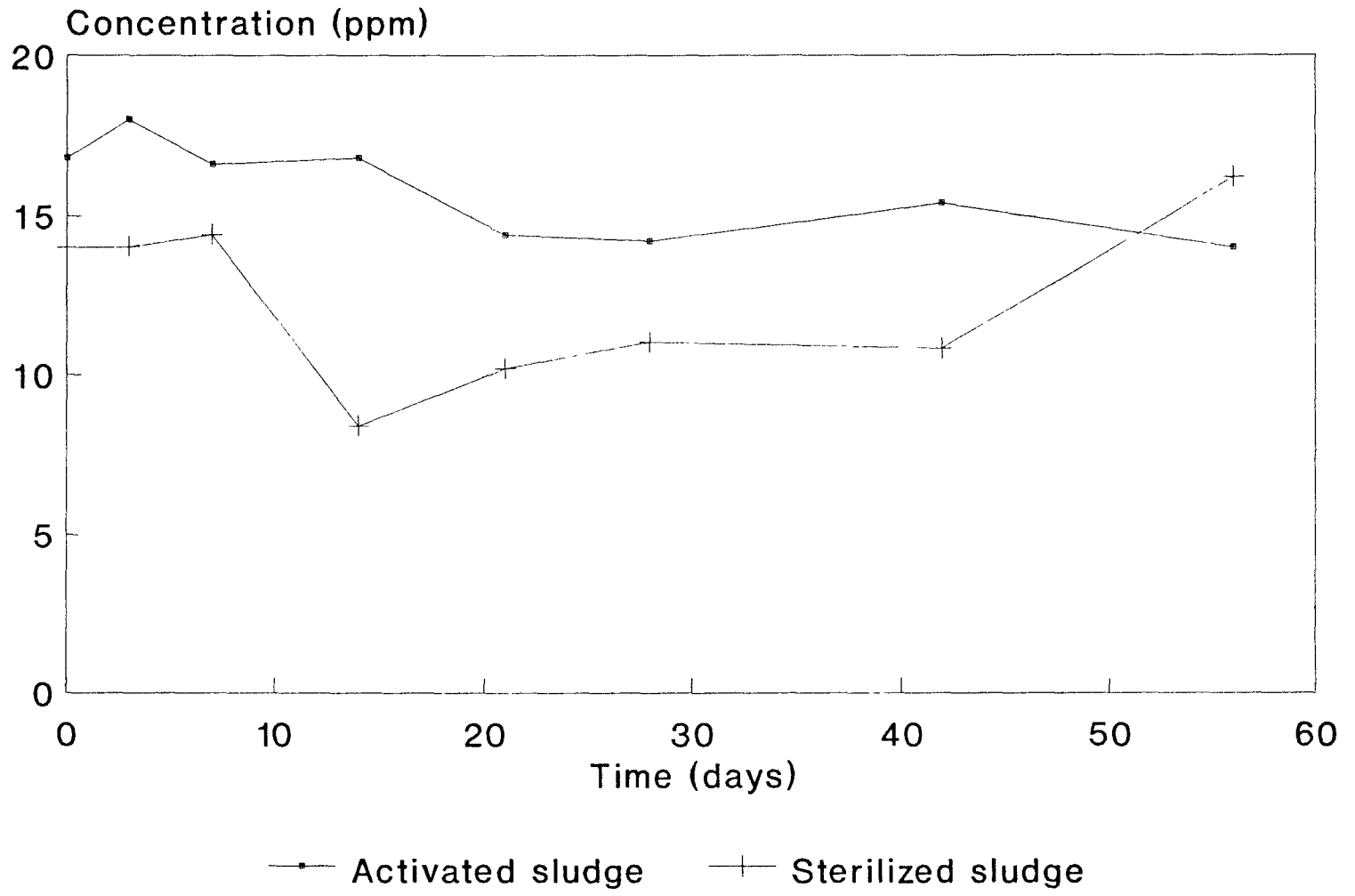


Figure 6-3

Analysis of HCB (Time vs Concentration) Butyl Rubber Stopper and Sludge Analysis

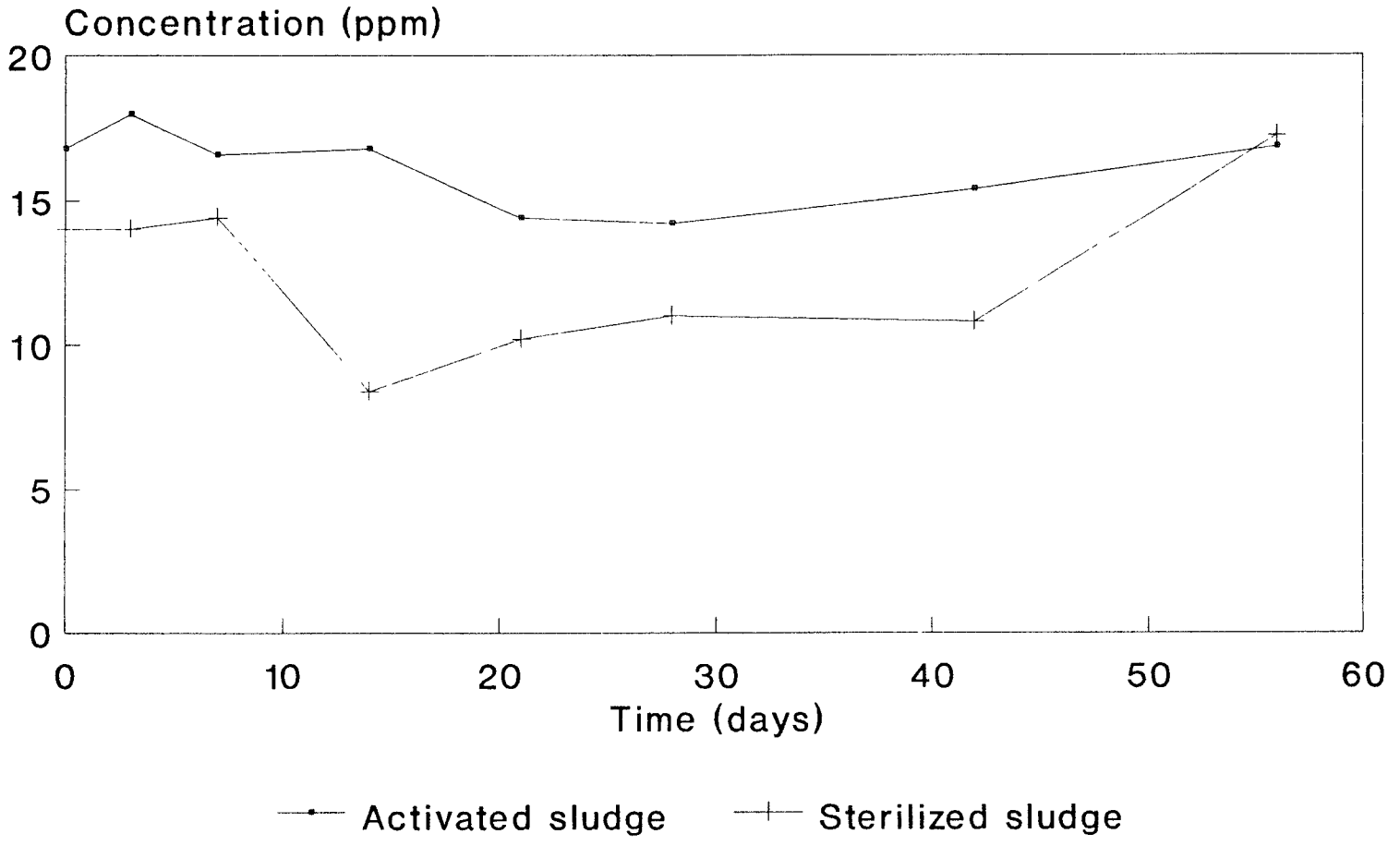


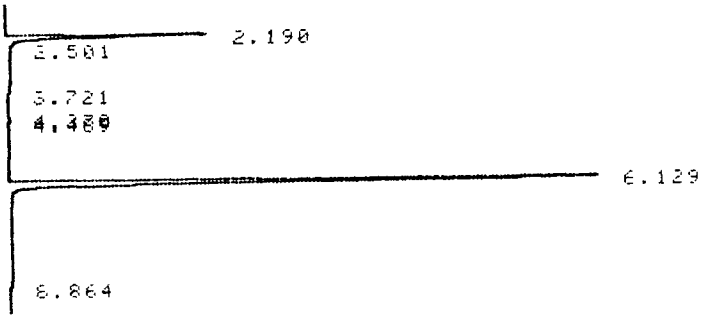
Figure 6-4

Table 6-4 Results of Pentachlorobenzene Analysis (20 ppm)

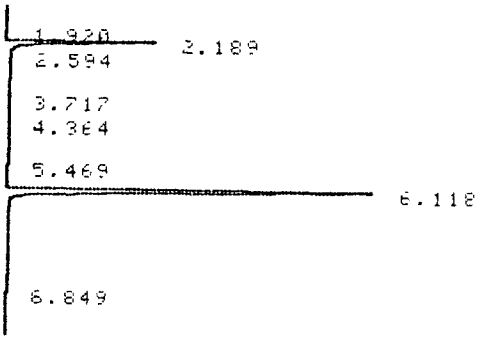
Time of Incubation (days)	Activated sludge plus compound (ppm)	mg	% Recovery
0	8.6	0.86	86
56	5.4	0.54	54
Time of Incubation (days)	**Sterilized sludge plus compound (ppm)**	**mg**	**% Recovery**
0	8.8	0.88	88
56	5.6	0.56	56

the concentrations of QCB in the bottles.

Once again there is a decrease in extraction efficiency with an increase in incubation time in both the activated and sterilized sludge samples. This is consistent with the results obtained from analysis of the HCB bottles. However, the concentrations of QCB in the activated and sterilized sludge bottles are almost identical as compared to those of the HCB bottles. Therefore, there is no possibility of any form of chemical breakdown of the QCB as a result of autoclaving the compound to serve as sterile controls. On the other hand, this decrease in extraction efficieny with increased incubation time again suggested the possibility of the QCB being more tightly bound to the sludge particles over time. However, the butyl rubber stoppers of the QCB bottles were not extracted. As a result, it is not known if the QCB absorbed into the stoppers. Nevertheless, it is assumed that QCB behaves similar to HCB and that the compound did absorb into the stoppers yielding results that

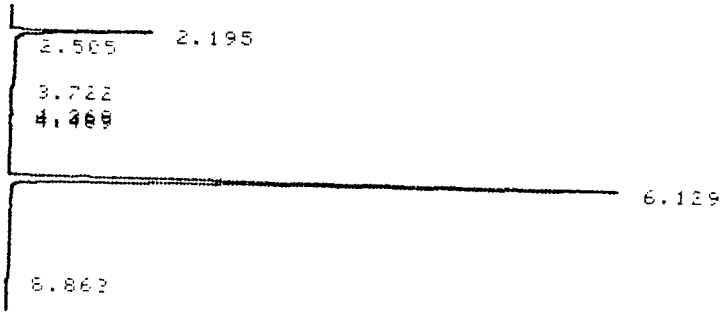


a. Activated sludge plus 20 ppm QCB at 0 day

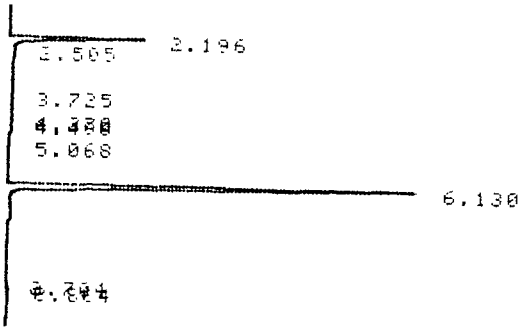


b. Activated sludge plus 20 ppm QCB at 56 days

Figure 6-5



c. Sterilized sludge plus 20 ppm QCB at 0 day



d. Sterilized sludge plus 20 ppm QCB at 56 days

Figure 6-5 (Continued)

Table 6-5 Results of 1,2,3,4-Tetrachlorobenzene Analysis (20 ppm)

Time of Incubation (days)	Activated sludge plus compound (ppm)	mg	% Recovery
0	3.8	0.38	38
70	**0.1**	**0.01**	**1**
Time of Incubation (days)	Sterilized sludge plus compound (ppm)	mg	% Recovery
0	5.5	0.55	55
70	4.2	0.42	42

would be similar to the results for HCB. This factor in addition to the absence of dechlorination products reveals no biodegradation of QCB at a concentration of 80 uM (20 ppm) (Figure 6-6).

6.3 Outcome of 1,2,3,4-Tetrachlorobenzene Study

The bottles containing 93 uM (20 ppm) 1,2,3,4-TetCB showed no biodegradation of 1,2,3,4-TetCB when compared to the sterile control. There were no dechlorination products detectable (Table 6-5 and Figure 6-7). These bottles, activated sludge plus compound and sterilized sludge plus compound, were prepared utilizing an extended extraction procedure (extension to the procedure used in the QCB and day 56 HCB analysis). In this case, the bottles were thawed and a 5 ml:5 ml hexane/acetone solution was added. The bottles were then shaken for 1 hour on the incubator shaker. Following this, the contents of the bottles were divided into 3 different glass centrifuge tubes and centrifuged. After decanting the solvent layer, the remaining portion was

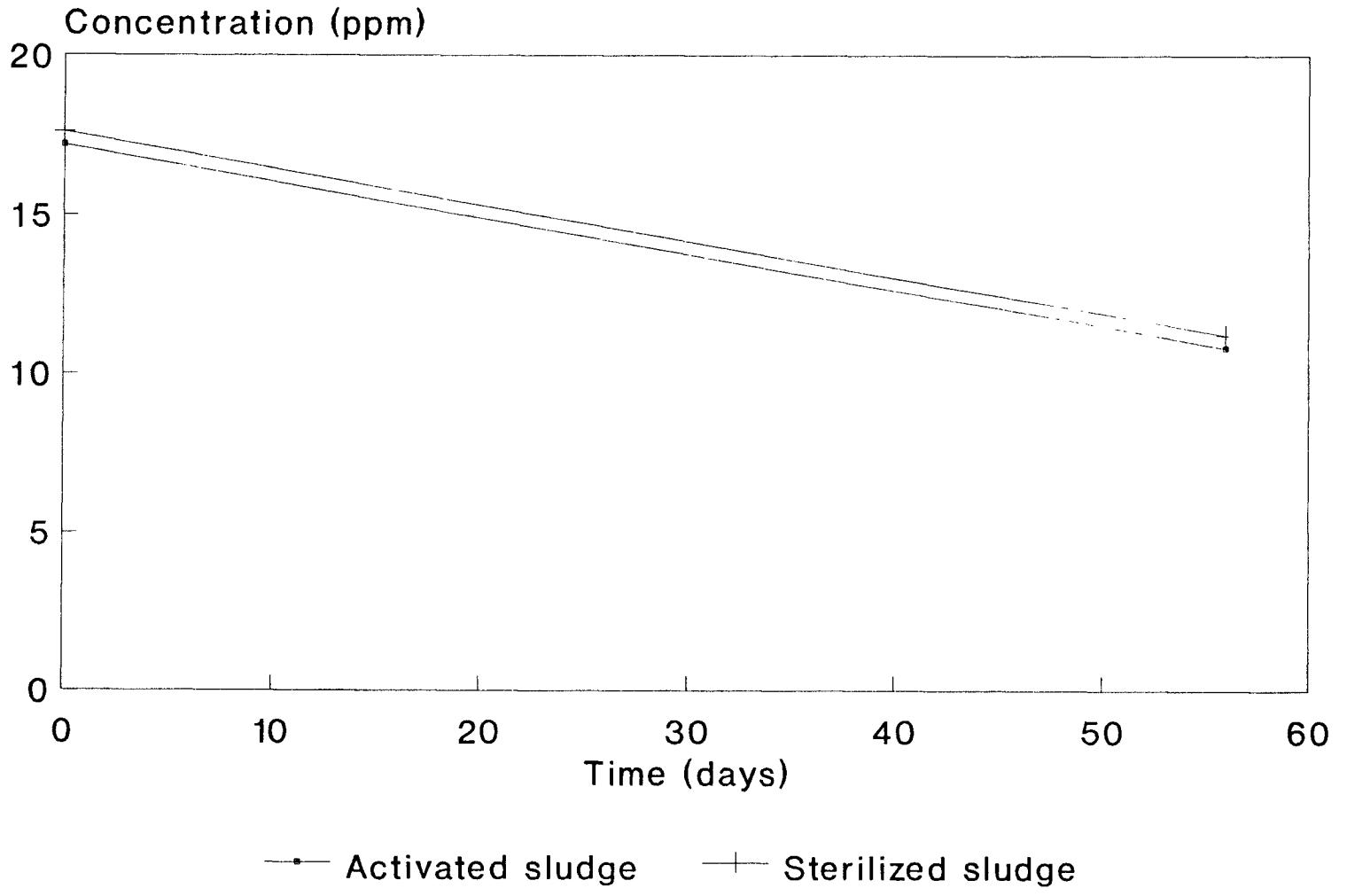
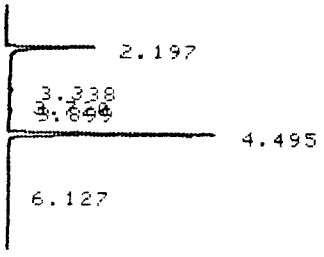
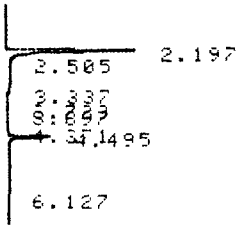

Analysis of QCB (Time vs Concentration)
Concentration (ppm)
20
15
10
5
0
0
10
20
30
40
50
60
Time (days)
Activated sludge
Sterilized sludge

Figure 6-6

a.

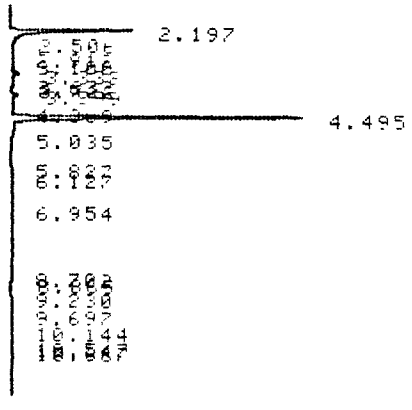


b.

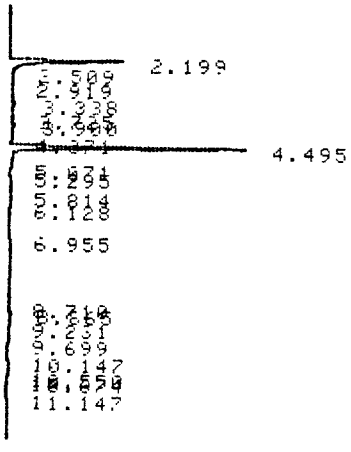


a. Activated sludge plus 20 ppm 1,2,3,4-TetCB at 0 day
b. Activated sludge plus 20 ppm 1,2,3,4-TetCB at 70 days

c.



d.



c. Sterilized sludge plus 20 ppm 1,2,3,4-TetCB at 0 day
d. Sterilized sludge plus 20 ppm 1,2,3,4-TetCB at 70 days

Figure 6-7

rinsed with 5 ml:5 ml hexane and acetone, ultrasonicated, and centrifuged again. After decanting the solvent layer, the water was then removed and discarded from the samples and the solid material remaining as the pellet was rinsed with 5 ml:5 ml hexane and acetone, ultrasonicated, centrifuged, and the solvent layer decanted. Again, the pellet was rinsed with 10 ml hexane, ultrasonicated, centrifuged, and the solvent layer decanted. This was done two times. Finally, the five solvent layers were combined to a volume of 100 ml and analyzed. Appendix C contains the standard calibration curve for 1,2,3,4-TetCB used in determining the concentrations of 1,2,3,4-TetCB in the bottles.

Again there is a decrease in extraction efficiency with an increase in incubation time even with an extended extraction procedure. In addition, the efficiencies to begin with are much lower than those of the HCB and QCB bottles. The day 70 activated sludge plus compound sample contained very little 1,2,3,4-TetCB, suggesting once again that either it is more tightly bound to the sludge particles making it harder to extract or that it is going somewhere else. Therefore, the rubber stoppers of all the bottles were analyzed for 1,2,3,4-TetCB using the extraction procedure described earlier and comparing the results to the blank stopper result (Table 6-6 and Figure 6-8). It can be seen that 1,2,3,4-TetCB did absorb into the butyl rubber stopper based on the appearance of a definite peak and the

Table 6-6 Butyl Rubber Stopper Analysis
20 ppm 1,2,3,4-TetCB

Time (days)	Activated sludge plus compound (ppm)	mg	Sterilized sludge plus compound (ppm)	mg
0	2.4	0.06	3.6	0.09
70	19.0	0.475	8.9	0.2225

Table 6-7 Total Extraction Efficiency of 1,2,3,4-Tetrachlorobenzene (Butyl Rubber Stopper and the Sludge)

	Activated sludge plus compound (ppm) 0 day	Sterilized sludge plus compound (ppm) 0 day
Stopper	0.06	0.09
Sludge	0.38	0.55
Total extracted	0.44	0.64
% Recovery	44.00	64.00
	70 day	70 day
Stopper	0.475	0.2225
Sludge	0.01	0.42
Total extracted	0.485	0.6425
% Recovery	49.00	65.00

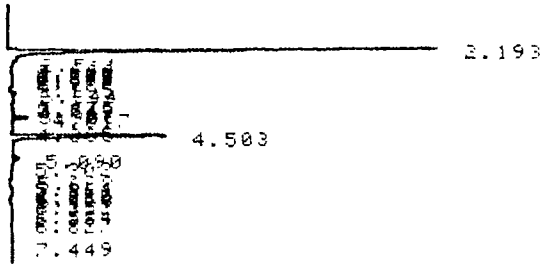
absence of a peak in the control butyl rubber stopper from Figure 6-2. The next step was to add the amount that absorbed into the stopper with the amount extracted from the sludge to determine the total efficiency of extraction (Table 6-7). Upon completion of this step, the extraction efficiencies for both the activated and sterilized sludge samples are somewhat similar and consistent. This factor in addition to the absence of dechlorination products reveals

no biodegradation of 1,2,3,4-TetCB at a concentration of 93 uM (20 ppm) (Figure 6-9, 6-10).

6.4 Outcome of Experiments with Longest Incubated Bottles

The first series of enrichments prepared with the compounds were still available. These enrichments contained activated sludge plus HCB and sterilized sludge plus HCB, activated sludge plus QCB and sterilized sludge plus QCB, and activated sludge plus 1,2,3,4-TetCB and sterilized sludge plus 1,2,3,4-TetCB. All of these bottles have been incubating at 30^{o}C for 98 days. These bottles were all prepared following the same procedure as described in section 5.3. They contain 70 uM (20 ppm), 80 uM (20 ppm), and 93 uM (20 ppm), respectively. Analysis of these bottles following the same extended extraction procedure as done with the 1,2,3,4-TetCB bottles revealed no biodegradation after 98 days (14 weeks). This result is based on the absence of dechlorination products (Table 6-8 and Figure 6-11). However, sterile control bottles of these older bottles were not analyzed. The rubber stoppers were also extracted and the total extraction efficiency determined (Table 6-9 and Figure 6-12).

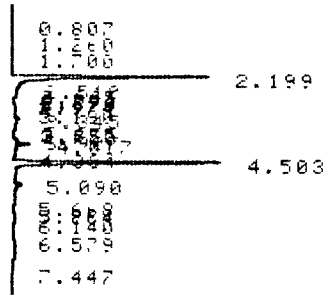
With these sets of bottles, the extraction efficiences of both the 1,2,3,4-TetCB and QCB are essentially zero. However, the HCB bottle had a recovery of 91% which is even higher than the set of HCB samples whose results are shown



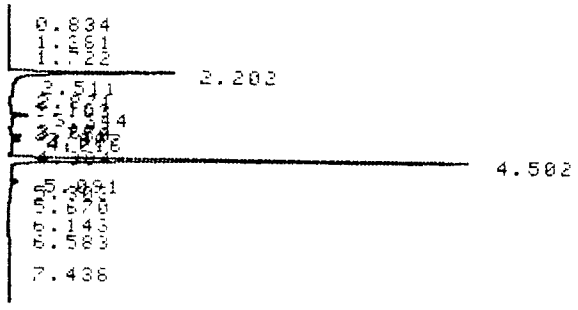
a. Butyl rubber stopper of activated sludge plus 20 ppm 1,2,3,4-TetCB at 0 day

b. Butyl rubber stopper of activated sludge plus 20 ppm 1,2,3,4-TetCB at 70 days

Figure 6-8



c. Butyl rubber stopper of sterilized sludge plus 20 ppm 1,2,3,4-TetCB at 0 days



d. Butyl rubber stopper of sterilized sludge plus 20 ppm 1,2,3,4-TetCB at 70 days

Figure 6-8 (Continued)

Analysis of 1,2,3,4-TetCB (Time vs Concentration)

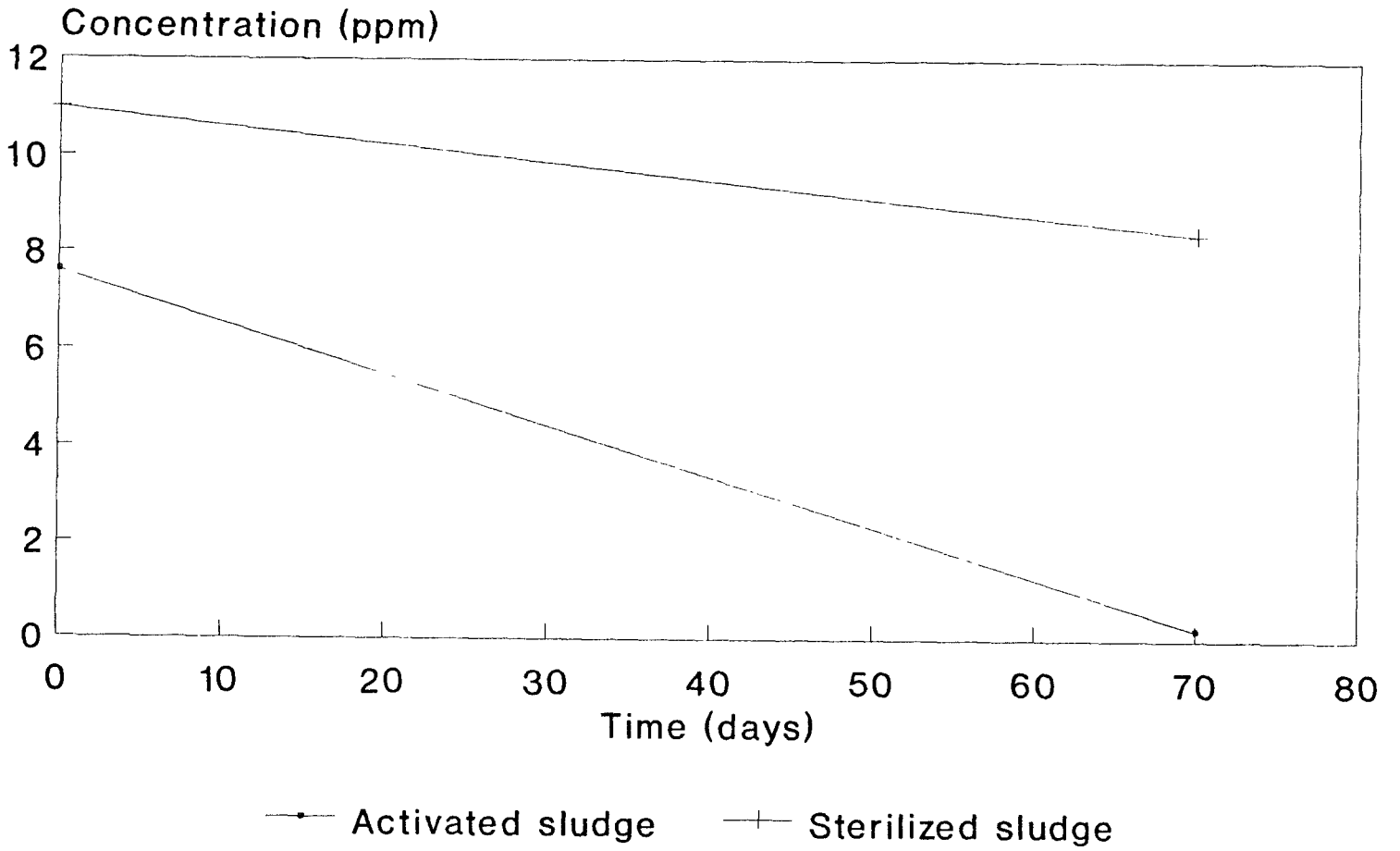


Figure 6-9

Analysis of 1,2,3,4-TetCB
Butyl Rubber Stopper and Sludge Analysis

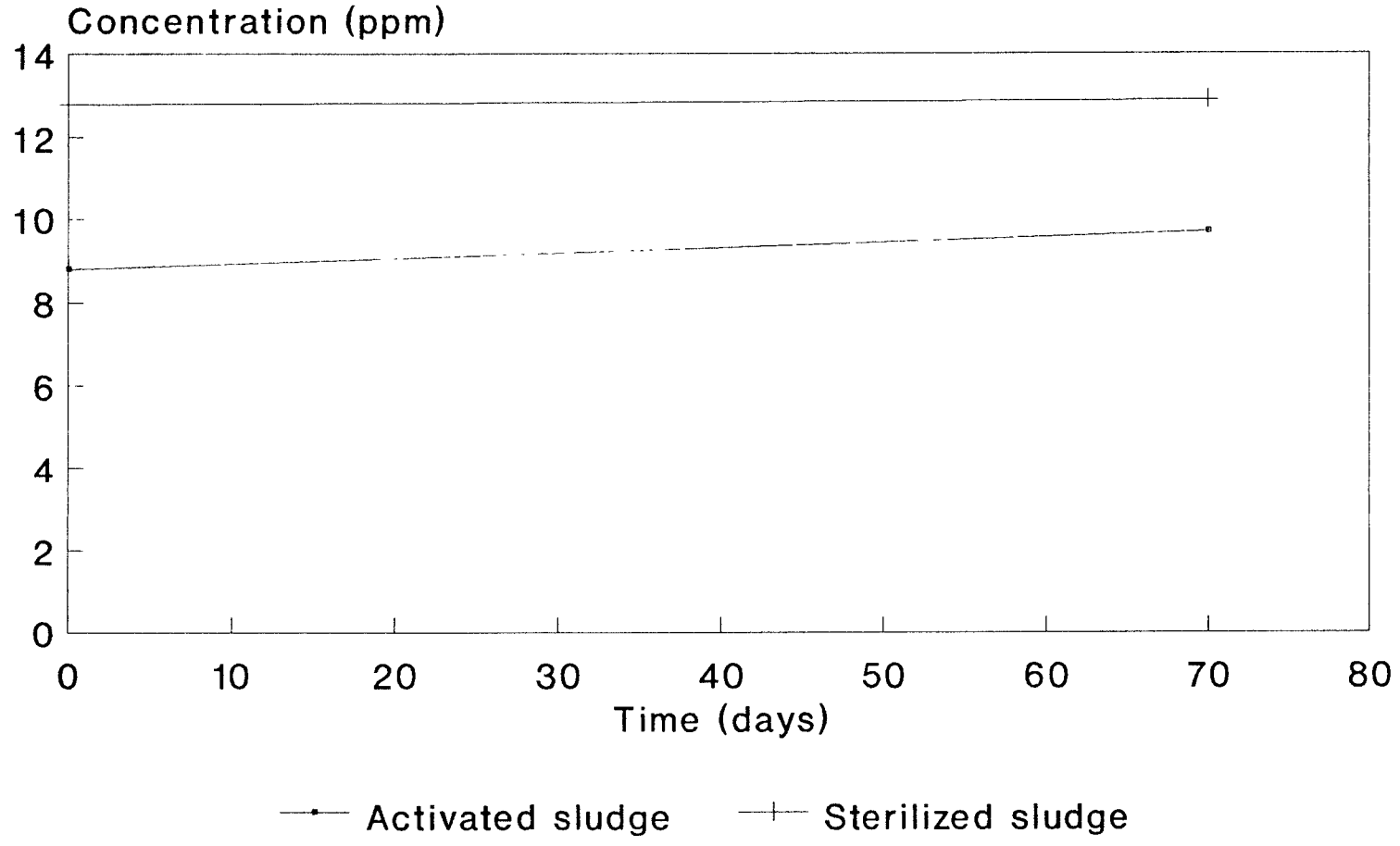


Figure 6-10

Table 6-8 Results of Longest Incubated Systems (all 20 ppm)

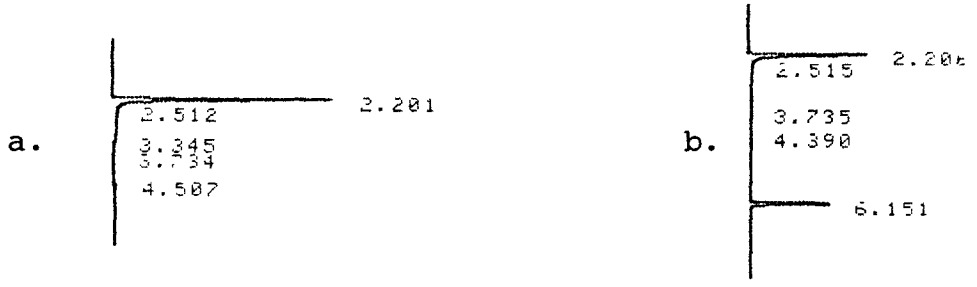
	Compound	Time (days)	ppm extracted	mg	% Recovery
Activated sludge	HCB	98	9.1	0.91	**91.00**
	QCB	98	0.62	0.062	**6.2**
	TetCB	98	0.0	0.0	**0.0**

Table 6-9 Butyl Rubber Stopper Analysis of Longest Incubated Systems (20 ppm) and Total Extraction Efficiencies

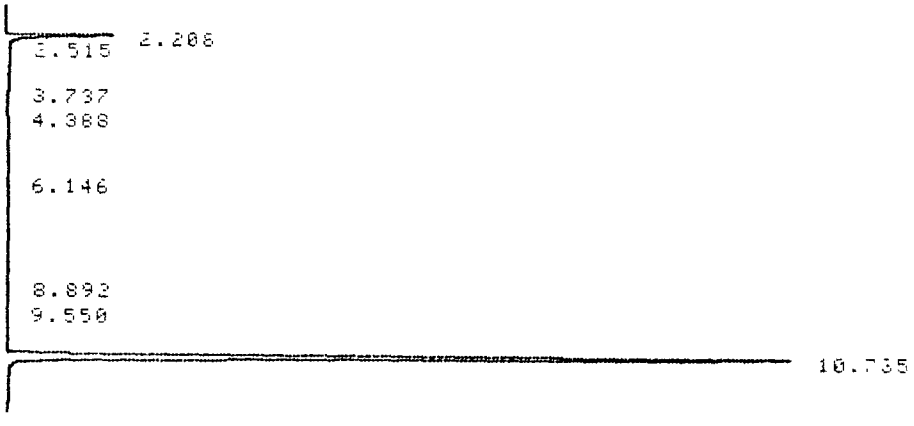
	HCB (mg)	QCB (mg)	TetCB (mg)
Stopper	0.1725	1.1625	0.47
Sludge	0.91	0.062	0.0
Total extracted	1.0825	1.2245	0.47
% Recovery	108.25	122.45	47.00

in section 6.1. This 91% is even higher than day 0 of the set in section 6.1 indicating strong evidence that with increased incubation time, the compound does not bind more tightly to the sludge particles. In order to test this hypothesis, bottles from the set described in section 6.1 at a time of 77 days incubation were frozen and then analyzed following the extended procedure as explained above with the 1,2,3,4-TetCB and longest incubated bottles (Table 6-10) (Figure 6-13). The stoppers of each were also extracted and the total extraction efficiency determined (Table 6-11) (Figure 6-14).

As can be seen, the extraction efficiency for the activated sludge plus compound sample remained almost unchanged while the extraction efficiency for the sterilized

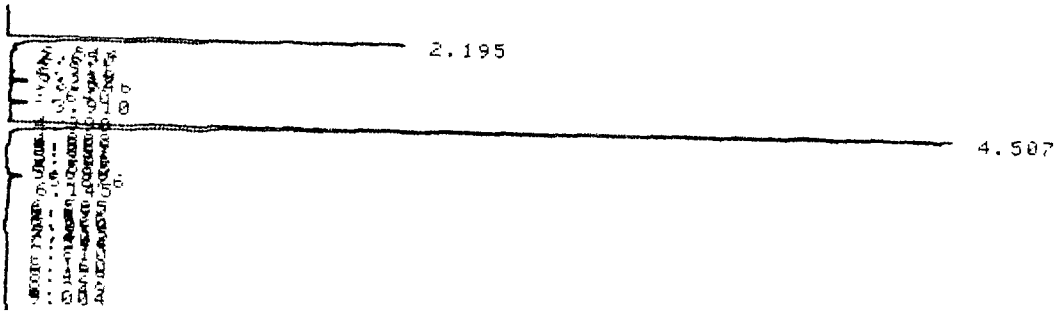


a. Activated sludge plus 20 ppm 1,2,3,4-TetCB at 98 days
b. Activated sludge plus 20 ppm QCB at 98 days

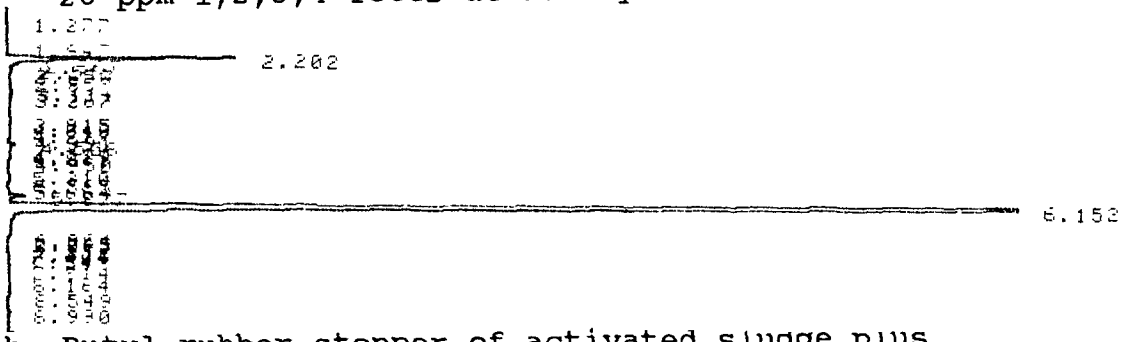


c. Activated sludge plus 20 ppm HCB at 98 days

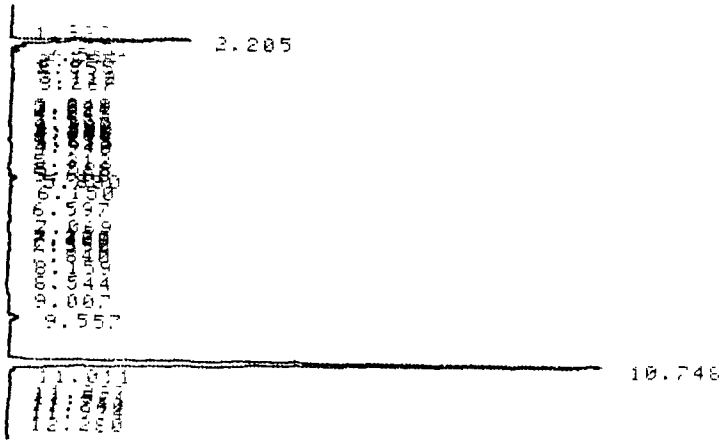
Figure 6-11



a. Butyl rubber stopper of activated sludge plus 20 ppm 1,2,3,4-TetCB at 98 days



b. Butyl rubber stopper of activated sludge plus 20 ppm QCB at 98 days



c. Butyl rubber stopper of activated sludge plus 20 ppm HCB at 98 days

Figure 6-12

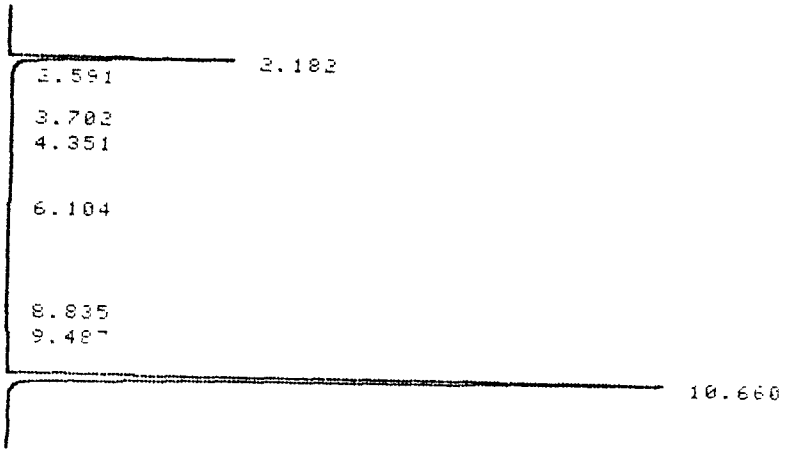
Table 6-10 Results of Hexachlorobenzene Analysis (20 ppm) at day 77

Activated sludge plus compound (ppm)	mg	% Recovery
7.6	0.76	76
Sterilized sludge plus compound (ppm)	**mg**	**% Recovery**
5.8	0.58	58

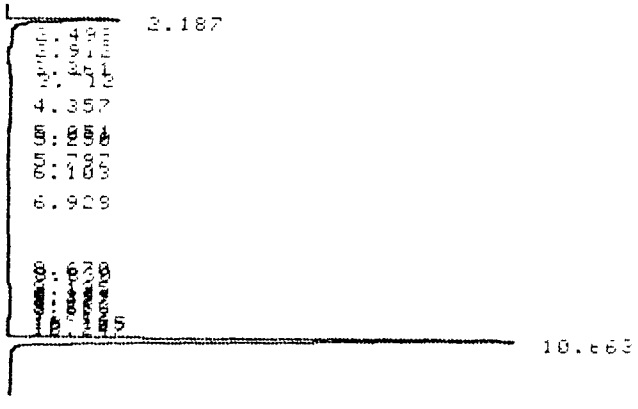
Table 6-11 Butyl Rubber Stopper Analysis of HCB (20 ppm) at day 77 and Total Extraction Efficiencies

	Activated sludge plus compound (mg)	Sterilized sludge plus compound (mg)
Stopper	0.1125	0.1875
Sludge	0.76	0.58
Total extracted	0.8725	0.7675
% Recovery	87.25	76.75

sludge plus compound sample decreased dramatically (see Table 6-3 for analysis at day 56). This now becomes very difficult to explain since the activated and sterilized sludge plus compound samples are not similar. In the case of the activated sludge plus compound sample, it would suggest that the extraction procedure had no effect on removal efficiency and that the particles are not more tightly bound to the sludge particles with increased incubation time. However, the sterilized sludge plus compound sample would suggest just the opposite. In this case, the extraction efficiency decreased 10 percentage

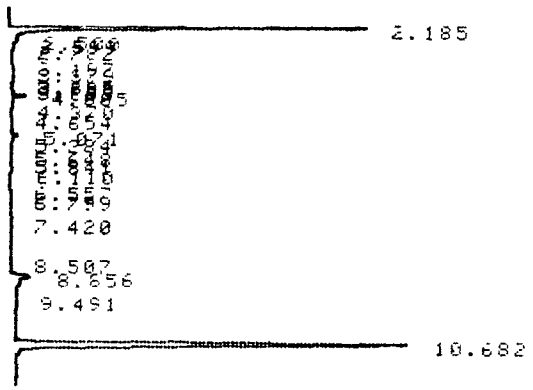


a. Activated sludge plus 20 ppm HCB at 77 days

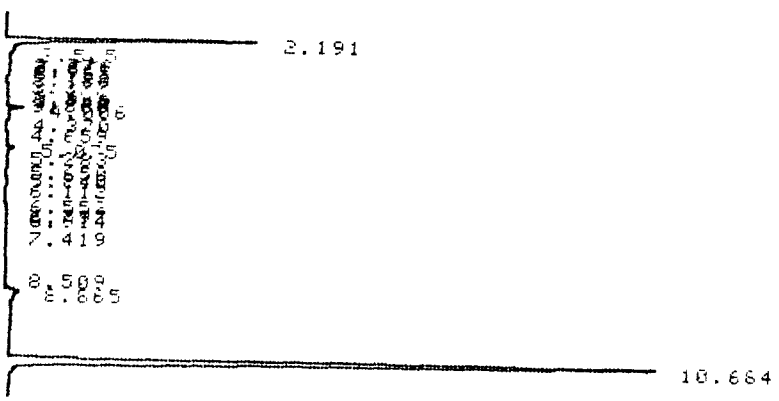


b. Sterilized sludge plus 20 ppm HCB at 77 days

Figure 6-13



a. Butyl rubber stopper of activated sludge plus 20 ppm HCB at 77 days



b. Butyl rubber stopper of sterilized sludge plus 20 ppm HCB at 77 days

Figure 6-14

Table 6-12 Results of Toluene Removal Process

Bottle	Time (min)	ppm Extracted	mg	% Recovery
1	0	8.6	0.86	86
2	2	9.4	0.94	94
3	4	9.1	0.91	91
4	5 (end)	9.5	0.95	95

points indicating that the compound is more tightly bound to the particles and thus harder to extract, or that the extended extraction procedure has some type of adverse affect on removing the HCB.

Another factor considered was the possibility that when the toluene was evaporated by deoxygenated N_2, that some of the compound was removed along with the toluene. However, this possibility was ruled out by an experiment. Several bottles containing a solution of 20 ppm HCB had the toluene removed utilizing the modified Hungate procedure as described earlier. The contents of the bottles were analyzed at different time intervals of the removal process to determine if any of the HCB had been blown away during the process. All the samples were diluted to a known concentration in toluene for quantification and analyzed by GC (Table 6-12 and Figure 6-15).

6.5 Outcome of Experiments with Sludge and No Compound

The control bottles of activated sludge plus no compound and sterilized sewage sludge plus no compound were also analyzed for HCB, QCB, 1,2,3,4-TetCB following the same extraction procedure described above in the previous section. The sewage sludge contained none of the compounds.

Results of Toluene Removal by Nitrogen Utilizing the Modified Hungate Technique

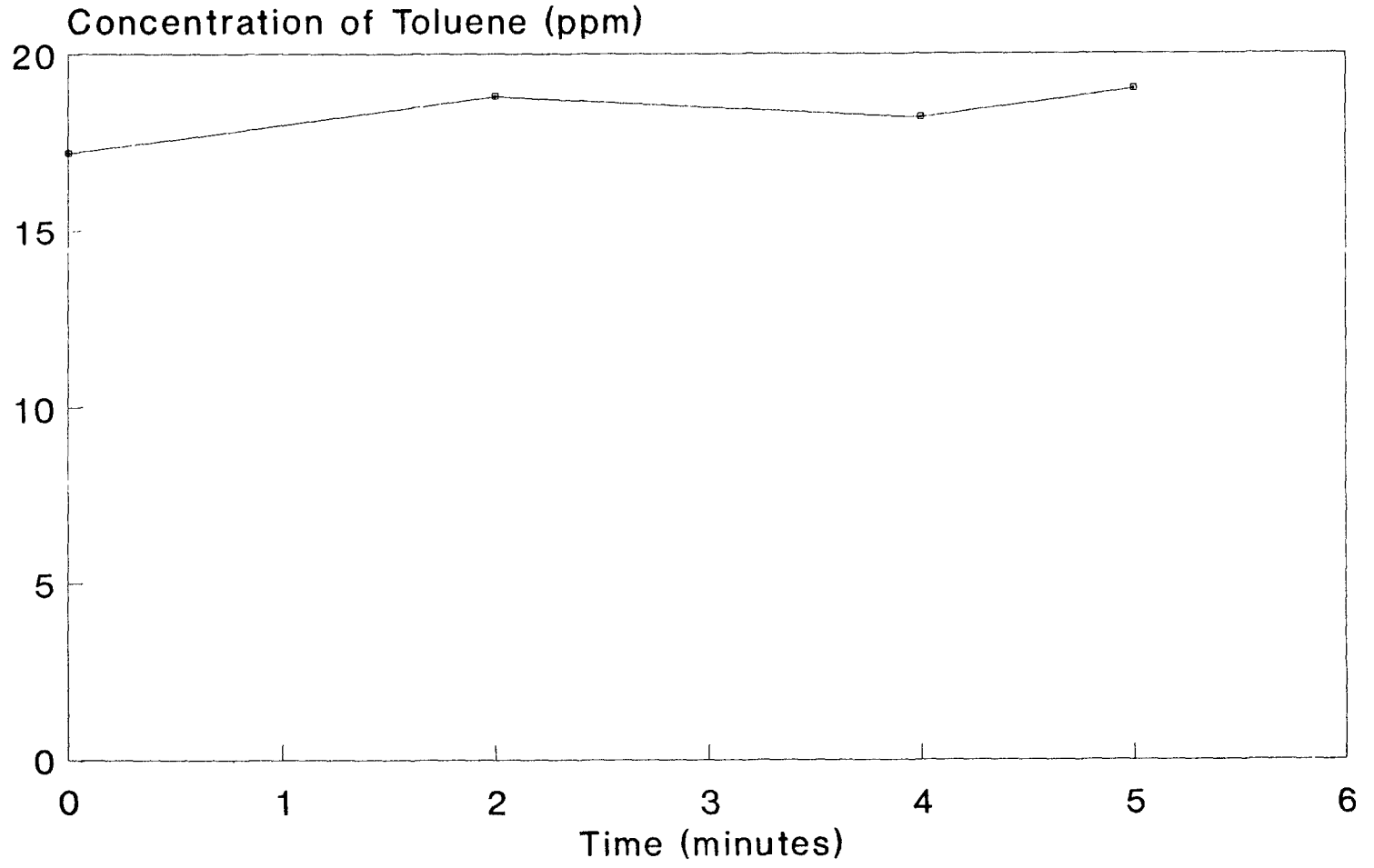


Figure 6-15

7. CONCLUSIONS

7.1 Conclusions of Hexachlorobenzene Study

Based on the analyses of the incubation system of bottles containing HCB, no biodegradation of HCB was observed. This is apparent from the absence of degradation products and the control bottles containing sterilized sludge and HCB. Evidence confirming this observation is shown in Figures 6-1, 6-3, and 6-4. There is a similar relationship between the activated sludge plus HCB bottles and the sterilized sludge plus HCB bottles. This suggests no biodegradation of HCB because the sterilized sludge plus HCB bottles contain no active microorganisms capable of utilizing HCB as a substrate. If any biodegradation would have occurred, there would have been a decrease in the amount of HCB over the 56 day incubation period in the activated sludge plus HCB bottles, whereas the amount of HCB would have remained the same in the sterilized sludge plus HCB bottles.

Regarding the possibility that autoclaving the sludge and HCB simulataneously resulted in some form of chemical breakdown of HCB in the sterilized sludge plus HCB bottles, analysis has disproven this possibility. This hypothesis would have accounted for the difference in the activated and sterilized sludge plus HCB bottles from day 0 through day 42. However, when further extraction was completed on the bottles of day 56, the recovery efficiency of the sterilized

sludge plus HCB bottle increased almost 30 percentage points. This seems to suggest that autoclaving had no adverse affect on HCB. If it would have, the recovery efficiency would have been expected to remain the same.

HCB becoming more tightly bound to the sludge particles with increased incubation time was one possible solution to the question of why the recovery efficiencies of HCB were decreasing with increased incubation time. However, it was discovered via extraction of the butyl rubber stoppers used to seal the serum bottles that HCB was absorbed into the stoppers. After adding the amounts of HCB in the stoppers and in the bottles containing the sludge together, the total amount of HCB recovered was obtained. This is evident from Table 6-1 and Table 6-3. Inspection of the tables reveals that after combining the amounts in the stoppers and sludge that the recovery efficiencies of day 56 are similar to what they were at day 0. This discovery of HCB absorbing into the stoppers accounts for why less HCB was recovered from the bottles with increased time and not that HCB is more tightly bound to the sludge particles with increased incubation time. Nonetheless, the possibility of HCB being more tightly bound to the sludge particles is not completely abandoned. It is well-known that suspended solids in water adsorb hydrophobic pollutants (Ekelund *et al.*, 1987), and HCB is hydrophobic.

In an effort to increase the extraction efficiency of HCB from the bottles containing the sludge, an additional

step was added to the original extraction procedure and completed on day 56 bottles. As stated above, this modified extraction procedure increased the extraction efficiency of the sterilized sludge plus HCB bottle almost 30 percentage points. However, the activated sludge plus HCB bottle remained about the same. Therefore, the extraction of HCB from the sludge has proven to be a very critical component of analysis.

7.2 Conclusions of Pentachlorobenzene Study

Based on the analytical analysis of the incubation system of bottles containing QCB, no biodegradation of QCB was observed. This is apparent from the absence of degradation products and the control bottles containing sterilized sludge and QCB. Evidence confirming this observation is shown in Figures 6-5 and 6-6. Again there is a similar relationship between the activated sludge plus QCB bottles and the sterilized sludge plus QCB bottles.

The butyl rubber stoppers used to seal these bottles were not analyzed. However, it is assumed that the QCB was also absorbed into the stoppers as in the case of HCB. This assumption is based on the similarity of the QCB and HCB results and results from the longest incubated bottles which show QCB being absorbed into the stopper. As seen with the HCB system, the recovery efficiences of QCB decrease with increasing incubation time. This suggests that the QCB has also absorbed into the stoppers. In addition, the QCB may

be more tightly bound to the sludge particles due to the hydrophobic nature of QCB.

7.3 Conclusions of 1,2,3,4-Tetrachlorobenzene Study

Once again, no biodegradation of 1,2,3,4-TetCB was observed based on the analytical analysis of the incubation system of bottles. This is apparent from the absence of degradation products and the control bottles containing sterilized sludge and 1,2,3,4-TetCB. Evidence confirming this observation is shown in Figures 6-7, 6-9, and 6-10. Again, there is a similar trend between the activated sludge plus 1,2,3,4-TetCB bottles and the sterilized sludge plus 1,2,3,4-TetCB bottles.

Analysis of the butyl rubber stoppers used to seal the bottles revealed the same trend as the HCB stoppers. It was discovered that the 1,2,3,4-TetCB does absorb into the stoppers with increased incubation time. In addition, 1,2,3,4-TetCB is also hydrophobic.

Again, an additional step to the extraction procedure was added in an effort to increase extraction efficiency. Nevertheless, there was a decrease in extraction efficiency with increased incubation time indicating that the compounds are either getting more difficult to extract or that the compounds are going somewhere else. One possibility is that the compounds are going straight through the stopper into the atmosphere in addition to being absorbed into the stopper. This may account for the the trend seen in Table

Table 7-1 Comparison of the Amounts Extracted of each of the Compounds

	Time (days)	mg Extracted
HCB		
Activated sludge	77	0.76
Sterilized sludge	77	0.58
QCB		
Activated sludge	56	0.54
Sterilized sludge	56	0.56
1,2,3,4-TetCB		
Activated sludge	70	0.01
Sterilized sludge	70	0.42

7-1. Notice how the concentration of compound recovered from each system decreases as the degree of chlorination decreases. This is based on the volatilities of each compound. The higher the degree of chlorination, the lower the volatility. Therefore, HCB which is the least volatile of the three had the highest concentration recovered because less went out through the stopper. On the other hand, 1,2,3,4-TetCB has the highest volatility of the three and the lowest concentration recovered suggesting that most of it went into the stopper and out into the atmosphere.

7.4 Conclusions of the Longest Incubated Systems

After 98 days, there appears to be no biodegradation of HCB, QCB, or 1,2,3,4-TetCB based on the absence of degradation products as seen in Figure 6-11. Sterilized sludge plus compound controls of these bottles were not

completed. However, it is assumed that similar trends would have been seen with these sterilized sludge plus compound bottles as the groups described above.

As seen with HCB and 1,2,3,4-TetCB, the compounds did go into the butyl rubber stoppers. In addition, that same trend of volatility can be seen in Table 6-18.

7.5 Summary of Investigations

No microbial biodegradation occurred in the incubation systems of HCB, QCB, and 1,2,3,4-TetCB and longest incubated systems under anaerobic conditions utilizing anaerobic digested sewage sludge from the Livingston Water Pollution Control Facility. This may be the result of the sludge being residential rather than industrial, thereby having a microbial population not selected for toxic compound degradation. It may be the result of the low bioavailability of these compounds. Hydrophobic pollutants deposited in sediments usually have a low bioavailability (Ekelund *et al.*, 1987). HCB has been reported to be resistant to hydrolysis and biodegradation (USEPA (1985a)), and resistant to biodegradation by soil microbes (Hamdy, 1988). It may be due to the conditions of which the bottles were subject. Perhaps they were lacking essential nutrients. It may also be due to a very long acclimation period by the microbes present in the bottles.

Based on the results obtained from this investigation, several parameters of this procedure must be altered. To

begin with, teflon-lined rubber stoppers would be the best choice to seal the bottles. This would ensure that the compounds do not absorb and possibly pass through the stoppers. A modified procedural set up would also be suggested. Sacrificing the entire bottle for analysis required many different bottles being set up. Also, based on the results, the inconsistencies in recovery efficiencies in some of the incubation systems may be directly related to this. It may be possible that each individual bottle reacted differently to the conditions. Perhaps, when the sterilized sludge plus compound bottles were autoclaved they all reacted differently. This would account for the differences from day to day between the same series of bottles. Nonetheless, a system of sampling from the same bottle in some way may serve to be better, more consistent, and reliable.

In addition, perhaps the most important component to the whole study is the extraction procedure. It is crucial that an extraction procedure be developed which produces reproducible results and maximum extraction efficiency. This will involve an entire analysis of just extraction procedure.

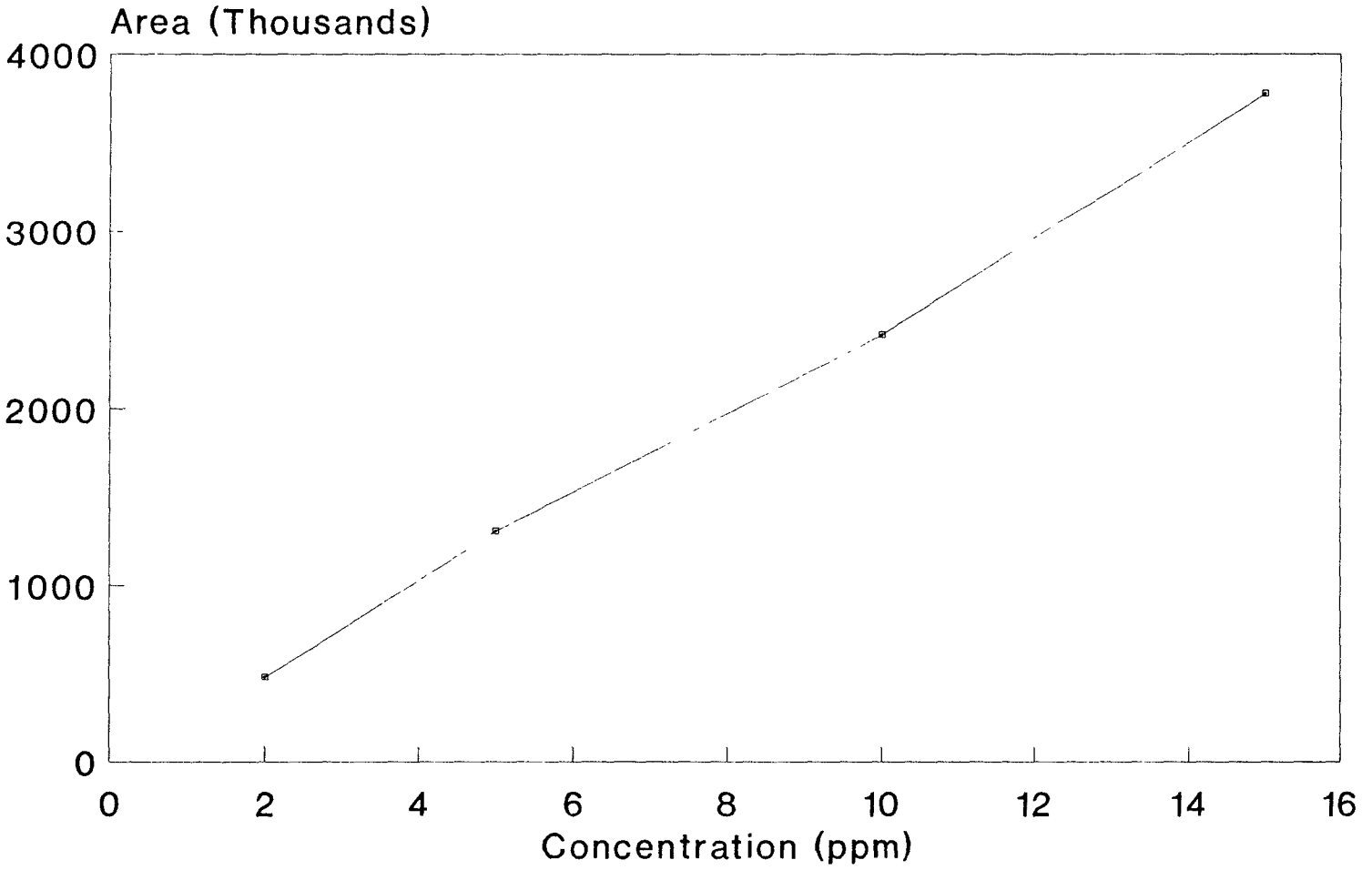
APPENDIX

APPENDIX A

Hexachlorobenzene Calibration Data

HCB (ppm)	Area
2	472280
5	1305080
10	2417448
15	3779502

Hexachlorobenzene Calibration Curve

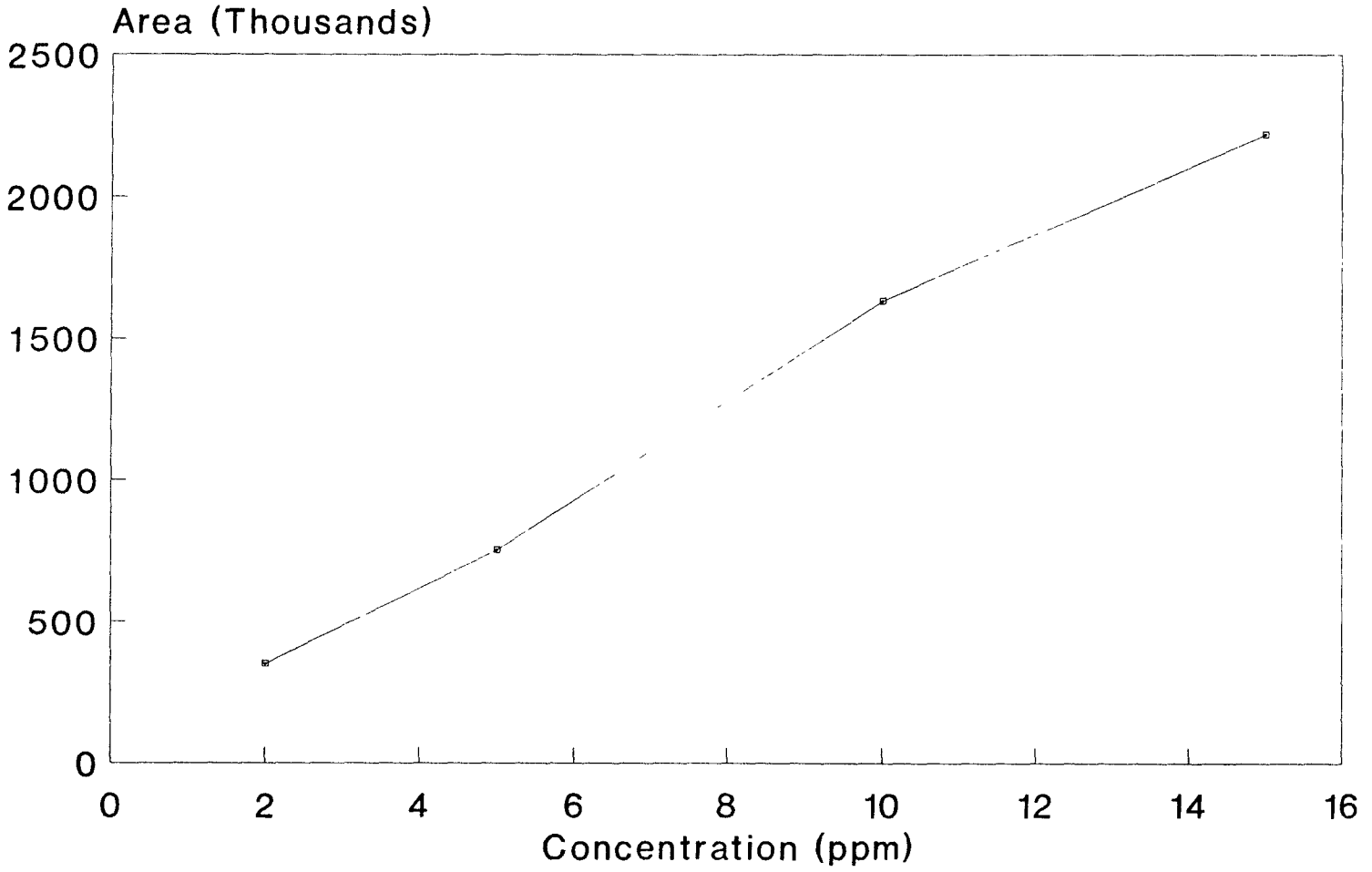


APPENDIX B

Pentachlorobenzene Calibration Data

QCB (ppm)	Area
2	350121
5	751754
10	1630824
15	2220838

Pentachlorobenzene Calibration Curve

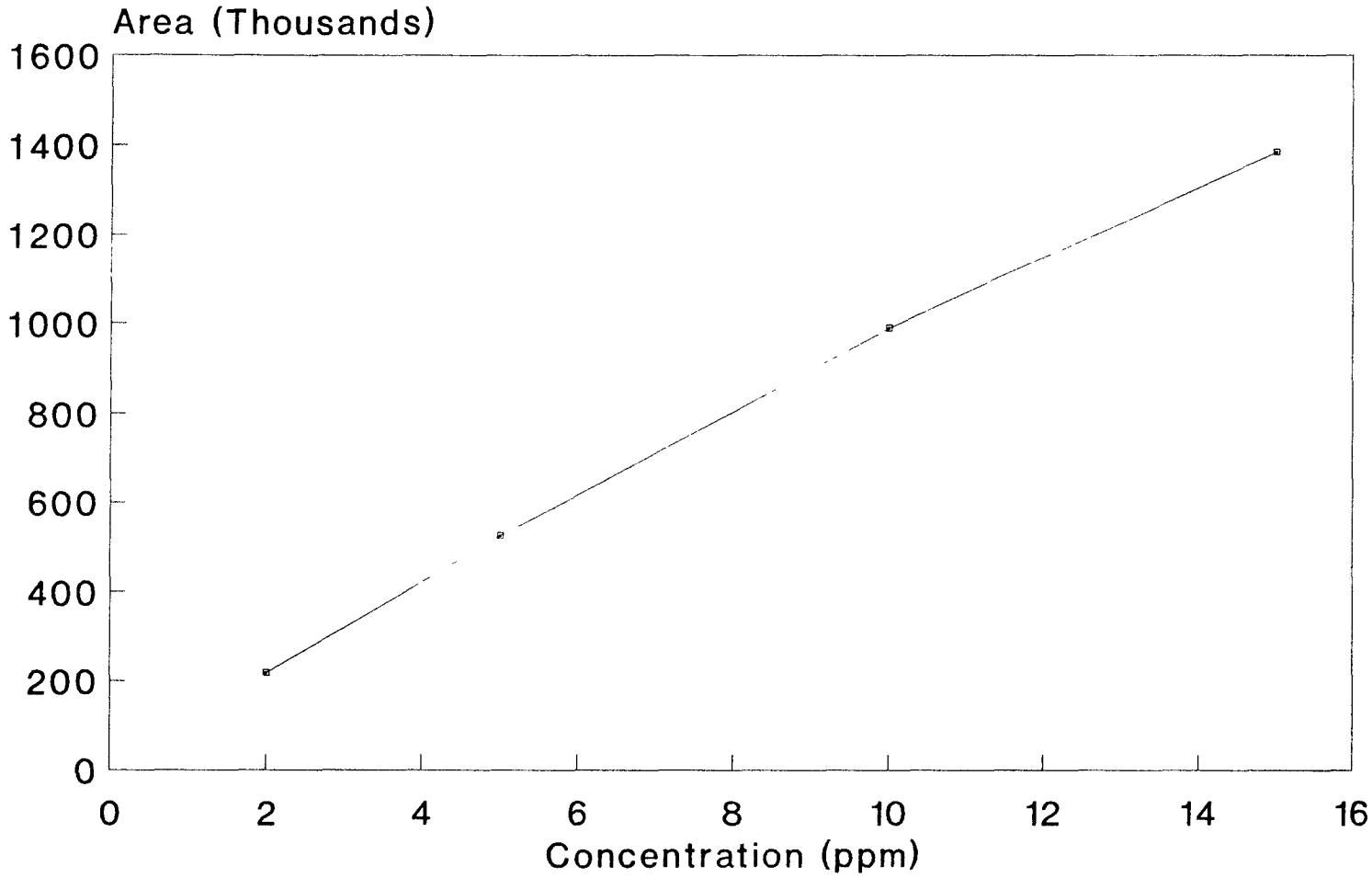


APPENDIX C

1,2,3,4-Tetrachlorobenzene Calibration Data

1,2,3,4-TetCB (ppm)	Area
2	217223
5	524380
10	989311
15	1384276

1,2,3,4-Tetrachlorobenzene Calibration Curve



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