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BIODEGRADATION AND BIOREMEDIATION OF HEXACHLOROCYCLOHEXANE ISOMERS, CHLORINATED ETHENES, CHLORINATED BENZENES AND BENZENE

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BIODEGRADATION AND BIOREMEDIATION
OF HEXACHLOROCYCLOHEXANE ISOMERS, CHLORINATED ETHENES,
CHLORINATED BENZENES AND BENZENE

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Engineering and Earth Science

by
Vijaikrishnah Elango
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ABSTRACT

Groundwater at an industrial site is contaminated with $\alpha$-hexachlorocyclohexane (HCH) and $\gamma$-HCH (i.e., lindane) (0.3-0.5 ppm). Other contaminants in the 1-15 ppm range include 1,2,4-trichlorobenzene (TCB), 1,2-dichlorobenzene (DCB), 1,3-DCB, 1,4-DCB, chlorobenzene (CB), benzene, trichloroethene (TCE) and $cis$-1,2-dichloroethene (cDCE). The aquifer consists of a shallow layer of soil over fractured dolomite, where most of the contaminant mass resides. The objective of this study was to compare 1) anaerobic reductive dechlorination of the polychlorinated contaminants, followed by aerobic biodegradation of the daughter products (mainly DCBs, CB, and benzene); and 2) aerobic biodegradation of $\alpha$- and $\gamma$-HCH, TCB, DCBs, CB and benzene, followed by anaerobic reduction of TCE and cDCE to ethene. Conventional wisdom suggests that sequential anaerobic and aerobic conditions are desirable for bioremediating sites contaminated by mixtures of polychlorinated organics. The results of this microcosm study suggest that a sequential aerobic and anaerobic approach may be more successful, although implementing this in the field presents some major challenges. In the dolomite microcosms incubated initially under aerobic conditions (59 days), $\alpha$- and $\gamma$-HCH were biodegraded close to the maximum contaminant level for lindane; all of the aromatic compounds were consumed; and there was partial removal of TCE and cDCE (presumptively via cometabolism). The subsequent switch to anaerobic conditions (day 101) yielded reductive dechlorination of the remaining TCE; a significant level of ethene was produced, although some cDCE and VC persisted. In contrast, sequential anaerobic (393 days) and aerobic treatment (498 days) for the dolomite microcosms was ineffective
in completely removing the aromatic compounds, α-HCH, cDCE and VC. For the soil microcosms, both treatment sequences were effective, most likely reflecting a greater abundance of the necessary microbes and electron donor in this part of the site.

In the microcosm study for an industrial site contaminated with HCH isomers (predominantly γ-HCH), we observed rapid anaerobic biodegradation of γ-HCH to benzene and chlorobenzene. The pattern and rate of activity suggested that γ-HCH may be used as a terminal electron acceptor. Using inoculum from microcosms that exhibited high rates of γ-HCH reduction, enrichment cultures were developed in groundwater from the industrial site and subsequently transferred to an anaerobic mineral medium without loss of γ-HCH dechlorination. Analysis of the enrichment culture by denaturant gradient gel electrophoresis and sequencing revealed the presence of a Desulfomicrobium sp. and several uncultured bacteria. The absence of a Dehalobacter sp. (previously reported to anaerobically biodegrade β-HCH) was verified by DNA amplification with Dehalobacter specific primers. The culture was further enriched in a sulfate free media with two different types of buffers (bicarbonate and HEPES) and γ-HCH as the only terminal electron acceptor and hydrogen as electron donor. Electron balance calculations in the bicarbonate buffered enrichment cultures revealed that only a small fraction of the hydrogen was involved in γ-HCH dechlorination; most was consumed for acetogenesis. Based on the fraction of electron equivalents used for γ-HCH dechlorination in HEPES-buffered enrichment culture and the ability to transfer this culture with γ-HCH as the sole terminal electron acceptor, this study is the first to demonstrate chlororespiration of γ-HCH. Molecular analysis of enrichment cultures (in bicarbonate and HEPES buffered
medium) in this study did not provide sufficient information to associate a specific microbe with chlororespiration of γ-HCH. The development of γ-HCH dechlorinating culture in this study will improve our understanding of remediation of γ-HCH by natural attenuation and engineered approaches. However, γ-HCH dechlorination will have to be coupled with improvements in anaerobic bioremediation of the terminal dechlorination products, benzene and chlorobenzene, or aerobic treatment of dechlorination products that would ensure that the endpoint is environmentally acceptable.

Using inoculum from a microcosm that exhibited aerobic transformation of cis-dichloroethene (cDCE) and trichloroethene (TCE) commensurate with biodegradation of the monoaromatic compounds, enrichment cultures were developed in groundwater by providing benzene, chlorobenzene, dichlorobenzene isomers and 1,2,4-trichlorobenzene as carbon and energy sources. These enrichments were subsequently transferred to a mineral salt medium and were grown on each of the monoaromatic compounds separately and were successfully maintained through several transfers. Isolates growing on benzene, chlorobenzene, 1,2-dichlorobenzene and 1,3-dichlorobenzene were identified as *Rhodococcus*, *Ralstonia*, *Variorax*, and *Ralstonia*, respectively, by their 16S rRNA gene sequences. The yield measured on the isolates growing on corresponding substrates ranged from 0.36-0.45 mg biomass/mg substrate, with highest yield on benzene and lowest yield on 1,3-dichlorobenzene. Cometabolic transformation of cDCE and TCE evaluated based on pseudo-first order cometabolic degradation rate constant, transformation capacity and transformation yield for resting cells were observed to be on the low end of the reported values on phenol or toluene grown isolates. Cometabolic
transformation of cDCE and TCE was also evaluated with growing cells of each isolate and were observed to be on low end of reported values for TCE and this is the first study to report for cDCE. In general, the cometabolic transformation parameters observed for cDCE were greater than TCE. The results of this study confirm the potential for cometabolism of cDCE and TCE during aerobic growth on benzene, CB, 1,2-DCB and 1,3-DCB. This is especially relevant for natural attenuation scenarios when these compounds occur as co-contaminants and may be transported from an anaerobic to an aerobic environment. Although it may seem reasonable to assume that all aerobically biodegradable monoaromatic compounds may serve as primary substrates for cDCE and TCE cometabolism, the wide range of dioxygenases involved indicates this is not necessarily the case.

The major findings from this dissertation are; (i) aerobic/anaerobic treatment should be considered as an alternative to anaerobic/aerobic treatment for bioremediation of complex mixtures of chlorinated contaminants, (ii) chlororespiration of γ-HCH was demonstrated for the first time and this improves the opportunities for in situ bioremediation, and (iii) aerobic cometabolism of cDCE and TCE with aromatic contaminants helps to explain the potential pathways for natural attenuation of complex mixtures.
DEDICATION

This dissertation is dedicated to my father, Elango Veeraswamy, my mother, Mayavathi Elango, my wife, Bagya and my brother Rajarajan.
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TABLE OF CONTENTS

TITLE PAGE .................................................................................................................................................. i
ABSTRACT .................................................................................................................................................... ii
DEDICATION .................................................................................................................................................. vi
ACKNOWLEDGEMENTS ................................................................................................................................. vii
LIST OF TABLES ........................................................................................................................................... xi
LIST OF FIGURES .......................................................................................................................................... xi

1. INTRODUCTION ........................................................................................................................................ 1
   1.1 BACKGROUND ......................................................................................................................................... 1
   1.2 HEXACHLOROCYCLOHEXANE ISOMERS and LINDANE ............................................................................. 2
   1.3 CHLORINATED ETHENES ......................................................................................................................... 7
   1.4 BENZENE and CHLORINATED BENZENES ............................................................................................... 10
   1.5 OPPORTUNITIES for BIOREMEDIATION at a SPECIFIC SITE .............................................................. 12
   1.6 OBJECTIVES ........................................................................................................................................... 14
   1.7 REFERENCES .......................................................................................................................................... 16

2. BIOREMEDIATION OF HEXACHLOROCYCLOHEXANE ISOMERS, CHLORINATED BENZENES AND
   CHLORINATED ETHENES IN SOIL AND FRACTURED DOLOMITE ........................................................... 43
   2.1 ABSTRACT ............................................................................................................................................. 43
   2.2 INTRODUCTION ..................................................................................................................................... 44
   2.3 MATERIALS AND METHODS ............................................................................................................... 47
   2.4 RESULTS ................................................................................................................................................ 55
   2.5 DISCUSSION ......................................................................................................................................... 65
   2.6 REFERENCES ........................................................................................................................................ 71

3. USE OF γ-HEXACHLOROCYCLOHEXANE AS A TERMINAL ELECTRON ACCEPTOR BY ANAEROBIC
   ENRICHMENT CULTURES ......................................................................................................................... 88
   3.1 ABSTRACT ............................................................................................................................................. 88
   3.2 INTRODUCTION ..................................................................................................................................... 89
   3.3 MATERIAL and METHODS .................................................................................................................... 92
   3.4 RESULTS ................................................................................................................................................ 99
   3.5 DISCUSSION ......................................................................................................................................... 104
3.6 REFERENCES ...........................................................................................................109

4. AEROBIC COMETABOLISM OF TRICHLOROETHENE AND cis-DICHLOROETHENE WITH BENZENE AND CHLORINATED BENZENES AS GROWTH SUBSTRATES.................................................................123
  4.1 ABSTRACT.............................................................................................................123
  4.2 INTRODUCTION ..................................................................................................124
  4.3 MATERIAL and METHODS ................................................................................127
  4.4 RESULTS .............................................................................................................135
  4.5 DISCUSSION .......................................................................................................138
  4.6 REFERENCES .....................................................................................................142

5. CONCLUSIONS AND RECOMMENDATION FOR FUTURE RESEARCH ......154
  5.1 CONCLUSIONS ..................................................................................................154
  5.2 RECOMMENDATIONS for FUTURE RESEARCH.................................................157

APPENDICES .............................................................................................................159
Appendix 6.1: Preparation of Calibration Standards and Analytical Results ..........160
Appendix 6.2: Supplementary Results for Chapter 2 ..................................................166
Appendix 6.3: Supplementary Results for Chapter 3 ..................................................185
Appendix 6.4: Supplementary Results for Chapter 4 ..................................................233
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Global use of HCH from 1948 to 1997 and Year of Ban&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25</td>
</tr>
<tr>
<td>1.2 Global Distribution of HCH</td>
<td>26</td>
</tr>
<tr>
<td>1.3 Health Effects and Carcinogenicity Assessment</td>
<td>27</td>
</tr>
<tr>
<td>1.4 Physical and Chemical Properties of HCH Isomers</td>
<td>28</td>
</tr>
<tr>
<td>1.5 Aerobic Cometabolism of Chlorinated Ethenes</td>
<td>29</td>
</tr>
<tr>
<td>2.1 Average Initial Contaminants Concentration for the Microcosm Study</td>
<td>77</td>
</tr>
<tr>
<td>2.2 Incubation Times and Average Percent Removals for Killed Controls and As-Is Treatments</td>
<td>78</td>
</tr>
<tr>
<td>3.1 Mass Balance between Hydrogen Consumed and Benzene and CB Produced from γ-HCH Dechlorination</td>
<td>117</td>
</tr>
<tr>
<td>3.2 16S rRNA Sequencing Results for Bands Excised from the DGGE Gel Shown in Figure 3.4</td>
<td>118</td>
</tr>
<tr>
<td>4.1 Identification of Isolates and Yields</td>
<td>148</td>
</tr>
<tr>
<td>4.2 Cometabolic Transformation of cDCE and TCE by Resting Cells</td>
<td>149</td>
</tr>
<tr>
<td>4.3 Cometabolic Transformation of cDCE and TCE by Growing Cells</td>
<td>150</td>
</tr>
<tr>
<td>4.4 Dioxygenases Involved in the Metabolism of Substrates Evaluated in this Study</td>
<td>151</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Structure of HCH isomers. Axial (a) and equatorial (e) position of the chlorine atoms are identified for each isomer. Adapted from Willet et al. (60).</td>
</tr>
<tr>
<td>1.2</td>
<td>Proposed pathway for anaerobic biodegradation of β-HCH; adapted from Middeldorp et al. (37). TeCCH = 3,4,5,6-tetrachlorocyclohexane; DCCH = dichlorocyclohexadiene; [H] = H⁺ + e⁻.</td>
</tr>
<tr>
<td>1.3</td>
<td>Proposed aerobic biodegradation pathway for γ-HCH by Sphingomonas paucimoblis UT26, adapted from Phillips et al. (43). PCCH = pentachlorocyclohexene; 2,4,5-DNOL = 2,4,5-trichloro-2,5-cyclohexadiene-1-ol; 2,5-DDOL = 2,5-dichloro-2,5-cyclohexadiene-1,4-diol; 2,5-DCP = 2,5-dichlorophenol; 2,5-DCHQ = 2,5-dichlorohydroquinone; CHQ = chlorohydroquinone; HQ = hydroquinone; HSMA = hydroxymuconic semialdehyde; [H] = H⁺ + e⁻.</td>
</tr>
<tr>
<td>1.4</td>
<td>Cometabolism of TCE via a toluene monooxygenase.</td>
</tr>
<tr>
<td>1.5</td>
<td>Cometabolism of TCE via a toluene dioxygenase; adapted from Li and Wackett (28).</td>
</tr>
<tr>
<td>1.6</td>
<td>Cometabolism of TCE via methane monooxygenase; adapted from Fox et al. (18).</td>
</tr>
<tr>
<td>1.7</td>
<td>Cometabolism of cDCE via monooxygenase.</td>
</tr>
<tr>
<td>1.8</td>
<td>Initial step during aerobic biodegradation of benzene (9).</td>
</tr>
<tr>
<td>1.9</td>
<td>Initial step during aerobic biodegradation of chlorobenzene (9).</td>
</tr>
<tr>
<td>1.10</td>
<td>Initial step during aerobic biodegradation of 1,4-dichlorobenzene (9).</td>
</tr>
<tr>
<td>1.11</td>
<td>Initial step during aerobic biodegradation of 1,2,4-trichlorobenzene (9).</td>
</tr>
</tbody>
</table>
1. 12 Potential biodegradation pathways for a complex mixture of contaminants when subjected to sequential anaerobic and then aerobic treatment. .......................41

1. 13 Potential biodegradation pathways for a complex mixture of contaminants when subjected to sequential aerobic and then anaerobic treatment. .......................42

2. 1 Experimental design for the treatments subjected to anaerobic/aerobic conditions (Sequence I) and aerobic/anaerobic conditions (Sequence II) ..........................79

2. 2 Results for soil microcosms with low initial concentrations of TCE and cDCE under as-is anaerobic conditions (a, b, c; average of three bottles) and anaerobic biostimulation with lactate followed by a switch to aerobic conditions on day 136 (d, e, f; average of six bottles) ↓=lactate addition; O2=oxygen addition . 80

2. 3 Results for soil microcosms with low initial concentrations of TCE and cDCE under as-is aerobic conditions (a, b, c; average of three bottles) and aerobic conditions followed by anaerobic biostimulation with lactate on day 137 (d, e, f; average of six bottles) ↓=lactate addition .................................................................81

2. 4 Results for dolomite microcosms with low initial concentrations of TCE and cDCE under as-is anaerobic conditions (a, b, c; average of three bottles) and anaerobic bioaugmentation followed by a switch to aerobic conditions on day 393 (d, e, f; average of three bottles) ↓=lactate addition; ★ =bioaugmentation; O2= oxygen addition ..............................................................................................82
2. 5 Results for dolomite microcosms with low initial concentrations of TCE and cDCE under as-is aerobic conditions (a, b, c; average of three bottles) and aerobic conditions followed by anaerobic bioaugmentation on day 101 (d, e, f; average of three bottles); ↓=lactate addition; ★ = bioaugmentation ..........83

2. 6 First order biodegradation rate coefficients comparing aerobic conditions for sequence I (anaerobic/aerobic) versus sequence II (aerobic/anaerobic) at (a) low and (b) high levels of TCE and cDCE. .................................................................84

2. 7 First order biodegradation rate coefficients comparing the highest aerobic rate versus the highest anaerobic rate at low and high levels of TCE for (a) soil and (b) dolomite microcosms .................................................................85

2. 8 First order biodegradation rate coefficients comparing the effect of low and high levels of TCE and cDCE in (a) soil and (b) dolomite microcosms. ...............86

2. 9 First order biodegradation rate coefficients comparing soil and dolomite microcosms under (a) anaerobic conditions from sequence I (anaerobic/aerobic) and (b) aerobic conditions from sequence II (aerobic/anaerobic). .........................87

3.1 γ-HCH dechlorination by enrichment culture I with methanol and lactate as electron donors ........................................................................................................119

3.2 γ-HCH dechlorination by enrichment culture III ............................................120

3.3 γ-HCH dechlorination by enrichment culture IV ............................................121

3.4 DGGE profile of enrichment cultures in HEPES and bicarbonate buffered media..122

4.1 Cometabolism of cDCE and TCE by resting cells ...........................................152
4.2 Cometabolism of cDCE and TCE by growing cells ..........................................................153
1. INTRODUCTION

1.1 BACKGROUND

One of the greatest challenges for remediation of hazardous waste sites is devising strategies to treat complex mixtures of contaminants. The focus of this dissertation is the development of bioremediation approaches that may be used to treat complex mixtures of contaminants, with particular attention given to following compounds:

- Hexachlorocyclohexane (HCH) isomers, including the pesticide lindane (γ-HCH);
- Chlorinated ethenes, including trichloroethene (TCE) and cis-1,2-dichloroethene (cDCE); and
- Monoaromatics, including benzene, chlorobenzene (CB), 1,2-dichlorobenzene (DCB), 1,3-DCB, 1,4-DCB and 1,2,4-trichlorobenzene (TCB).

The motivation to evaluate this particular group of contaminants is a hazardous waste site where all of these compounds co-occur. In addition to the array of chemicals present, the site geology also poses significant challenges to remediation, since it is dominated by fractured dolomite. Use of bioremediation at this site will require a thorough understanding about the biodegradation processes that govern each contaminant individually, as well as in mixtures.

Details about the hazardous waste site are presented later in the Introduction. In order to appreciate the broader context for this dissertation, information about the
environmental distribution, health effects, biodegradation pathways and properties of HCH isomers are presented first. This is followed by a description of the occurrence and biodegradation of chlorinated ethenes, and biodegradation of benzenes and chlorinated benzenes. Understanding the biodegradation of the individual compounds affords an opportunity to describe how various strategies may be employed for the hazardous waste site mentioned above. Chapter 1 then concludes with the specific objectives for this dissertation.

The following three chapters address the specific objectives. These chapters are written as independent manuscripts for publication; each includes an abstract, introduction, materials and methods, results, discussion, and references. The final chapter of this thesis presents the overall conclusions as well as recommendations for additional research. The appendices provide extensive supplemental material for Chapters 2, 3, and 4.

1.2 HEXACHLOROCYCLOHEXANE ISOMERS and LINDANE

HCH is a highly chlorinated, recalcitrant pesticide and is mainly composed of four isomers (α, β, γ, and δ). HCH is commercially available in two grades: technical HCH and lindane (once erroneously known as benzene hexachloride, BHC). The technical grade HCH is composed of α-HCH (55-80%), β-HCH (5-14%), γ-HCH (8-15%), δ-HCH (2-16%) and ε-HCH (3-4%) (26). Lindane contains more than 90% of the γ-HCH isomer and trace levels of other isomers. HCH isomers differ in the arrangement of their chlorine atoms (Figure 1.1). In α-HCH, two chlorine atoms are at the equatorial orientation and the other chlorine atoms are in the axial position, whereas γ- and δ-HCH
have three and five equatorially oriented chlorine atoms, respectively. Axial chlorine atoms are known as sites of active enzymatic biodegradation and the presence of all equatorial chlorine atoms in β-HCH makes it persistent in the environment (43).

HCH isomers are manufactured by photochlorination of benzene under UV light. Further treatment with methanol or acetic acid with fractional recrystallization yields lindane (43). Only γ-HCH is known to exhibit insecticidal properties, suggesting that lindane would be used most frequently. However, technical grade HCH has also been used worldwide over the last five decades (60). The global usage of HCH between 1948 and 1997 has been estimated at 10 million metric tons (Table 1.1). The maximum concentration of HCH reported for different media and locations are 10,000 pg/m$^3$ in air above the Bay of Bengal and Arabian sea; 1,000 pg/L in the surface waters of the Bering Sea, Gulf of Alaska, Chucki Sea and Sea of Japan; and 1,100E3 pg/g in sediment in India (58, 60) (Table 1.2).

Exposure to high concentrations of lindane results in stimulation and subsequent damage to the central nervous system, while α-, β-, and δ-HCH are known to be central nervous system depressants (Table 1.3). According to a carcinogenicity assessment by the United States Environmental Protection Agency, technical grade HCH and α-HCH are probable human carcinogens (i.e., Class B2), β-HCH is a possible human carcinogen (i.e., Class C), and data for δ-HCH are not adequate to make a determination (Table 1.3). The manufacture and use of HCH has been banned in Japan, the United States, France, China, the former Soviet Union, and India, although lindane is still being manufactured and used worldwide (29). According to the national primary drinking water regulations
of the United States Environmental Protection Agency, the maximum contaminant level (MCL) for lindane is 0.2 µg/L. HCH isomers exhibit varying physical and chemical properties (Table 1.4). Henry’s law constant is lowest for β-HCH and highest for γ-HCH. The higher Henry’s law constants and vapor pressures for α- and γ-HCH are consistent with the higher concentration of α- and γ-HCH in the atmosphere (58).

Biodegradation of lindane and other HCH isomers under anaerobic conditions has been widely reported (43) with pure cultures (10, 24, 35, 40), enrichment cultures (10, 37, 55), and soil slurries (8). Benzene and CB are the predominant end products of lindane and HCH anaerobic biodegradation (Figure 1.2). The first step involves a dihaloelimination reaction to form 3,4,5,6-tetrachlorocyclohexene. A second dihaloelimination reaction is thought to form dichlorocyclohexadiene, although this has not yet been proven. Dichlorocyclohexadiene undergoes an additional dihaloelimination reaction to form benzene or it is spontaneously converted to CB via dehydrohalogenation. Several other intermediates or end products have also been reported, including tetrachlorobenzene, TCB, dichlorobenzene isomers, tetrachlorocyclohexadiene, and pentachlorocyclohexadiene (37, 44).

The earliest reported lindane degrading anaerobic organism, Clostridium sphenoides (35), was initially thought to use lindane as a sole carbon and energy source. Later studies demonstrated that lindane is used as an electron acceptor under anaerobic conditions rather than as a carbon source (24). Studies with radiolabeled ³H/³⁶Cl demonstrated the release of ³⁶Cl but not ³H, indicating that the cyclic structure was not cleaved to release hydrogen or carbon (24) and other studies have also demonstrated the
need for an exogenous electron donor and carbon source for anaerobic biodegradation of lindane (43).

HCH isomers (α, β, γ and δ) are biodegraded under methanogenic conditions (8, 37-38, 55) and under sulfate reducing conditions, with benzene and CB as the primary daughter products (10, 46). In experiments with marine sediments collected from around burrows of Saccoglossus kowalevskii (a tribromopyrrole-producing marine hemichordate), Boyle et al. (10) demonstrated lindane biodegradation under sulfate reducing conditions with short chain fatty acids serving as the carbon and energy source. Molybdate inhibited lindane biodegradation, suggesting that sulfate reduction plays a central role in this activity. CB and benzene were detected as products. Pure cultures of Desulfovibrio gigas, Desulfovibrio africanus and Desulfococcus multivorans were also able to dehalogenate lindane to benzene and CB. Nevertheless, no attempt was made to determine if lindane was being used as a terminal electron acceptor. Also, no attempt was made to reconcile potential competition between use of sulfate and lindane as terminal electron acceptors. Although sulfate reducing bacteria clearly played an important role in lindane biodegradation in the study by Boyle et al. (10), what that role is remains to be determined.

Biodegradation of lindane (46) and α- and β-HCH (8) is inhibited under denitrifying conditions. Lindane biodegradation studies with Clostridium species also revealed the inhibitory effects of nitrate reducing conditions, while the presence of sulfate did not inhibit lindane biodegradation (46). The cause of this inhibition is not well understood, although it is consistent with the absence of reductive dechlorination of other
polychlorinated organic compounds under denitrifying conditions, including chlorinated ethenes. The elevated redox level associated with nitrate as a terminal electron acceptor may be the main factor restricting reductive dechlorination reactions.

Under aerobic conditions, α-, γ-, β- and δ-HCH are biodegraded but only lindane and α-HCH are known to serve as sole sources of carbon and energy (Figure 1.3). The aerobic biodegradation of HCH isomers is well documented with respect to the microbes *Sphingomonas paucimobilis* UT26 (39) and *Sphingomonas paucimobilis* strain B90A (49). According to Philips et al. (43), the biodegradation pathway for strains UT26 and B90A begins with two dehydrochlorination reactions to form 1,3,4,6-tetrachlorocyclohexene via γ-pentachlorocyclohexene, followed by two hydrolytic dechlorination reactions to form 2,5-dichloro-2,5-cyclohexadiene-1,4-diol via 2,4,5-trichloro-2,5-cyclohexadiene-1-ol (Figure 1.3). The 2,5-dichloro-2,5-cyclohexadiene-1,4-diol undergoes dehydrogenation to form 2,5-dichlorohydroquinone, followed by two reductive dechlorination reactions to form hydroquinone. The transformations up to this point do not involve oxygen as a reactant. Cleavage of the hydroquinone ring is mediated by a dioxygenase, producing maleylacetate as a product, which enters central metabolic pathways. Since oxygen is not used as a reactant during lindane biodegradation until the formation of hydroquinone, it is possible that these reactions also occur under anaerobic conditions.

Variations in the biodegradation pathway described above includes spontaneous elimination of HCl from 1,3,4,6-tetrachlorocyclohexene and 2,4,5-trichloro-2,5-cyclohexadiene-1-ol to from 1,2,4-TCB and 2,5-dichlorophenol, respectively, which are
also biodegraded under aerobic conditions. Although strains UT26 and B90A appear to use similar biodegradation pathways for lindane, they differ in their rates of biodegradation and also in their abilities to aerobically biodegrade other HCH isomers (25).

1.3 CHLORINATED ETHENES

TCE is mainly used as a solvent for degreasing metals and also as a constituent in adhesives, paint removers, and spot removers (7). cDCE does not have significant industrial applications (7). TCE has entered the environment through various pathways, including improper disposal practices and leaking storage tanks. TCE is also found in anaerobic environments as a reductive dechlorination product from tetrachloroethene. The presence of cDCE in the environment is predominantly due to reductive dechlorination of TCE. According to the United States Environmental Protection Agency, the MCL for TCE and cDCE are 5 µg/L and 70 µg/L, respectively (52). Health issues associated with exposure to TCE include an increased risk of cancer and liver problems, while exposure to cDCE is associated with liver problems. Out of 1430 sites on the National Priority List, TCE and cDCE are found at 852 and 146 sites, respectively (7).

TCE and cDCE are known to biodegrade aerobically via cometabolism, with primary substrates that include several monoaromatic compounds (5, 22). cDCE may also be degraded aerobically as a sole substrate (11, 14). Studies with numerous pure and mixed cultures have demonstrated aerobic cometabolism of TCE using a wide range of growth substrates for which metabolism induces oxygenase activity, including toluene,
phenol, methane, butane, propane, propene and ammonia (5-6). Of these growth substrates, toluene and methane are among the most intensively evaluated. Cometabolic transformations are defined as reactions that do not yield the energy or carbon needed for cell growth (30). Consequently, cometabolic transformation of chlorinated ethenes relies on microbial metabolism of a growth substrate that induces the activity of nonspecific oxygenases.

Aerobic oxidation of toluene and cometabolic transformation of TCE is supported by three types of toluene-monooxygenases (Figure 1.4) and one toluene-dioxygenase (Figure 1.5), depending on the microorganism used. The three types of monooxygenase enzymes and corresponding organisms that support oxidation of toluene and cometabolic transformation of TCE are toluene 2-monooxygenase in *Burkholderia cepacia* G4 (17), toluene 4-monooxygenase in *Pseudomonas mendocina* KR1 (27) and toluene 3-monooxygenase in *Ralstonia pickettii* PKO1 (27). *Pseudomonas putida* F1 (57) uses a dioxygenase enzyme to oxidize toluene and transform TCE by cometabolic reactions. Aerobic cometabolic transformation of TCE with methane as the growth substrate is catalyzed by soluble methane monooxygenase (sMMO) (Figure 1.6) and particulate methane monooxygenase (pMMO) (32, 50). *Methylosinus trichosporium* OB3b is perhaps the best characterized methanotroph that supports aerobic transformation of TCE (42). Several mixed and enrichment culture studies (3, 5, 16) have successfully demonstrated the aerobic cometabolic transformation of TCE with methane serving as the growth substrate. In addition to strain OB3b, other methanotrophs that support TCE cometabolism include strains 46-1 (30) and CAC1 (12). Aerobic cometabolic
transformation of TCE, based on a pseudo-first order cometabolic degradation rate constant ($k_1$), was faster in studies with sMMO in strain OB3b than with pMMO (5).

Numerous microbes that grow on toluene, phenol, and methane, and support TCE cometabolism are also known to transform cDCE; representative strains are shown in Table 1.5. cDCE epoxide was identified as a transient intermediate during aerobic cometabolism of cDCE by methane-grown strain OB3b when sMMO activity was induced (56). Although cDCE-epoxide was observed during cDCE transformation by strain OB3b, less is known about the end products. Overall, the pathways for cDCE cometabolism are less well understood than those for TCE transformation (Figure 1.7). Aerobic cometabolism of cDCE was also demonstrated with methane grown strain CAC1 (12) and toluene grown strain F1 (57), although the cDCE transformation pathway was not identified.

The effectiveness of a compound to serve as growth substrate for cometabolic transformation of chlorinated ethenes is commonly evaluated based on its transformation capacity ($T_c$) and transformation yield ($T_y$) (4). $T_c$ is defined as the mass ratio of non-growth substrate removed (e.g., TCE and cDCE) to mass of resting cells grown on the primary substrate (e.g., monoaromatic compounds), while $T_y$ is the mass ratio of non-growth substrate removed to growth substrate consumed (4).

Complete biodegradation of TCE and cDCE to ethene is possible anaerobically (5, 22). During reductive dechlorination of the compounds, replacement of one or more chlorine atoms with hydrogen atoms is coupled to energy conservation by formation of ATP and the process is called chlororespiration (36). Several chlorinated compounds,
including PCE, TCE, polychlorinated biphenyls, chlorinated phenols, 1,2,4-TCB, and CB are known to be used as terminal electron acceptors during chlororespiration (1-2, 23, 36). In mixed cultures, chlororespiration of various compounds was demonstrated based on the fraction of electron equivalents from the electron donor used for reductive dechlorination ($f_e$); $f_e$ values of 0.6-0.7 were indicative of chlororespiration, while much lower values indicated that dechlorination was cometabolic (31). Biodegradation of HCH involves a series of steps whereby chlorine atoms are replaced by hydrogen atoms (37). However, none of the studies reviewed have demonstrated the use of $\gamma$-HCH isomers via chlororespiration and only one study demonstrated the use of $\beta$-HCH as terminal electron acceptor via chlororespiration (10, 37, 43, 54). An extensive review of the literature indicates that reductive dechlorination of $\gamma$-HCH has not yet been linked to chlororespiration and microbes with such capability have not been identified.

1.4 BENZENE and CHLORINATED BENZENES

Benzene is a part of crude oil, gasoline, cigarette smoke, and industrial applications, including use in manufacturing plastics, nylon, synthetic fibers, rubbers, lubricants and pesticides (7). Benzene enters the environment through industrial discharges, disposal of products containing benzene, gasoline leaks from underground storage tanks, among others. Benzene is classified as class A human carcinogen and its MCL is 5 $\mu$g/L. CB is mainly used as degreasing solvent in automobile parts, manufacture of pesticides and as a chemical intermediate in the manufacture of organic chemicals (7). Exposure to CB leads to headaches, nausea, sleepiness, numbness and
vomiting in humans. Animal studies have demonstrated that the liver, kidney and central nervous system are affected by exposure to CB. The MCL for CB is 100 µg/L.

DCB isomers are not found naturally and are mainly used in the production of herbicides, insecticides, medicine and dyes (7). Exposure to DCB isomers can result in breathing difficulties, irritation of the eyes and skin, headaches and liver problems. Only 1,4-DCB is regulated in drinking water, having an MCL of 75 µg/L. 1,2,4-TCB is used as a dye carrier, degreaser, lubricant, and a solvent in chemical manufacturing. Exposure to 1,2,4-TCB is mainly associated with affects on the adrenal glands; its MCL is 70 µg/L (7).

Benzene, CB, DCB isomers, and 1,2,4-TCB are readily biodegraded under aerobic conditions, serving as sole carbon and energy sources for numerous types of microbes (Figures 1.8-1.11) (15, 21, 45). It is noteworthy that oxygenase enzymes play a key role in ring fission of these aromatic compounds, since some oxygenases also catalyze cometabolic oxidation of chlorinated ethenes such as TCE and cDCE. Benzene, CB, DCB isomers and 1,2,4-TCB are converted to their corresponding catechol isomers by reactions catalyzed by dioxygenases and dehydrogenases (20, 48, 53, 59, 61), which is similar to the toluene 1,2-dioxygenase catalyzed aerobic mineralization of toluene and cometabolic transformation of TCE by Pseudomonas putida (47, 57). It is possible that the dioxygenase enzymes involved in the biodegradation of benzene, CB, DCB isomers and 1,2,4-TCB can catalyze aerobic cometabolism of TCE and cDCE when they are present as co-contaminants. However, no information was found in the literature that
specifically tested these compounds as growth substrates for aerobic cometabolism of chlorinated ethenes.

Under anaerobic conditions, 1,2,4-TCB is dechlorinated to dichlorobenzene isomers via chlororespiration by *Dehalococcoides* strain CBDB1 (2). Enrichment culture studies have demonstrated the anaerobic dechlorination of dichlorobenzene isomers and CB to benzene by a growth linked process but no pure culture has been isolated (19). Anaerobic biodegradation of benzene is not widely observed but a few studies have demonstrated the anaerobic oxidation of benzene with nitrate, sulfate and Fe (III) serving as terminal electron acceptors (13, 33-34).

### 1.5 OPPORTUNITIES for BIOREMEDIATION at a SPECIFIC SITE

The co-occurrence of contaminants at hazardous waste sites is the norm rather than the exception. When the mixtures include chemicals with widely differing properties it increases the difficulty of achieving cleanup. The co-occurrence of HCH isomers, chlorinated ethenes and monoaromatic compounds (including benzene and chlorinated benzenes) is an example of such a complex mixture. The frequency with which this particular combination occurs at hazardous waste sites is not known. However, it is a problem for at least one major industrial site located in the northeastern U.S., where a plume of TCE and cDCE intersected with a plume of HCH isomers, chlorinated benzenes, and benzene.

Most polychlorinated organic compounds, including HCH isomers, chlorinated ethenes, and chlorinated benzenes are subject to biodegradation under anaerobic conditions via reductive dechlorination. The kinetics of dechlorination tend to decrease
as more and more of the chlorine atoms are removed, raising the possibility that the lesser chlorinated daughter products may persist under anaerobic conditions. By contrast, organic compounds with one or two chlorine atoms generally undergo aerobic biodegradation quite readily, while more highly chlorinated compounds tend to be recalcitrant in the presence of oxygen. For this reason, complete bioremediation of many polychlorinated organic compounds is favored when redox conditions transition from anaerobic to aerobic (Figure 1.12). However, some compounds pose interesting exceptions. HCH isomers, for example, undergo biodegradation both aerobically and anaerobically, although via different pathways and with very different products (Figures 1.2 and 1.3). Benzene and CB are the predominant anaerobic daughter products, neither of which is an acceptable endpoint. Tri- and dichlorobenzenes also undergo biodegradation aerobically and anaerobically, although CB tends to be the terminal daughter product under anaerobic conditions. Both CB and benzene are readily biodegradable aerobically. TCE and cDCE undergo reductive dechlorination anaerobically, although both are known to be aerobically biodegraded via cometabolism, with primary substrates that include several monoaromatic compounds.

With a mixture of HCH isomers, benzenes, and chlorinated ethenes, sequential anaerobic and aerobic treatment would appear to be feasible. However, a significant potential drawback is the transient accumulation of benzene and CB from the HCH isomers, as well as from the di- and tri-chlorobenzenes. An alternative approach to avoid this concern would be to use sequential aerobic and anaerobic conditions (Figure 1.13). Under aerobic conditions, biodegradation kinetics may favor removal of the HCH
isomers and chlorinated benzenes without accumulation of hazardous daughter products. This would leave the chlorinated ethenes for removal via anaerobic reductive dechlorination, which is rapidly becoming a well-established in situ process. However, as will be described in the Chapter 2, no reports were found for field application of sequential aerobic and anaerobic treatment for a complex mixture of contaminants. Anaerobic biodegradation of γ-HCH isomers was observed in the microcosm study presented in Chapter 2 and, as described earlier, little is known about the use of γ-HCH as a terminal electron acceptor via chlororespiration. Also, aerobic transformation of cDCE and TCE was observed in our study in Chapter 2. Aerobic transformation of cDCE and TCE is known to occur by cometabolic reactions catalyzed by oxygenase enzymes induced by oxidation of a growth substrate. All of the aromatic contaminants that were part of contaminant mixture used in the study in Chapter 2 are known to induce oxygenase enzyme. However, the role of these aromatic contaminants in aerobic cometabolism of TCE or cDCE has not previously been reported.

1.6 OBJECTIVES

In order to address some of the issues raised in the preceding sections, three types of investigations were conducted. The first, covered in Chapter 2, was a microcosm study that was designed to compare a novel bioremediation strategy, i.e., sequential aerobic treatment followed by anaerobic treatment. The specific objective was to compare anaerobic reductive dechlorination of the contaminant mixture followed by aerobic biodegradation of the daughter products, versus aerobic biodegradation of α- and γ-HCH, TCB, DCBs, CB and benzene followed by anaerobic reduction of TCE and
cDCE to ethene. The comparison was made using microcosms prepared with soil, crushed dolomite, and groundwater from the industrial site mentioned above. The hypothesis for this investigation was: The novel approach of aerobic/anaerobic treatment will be preferable for this type of mixture based on the expected faster kinetics for mineralizing chlorinated benzenes and HCH, compared to reductive dechlorination under anaerobic conditions. After aerobic treatment, only TCE and cDCE are likely to persist and require bioremediation via reductive dechlorination.

As mentioned above, results from the microcosm study led to two additional investigations. In Chapter 3, the process of γ-HCH reductive dechlorination was investigated in detail. The specific objectives were to determine if γ-HCH can be used as a terminal electron acceptor via chlororespiration and to identify the microbes involved in this process. The hypothesis was that γ-HCH will join the growing list of chlorinated organic compounds that are known to serve as terminal electron acceptors. Previous studies have demonstrated that γ-HCH can be rapidly dechlorinated, but have fallen short of directly linking the dechlorination process to chlororespiration.

In Chapter 4, the potential for aerobic cometabolism of TCE and cDCE being linked to aromatic contaminants found at the hazardous waste site was investigated. The specific objectives were (i) to isolate and identify microbes that grow on benzene, CB, DCB isomers and 1,2,4-TCB, using as inoculum the aerobic microcosms described in Chapter 2; and (ii) to evaluate the ability of the isolates to cometabolize cDCE and TCE, with both resting cells grown on the various monoaromatic substrates and with cells that were actively consuming the monoaromatic compounds. The hypothesis was: Since
several monoaromatic compounds have been shown to serve as growth substrates that support aerobic cometabolism (e.g., toluene and phenol) via aromatic oxygenases, the aromatic compounds relevant to this research will also serve as primary substrates since their metabolic pathways also involves aromatic oxygenases.

1.7 REFERENCES


35. **MacRae, I. C., K. Raghu, and E. M. Bautista.** 1969. Anaerobic biodegradation of the insecticide lindane by *Clostridium* sp. Nature **221**:859-860.


Table 1.1 Global use of HCH from 1948 to 1997 and Year of Ban$^a$

<table>
<thead>
<tr>
<th>Country</th>
<th>Usage (Kt)</th>
<th>Year of Ban</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td>4,464</td>
<td>1983</td>
</tr>
<tr>
<td>India</td>
<td>1,057</td>
<td>1990</td>
</tr>
<tr>
<td>Former Soviet Union</td>
<td>693</td>
<td>1990</td>
</tr>
<tr>
<td>France</td>
<td>520</td>
<td>1988</td>
</tr>
<tr>
<td>Japan</td>
<td>400</td>
<td>1972</td>
</tr>
<tr>
<td>United States of America</td>
<td>343</td>
<td>1976</td>
</tr>
<tr>
<td>Total Global Usage</td>
<td>10,000</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Adapted from Li (29)
Table 1.2 Global Distribution of HCH

<table>
<thead>
<tr>
<th>Medium</th>
<th>Concentration</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>α- HCH 3.6 to 1,021 pg/m³</td>
<td>Northwest Pacific Ocean, Antarctica, Green Bay, Central Ontario, etc.</td>
<td>(58)</td>
</tr>
<tr>
<td></td>
<td>γ- HCH 1 to 580 pg/m³</td>
<td></td>
<td>(58)</td>
</tr>
<tr>
<td></td>
<td>10,000 pg/m³</td>
<td>Bay of Bengal and Arabian Sea</td>
<td>(60)</td>
</tr>
<tr>
<td></td>
<td>1,300 pg/m³</td>
<td>South China Sea</td>
<td>(60)</td>
</tr>
<tr>
<td></td>
<td>28-1,300 pg/m³</td>
<td>Northern Pacific</td>
<td>(60)</td>
</tr>
<tr>
<td>Surface Water</td>
<td>1,000 pg/L</td>
<td>Chucki Sea, Bering Sea, Gulf of Alaska, etc.</td>
<td>(60)</td>
</tr>
<tr>
<td></td>
<td>810-1,200 pg/L</td>
<td>Sea of Japan and Bering Sea</td>
<td>(60)</td>
</tr>
<tr>
<td>Sediments</td>
<td>1,100 E3 pg/g</td>
<td>India</td>
<td>(60)</td>
</tr>
</tbody>
</table>
Table 1.3 Health Effects and Carcinogenicity Assessment

<table>
<thead>
<tr>
<th>HCH Isomer</th>
<th>Health effect&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Carcinogenicity Assessment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CAS No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Hepatic nodules and hepatacellular carcinomas</td>
<td>B2 (Probable human carcinogen)</td>
<td>319-84-6</td>
</tr>
<tr>
<td>β</td>
<td>Environmental estrogen</td>
<td>C (Possible human carcinogen)</td>
<td>319-85-7</td>
</tr>
<tr>
<td>γ</td>
<td>Stimulates and damages central nervous system at high doses</td>
<td>No assessment</td>
<td>58-89-9</td>
</tr>
<tr>
<td>δ</td>
<td>Inadequate data from animal assay</td>
<td>D (not a human carcinogen)</td>
<td>319-86-8</td>
</tr>
<tr>
<td>Technical grade</td>
<td>Carcinogenicity observed in mice</td>
<td>B2 (Probable human carcinogen)</td>
<td>608-73-1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Willet et al. (60);
<sup>b</sup>Integrated risk information system (51).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>α- HCH</th>
<th>γ- HCH</th>
<th>β- HCH</th>
<th>δ- HCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCF human fat&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20 ± 8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>19 ± 9</td>
<td>527 ± 140</td>
<td>8.5</td>
</tr>
<tr>
<td>BCF aquatic animals&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.6 ± 0.5</td>
<td>2.5 ± 0.4</td>
<td>2.9 ± 0.3</td>
<td>NA&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Water solubility (mg/L)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10</td>
<td>17</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Vapor pressure (Pa) (20 or 25 °C)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6 ± 0.9 E-2</td>
<td>5.3 ± 1.4 E-3</td>
<td>4.2 ± 0.3 E-5</td>
<td>2.1 E-3</td>
</tr>
<tr>
<td>Henry’s law constant</td>
<td>2.41E-4</td>
<td>3.19E-4</td>
<td>1.44E-5</td>
<td>NA&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Willet et al. (60); BCF = bioconcentration factor;  
<sup>b</sup> ATSDR (7);  
<sup>c</sup> USEPA (51);  
<sup>d</sup> ± = standard errors;  
<sup>e</sup> NA = not available.
Table 1.5 Aerobic Cometabolism of Chlorinated Ethenes

<table>
<thead>
<tr>
<th>Representative Strain</th>
<th>PCE</th>
<th>TCE</th>
<th>cDCE</th>
<th>tDCE</th>
<th>VC</th>
<th>Ethene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>G4</td>
<td>NT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>✓&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>(17)</td>
</tr>
<tr>
<td>PKO1</td>
<td>NT</td>
<td>✓</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>(27)</td>
</tr>
<tr>
<td>KR1</td>
<td>NT</td>
<td>✓</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>(27)</td>
</tr>
<tr>
<td>F1</td>
<td>✗&lt;sup&gt;c&lt;/sup&gt;</td>
<td>✓</td>
<td>✓</td>
<td>NT</td>
<td>✗</td>
<td>✗</td>
<td>(57)</td>
</tr>
<tr>
<td>JS150</td>
<td>NT</td>
<td>✓</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>(21)</td>
</tr>
<tr>
<td>OB3b</td>
<td>✗</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>(41)</td>
</tr>
<tr>
<td>41-6</td>
<td>NT</td>
<td>✓</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>(30)</td>
</tr>
<tr>
<td>CAC1</td>
<td>✗</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>(12)</td>
</tr>
</tbody>
</table>

<sup>a</sup> NT = not tested;
<sup>b</sup> ✓ = cometabolism observed;
<sup>c</sup> ✗ = cometabolism not observed.
Figure 1.1 Structure of several HCH isomers. Axial (a) and equatorial (e) position of the chlorine atoms are identified for each isomer. Adapted from Willet et al. (60).
Figure 1.2 Proposed pathway for anaerobic biodegradation of β-HCH; adapted from Middeldorp et al. (37). TeCCH = 3,4,5,6-tetrachlorocyclohexane; DCCH = dichlorocyclohexadiene; [H] = H⁺ + e⁻.
Figure 1.3 Proposed aerobic biodegradation pathway for γ-HCH by *Sphingomonas paucimobilis* UT26, adapted from Phillips et al. (43). PCCH = pentachlorocyclohexene; 2,4,5-DNOL = 2,4,5-trichloro-2,5-cyclohexadiene-1-ol; 2,5-DDOL = 2,5-dichloro-2,5-cyclohexadiene-1,4-diol; DCP = dichlorophenol; DCHQ = dichlorohydroquinone; CHQ = chlorohydroquinone; HQ = hydroquinone; HSMA = hydroxymuconic semialdehyde; [H] = H⁺ + e⁻.
Figure 1.4 Cometabolism of TCE via a toluene monooxygenase.
Figure 1.5  Cometabolism of TCE via a toluene dioxygenase; adapted from Li and Wackett (28).
Figure 1.6 Cometabolism of TCE via methane monooxygenase; adapted from Fox et al. (18).
Figure 1.7 Cometabolism of cDCE via a monooxygenase.
Figure 1.8 Initial step during aerobic biodegradation of benzene (9).
Figure 1.9 Initial step during aerobic biodegradation of chlorobenzene (9).
Figure 1.10 Initial step during aerobic biodegradation of 1,4-dichlorobenzene (9).
Figure 1.11 Initial step during aerobic biodegradation of 1,2,4-trichlorobenzene (9).
Figure 1.12 Potential biodegradation pathways for a complex mixture of contaminants when subjected to sequential anaerobic and then aerobic treatment.
Figure 1.13 Potential biodegradation pathways for a complex mixture of contaminants when subjected to sequential aerobic and then anaerobic treatment.
2. BIOREMEDIATION OF HEXACHLOROCYCLOCYCLEXANE ISOMERS, CHLORINATED BENZENES AND CHLORINATED ETHENES IN SOIL AND FRACTURED DOLOMITE

2.1 ABSTRACT

Groundwater at an industrial site is contaminated with α- hexachlorocyclohexane (HCH) and γ-HCH (i.e., lindane) (0.3-0.5 ppm). Other contaminants in the 1-15 ppm range include 1,2,4-trichlorobenzene (TCB), 1,2-dichlorobenzene (DCB), 1,3-DCB, 1,4-DCB, chlorobenzene (CB), benzene, trichloroethene (TCE) and cis-1,2-dichloroethene (cDCE). The aquifer consists of a shallow layer of soil over fractured dolomite, where most of the contaminant mass resides. The objective of this study was to compare 1) anaerobic reductive dechlorination of the polychlorinated contaminants, followed by aerobic biodegradation of the daughter products (mainly DCBs, CB, and benzene); and 2) aerobic biodegradation of α- and γ-HCH, TCB, DCBs, CB and benzene, followed by anaerobic reduction of TCE and cDCE to ethene. Conventional wisdom suggests that sequential anaerobic and aerobic conditions are desirable for bioremediating sites contaminated by mixtures of polychlorinated organics. The results of this microcosm study suggest that a sequential aerobic and anaerobic approach may be more successful, although implementing this in the field presents some major challenges. In the dolomite microcosms incubated initially under aerobic conditions (59 days), α- and γ-HCH were biodegraded close to the maximum contaminant level for lindane; all of the aromatic compounds were consumed; and there was partial removal of TCE and cDCE.
(presumptively via cometabolism). The subsequent switch to anaerobic conditions (day 101) yielded reductive dechlorination of the remaining TCE and a significant level of ethene was produced, although some cDCE and VC persisted. In contrast, sequential anaerobic (393 days) and aerobic treatment (498 days) for the dolomite microcosms was ineffective in completely removing the aromatic compounds, α-HCH, cDCE and VC. For the soil microcosms, both treatment sequences were effective, most likely reflecting a greater abundance of the necessary microbes and electron donor in this part of the site.

2.2 INTRODUCTION

Many polychlorinated organic compounds are subject to biodegradation under anaerobic conditions via reductive dechlorination (3, 10, 26). The kinetics of dechlorination tends to decrease as each of the chlorine atoms are removed, raising the possibility that the lesser chlorinated daughter products may persist under anaerobic conditions. By contrast, organic compounds with one or two chlorine atoms generally undergo aerobic biodegradation quite readily, while more highly chlorinated compounds tend to be recalcitrant in the presence of oxygen. For this reason, sequential anaerobic/aerobic conditions are often favored for achieving complete bioremediation of many polychlorinated organic compounds (5, 12, 28).

A potential disadvantage with anaerobic/aerobic treatment is the high oxygen demand associated with excess addition of readily biodegradable electron donor (29). An alternative bioremediation approach that has received less attention is to sequence the redox environment from aerobic to anaerobic conditions. This avoids the problem with excess electron donor addition and may also take advantage of faster kinetic rates for
aerobic oxidation compared to reductive dechlorination (34). However, application of an aerobic/anaerobic strategy depends on having an appropriate mix of contaminants, i.e., a significant level of activity must occur aerobically and whatever contaminants are not consumed aerobically should be subject to complete anaerobic biodegradation.

Contaminant conditions at an industrial site suggested that both anaerobic/aerobic and aerobic/anaerobic bioremediation may be feasible. The site geology is characterized by an upper soil layer (1.5-3.0 m below the surface) consisting of heterogeneous fill material with an average of 0.8% organic matter. The soil is underlain by a fractured dolomite formation containing no measurable organic matter. The groundwater table ranges from 1.5-4.6 m below the surface. Both the soil and fractured dolomite are contaminated, although the majority of contaminant mass resides in the fractured dolomite. The contaminants include α-hexachlorocyclohexane (HCH) and γ-HCH (i.e., lindane), trichloroethene (TCE), cis-1,2-dichloroethene (cDCE), benzene, chlorobenzene (CB), 1,2-dichlorobenzene (DCB), 1,3-DCB, 1,4-DCB and 1,2,4-trichlorobenzene (TCB). The co-occurrence of contaminants at hazardous waste sites is the norm rather than the exception (36). Based on six years of field data from the site, groundwater characteristics for the soil and dolomite zones were similar; median values were 1.7 mg/L (range = 0 to 12) for dissolved oxygen, -3.0 mV (range = -300 to +490) for oxidation reduction potential, 7.4 (range = 2.6 to 13) for pH, 230 mg/L CaCO$_3$ for alkalinity, 8.3 mg/L for dissolved organic carbon, and 15°C.

HCH isomers undergo biodegradation both aerobically and anaerobically, although via different pathways and with different products (30). Benzene and CB are
the predominant anaerobic daughter products, neither of which is an acceptable endpoint (26). Tri- and dichlorobenzenes also undergo biodegradation aerobically and anaerobically, although CB tends to be the terminal daughter product under anaerobic conditions (2, 10). Both CB and benzene are readily biodegradable aerobically but not anaerobically (34). Complete reduction of TCE and cDCE to ethene is possible anaerobically (4, 17) and both are also known to biodegrade aerobically via cometabolism, with primary substrates that include several monoaromatic compounds (4, 17). cDCE may also be degraded aerobically as a sole substrate (8-9).

With a mixture of HCH isomers, chlorinated benzenes, benzene and chlorinated ethenes, sequential anaerobic and aerobic treatment would appear to be feasible. Reductive dechlorination of the polychlorinated contaminants (α- and γ-HCH, TCB, TCE and cDCE) would be followed by aerobic biodegradation of the daughter products (mainly DCBs, CB, and benzene). Using an aerobic/anaerobic approach, biodegradation of α- and γ-HCH, TCB, DCBs, CB and benzene, may be expected aerobically, followed by anaerobic reduction of TCE and cDCE to ethene. The objective of this study was to compare anaerobic reductive dechlorination of the contaminant mixture followed by aerobic biodegradation of the daughter products, versus aerobic biodegradation of α- and γ-HCH, TCB, DCBs, CB and benzene followed by anaerobic reduction of TCE and cDCE to ethene. The comparison was made using microcosms prepared with soil, crushed dolomite, and groundwater from the industrial site mentioned above.
2.3 MATERIALS AND METHODS

2.3.1 Field Samples and Chemicals

Samples of soil and dolomite from a contaminated industrial site were taken from 2.4-2.7 m and 3.7-8.2 m below the ground surface, respectively. Dolomite cores were crushed at the University of Waterloo using a hydraulic press equipped with a stainless steel piston head. The crushed rock and soil were shipped to Clemson University, where the rock was passed through an 850 µm sieve (number 20); pulverized dolomite less than 850 µm was used to prepare the microcosms. Groundwater was obtained from a single well in close proximity to where the soil and dolomite samples were obtained.

The sources and purity of chemicals used were: α-HCH (99%), γ-HCH (99.9%), 1,2,4-TCB (99%), 1,3-DCB (99%), 1,4-DCB (99%), CB (99.5%), cDCE (99%) from TCI America; vinyl chloride (VC; 99.5%) from Fluka; polymer grade ethene (99.9%) from Matheson; benzene (99%), 1,2-DCB (99.5%), TCE (99%), and methanol (99%) from Fisher Scientific; lactate from Mallinckrodt Baker, Inc. as a 60% (w/w) sodium lactate syrup; and hexane (99.9%) from Burdick&Jackson. All other chemicals used were reagent grade.

2.3.2 Experimental Design

Microcosms were prepared with groundwater and soil or dolomite that was obtained from the contaminated site. Two sequences of microcosms were prepared for both soil and dolomite. Sequence I was incubated first under anaerobic conditions and then switched to aerobic conditions. Sequence II was incubated first under aerobic conditions and then switched to anaerobic conditions (Figure 2.1). For each sequence,
six treatments were prepared: (i) Live “as-is” (AI) microcosms, designed to simulate in situ conditions, with a low concentration of TCE and cDCE present (AI-Low); (ii) Live microcosms biostimulated (BL) with lactate during anaerobic incubation, with a low concentration of TCE and cDCE present (BL-Low); (iii) Live microcosms bioaugmented (BA) and stimulated with lactate during anaerobic incubation, with a low concentration of TCE and cDCE present (BA-Low); (iv) Live “as-is” microcosms, designed to simulate in situ conditions, with a high concentration of TCE and cDCE present (AI-High); (v) Live microcosms biostimulated with lactate during anaerobic incubation, with a high concentration of TCE and cDCE present (BL-High); and (vi) Live microcosms bioaugmented and stimulated with lactate during anaerobic incubation, with a high concentration of TCE and cDCE present (BA-High).

Each of the treatments listed above was prepared in triplicate, yielding 36 live microcosms prepared with soil and 36 prepared with crushed dolomite. Triplicate killed controls were prepared with soil and with dolomite, along with one set of triplicate water controls.

2.3.2 **Microcosms**

Microcosms were prepared in 160 mL serum bottles with groundwater (75±0.2 mL) and soil or crushed dolomite (30±0.2 g). The pH of the groundwater was 7.1. The target concentrations of contaminants were based on a review of field monitoring data. After adding soil or crushed dolomite to the serum bottles, groundwater (37.5-38.8 mL) was added. Since the groundwater as received had lower concentrations of contaminants than the target levels, it was necessary to add each compound. Groundwater saturated
solutions of α-HCH, γ-HCH, 1,2,4-TCB, and 1,4-DCB were delivered using glass pipettes. The microcosms were then sealed with Teflon-faced red rubber septa and crimp caps. Water saturated solutions of benzene, CB, 1,2-DCB, 1,3-DCB, cDCE and TCE were injected into the microcosms, resulting in a total liquid volume of 75 mL. Because the latter compounds have comparatively higher aqueous phase solubility, use of water saturated solutions did not significantly alter the amount of groundwater used in the microcosms. Resazurin was added as a redox indicator (1 mg/L).

A saturated solution of α-HCH was prepared in groundwater as follows: 500 mL of groundwater was centrifuged (1 h at 12,000 rpm; Sorval 1500 centrifuge) and filtered (0.45 µm); 100 mg of α-HCH was added and the solution was agitated on a magnetic stirrer for a minimum of 48 h. The groundwater was filtered (0.45 µm) again to remove undissolved α-HCH and a sample was withdrawn to measure the aqueous concentration. Groundwater saturated solutions of γ-HCH and 1,4-DCB were prepared using the same procedure. Water saturated solutions of the other compounds were prepared by placing an excess of the neat liquid in contact with distilled deionized water for a minimum of 5 days in sealed containers. Measured initial concentrations and amounts per bottle for all contaminants are shown in Table 2.1.

The calculated amount of electron donor demand was 760 mg/L as chemical oxygen demand (COD), based on stoichiometric removal of nitrate (8.3 mg N/L) and sulfate (1090 mg/L), and reductive dechlorination of all of the chlorinated benzenes and the high concentrations of TCE and cDCE to nonchlorinated products. Electron donor
additions were made in 520 mg/L COD increments and the total amount added varied depending on the extent of dechlorination activity.

Microcosms that were initially incubated under anaerobic conditions were prepared in an anaerobic chamber, to exclude the introduction of oxygen and hasten the development of anaerobic conditions. A small amount of hydrogen was introduced from the chamber’s atmosphere, which consisted of <2% hydrogen, the balance being nitrogen. This amount of hydrogen was not enough to represent a significant source of electron donor for the concentration of contaminants being tested. Microcosms that were initially incubated under aerobic conditions were prepared on the bench top, so that the headspace contained 21% oxygen, providing an initial dissolved oxygen concentration of approximately 8 mg/L. The headspace oxygen concentration was measured periodically; if it fell below approximately 5%, pure oxygen was injected so that the amount was returned to 21%.

To switch anaerobic microcosms to aerobic conditions, pure oxygen was injected into the headspace, yielding an initial headspace concentration of 21%. To switch aerobic microcosms to anaerobic conditions, lactate was added in 520 mg/L COD increments. A change in the color of the resazurin (from pink to clear) made it possible to determine when low redox conditions were reached (i.e., below -110 mV). Further additions of lactate were made, as needed, to stimulate reductive dechlorination. Septa were changed when the switch from one condition to another was made; this was done quickly enough to avoid a detectable loss of the volatile compounds.
Two types of bioaugmentation cultures were used. To stimulate reductive dechlorination of TCE and cDCE, a chlororespiring enrichment culture characterized by Eaddy (13) was added (0.5 mL), containing approximately $10^9$ gene copies of *Dehalococcoides* per mL. To stimulate anaerobic reduction of the chlorinated benzenes in the dolomite microcosms, aliquots of slurry from the soil microcosms (Sequence I, AI-Low) were used (0.5 mL), since these exhibited robust activity.

Killed controls were prepared by autoclaving (1 hr for three consecutive days) anaerobic soil microcosms and anaerobic dolomite microcosms. For the dolomite microcosms, glutaraldehyde (1.05 g/bottle) was also added to further inhibit microbial activity (32). All controls received high concentrations of TCE and cDCE.

Serum bottles were stored at room temperature (22-24°C), in the dark, under quiescent conditions and in an inverted position (liquid and solids in contact with the septum), to minimize loss of volatiles by diffusion through punctures in the septa. Serum bottles that were operated under anaerobic conditions were incubated in an anaerobic chamber, to minimize introduction of oxygen. Serum bottles that were operated under aerobic conditions were incubated outside the anaerobic chamber.

For the aromatic compounds and ethenes, microcosm results are plotted in terms of the total amount per bottle (i.e., μmole), in order to directly reveal the stoichiometry of reductive dechlorination. The effect of adsorption was not included when measuring total amounts; abiotic losses (adsorption and diffusion) were assumed to be insignificant for the time zero measurements. Also, rates of abiotic losses were determined for the killed controls and used to adjust the biodegradation rates (see below). Conversion of
µmoles per bottle to aqueous phase concentrations (Table 2.1) was accomplished using Henry’s Law constants at 23°C (Benzene, 0.21; CB, 0.14; 1,2-DCB, 0.068; 1,3-DCB, 0.097; 1,4-DCB, 0.087; 1,2,4-TCB, 0.050; TCE, 0.38; and cDCE, 0.15) (35) and the volume of the aqueous phase (75 mL) and the gas phase (70 or 74 mL, taking into account the 15 or 11 mL occupied by the soil or crushed dolomite, respectively). Since α- and γ-HCH are much less volatile, their results are plotted in terms of aqueous phase concentrations, to facilitate a direct comparison to the maximum contaminant level (MCL) for lindane (0.2 µg/L, or 6.88E-4 µM).

2.3.4 Analytical Methods

α- and γ-HCH were analyzed by extracting an aqueous sample twice with hexane (19). Before taking samples, microcosms were placed upright and the soil was allowed to settle. A sample (200 µL) of the clarified aqueous phase was then transferred to a 2 mL glass vial; a preliminary evaluation determined that less than 12% of the HCH adsorbed to the sediment (Appendix 6.2.1). Hexane (500 µL) was added and the vial was closed with a screw cap and vortexed (Baxter Scientific, IL) for 2 min. The hexane was then transferred to a clean 2 mL glass vial. Additional hexane (500 µL) was added to the first vial and the process was repeated, to further extract HCH from the sample. Hexachlorobenzene was added (0.56 ng/sample, dissolved in hexane) to the combined 1 mL of hexane extract, to serve as an internal standard.

The hexane extract was analyzed by injecting 1 µL onto a Hewlett Packard 5890 Series II Plus gas chromatograph (GC) equipped with an HP 7673 autosampler, ZB 624 capillary column (30 m x 0.53 mm x 3.0 µm film; Zebron) and electron capture detector.
The injector, oven and detector temperatures were maintained at 220, 240 and 250°C, respectively. Helium (5.5 mL/min) was used as the carrier gas and nitrogen (30 mL/min) was the makeup gas. Elution times for hexachlorobenzene, α- and γ- HCH were 14.4, 15.5, and 18.4 min, respectively.

1,2,4-TCB, 1,2-DCB, 1,3-DCB, 1,4-DCB, CB, benzene and TCE were analyzed by injecting 500 µL of headspace samples onto a HP 5890 Series II Plus GC equipped with an RTX 5 column (30-m×0.53-mm×1.5-µm film; Restek Corp.) and flame ionization detector. The injector and detector temperatures were 250 and 325°C, respectively. The oven temperature program used was: 50°C for 4 min, increased at 10°C per min to 80°C, hold 10 min, increased at 10°C per min to 150°C, and hold for 1 min. Helium (5 mL/min) was used as the carrier gas and nitrogen (30 mL/min) was the makeup gas. The elution times were 3.3 min for benzene, 4.2 min for TCE, 7.9 min for CB, 14.8 min for 1,3-DCB, 15.3 min for 1,4-DCB, 17.1 min for 1,2-DCB, and 22.6 min for 1,2,4-TCB. Ethene, VC, cDCE, and TCE were analyzed by injecting 500 µL of a headspace sample onto a GC (HP 5890 Series II) equipped with a 60/80 Carbopak B column (Supelco) and flame ionization detector, as previously described (15).

Oxygen was analyzed by injecting 100 µL of a headspace sample onto a GC (HP 5890 Series II) equipped with thermal conductivity detector and molecular sieve 5A 60/80 column (1.8 m×3.1 mm; Alltech). The detector, oven, and injector temperatures were set at 120, 70 and 120°C, respectively. Helium (30 mL/min) was used as the reference gas and carrier gas. The elution time for oxygen was 0.79 min.
Nitrate and sulfate were measured on a Dionex AS50 ion chromatography system equipped with a CD25 conductivity detector and a Dionex guard column (AG9-HC, 4 mm×50 mm) followed by an IonPac® AS9-HC anion-exchange column (4 mm×250 mm). Eluant (9 mM Na₂CO₃) was delivered at 1.0 mL per min. Lactate, acetate and propionate were analyzed on a Waters 600E HPLC system composed of an autosampler (Waters 717 plus), pumping system (Waters 600), a UV/Vis detector (Model 490E) set at 210 nm, and an Aminex® HPX-87H ion exclusion column (300-mm×7.8-mm; BioRad) with 0.01N H₂SO₄ as eluant, delivered at 0.6 mL per min.

2.3.5 Calculation of Biodegradation Rate Constants

Pseudo-first order rate constants for the sequential anaerobic/aerobic microcosms (i.e., Figure 2.1, Sequence I) were calculated by regression of data pooled from each treatment. The aerobic rate constants for sequential anaerobic/aerobic microcosms were calculated by regression of data pooled from the biostimulation and bioaugmentation microcosms, from the day oxygen was added. For the sequential aerobic/anaerobic microcosms (i.e., Figure 2.1, Sequence II), aerobic rate constants were calculated by regression of data pooled from each treatment. For all treatments, net rates were calculated by subtracting the rates for killed controls. Comparisons of rate constants were made using the Student’s t-test.
2.4 RESULTS

2.4.1 Anaerobic/Aerobic Soil Microcosms, Low TCE and cDCE

In the anaerobic as-is microcosms with low levels of TCE and cDCE, the concentrations of α- and γ-HCH declined by 92-99% by the second sampling point on day 35 and nearly reached the MCL for lindane (0.2 µg/L) by day 67-187 (Figure 2.2a). It was not possible to detect benzene and CB as daughter products from biodegradation of α- and γ-HCH, since the initial amounts of benzene and CB (approximately 0.34 and 0.32 µmol/bottle, respectively) were considerably higher than for α- and γ-HCH (0.044 and 0.074 µmol/bottle, respectively). Even with stoichiometric conversion of α- and γ-HCH, the magnitude of increase in benzene and CB would not have been sufficient in comparison to the initial amounts present.

1,2,4-TCB and 1,2-DCB were completely degraded in the triplicate as-is anaerobic soil microcosms, with the majority of the decrease occurring during the first 56-78 days of incubation (Figure 2.2b). Coinciding with the decrease in 1,2,4-TCB, increases in 1,3-DCB and CB were observed, and the accumulated 1,3-DCB subsequently decreased under anaerobic conditions by 47% from its maximum concentration. The average decrease in 1,4-DCB was 46%. Once CB reached maximum levels from dechlorination of DCBs, there was no apparent decrease. The average decrease in benzene throughout the length of incubation was 31%. The total moles of aromatic compounds present at the end of the incubation period averaged 91% of the amount present on day 21 (i.e., after the initial decrease, likely due to abiotic losses). Complete dechlorination of TCE and cDCE to ethene was observed and the removal of TCE was
complete by day 56 (Figure 2.2c). VC and ethene were not monitored prior to day 112. At that point, the ethene was comparable to the combined amount of TCE and cDCE dechlorinated (i.e., a mass balance of 126%). These results confirmed that the soil contains an active population of microbes capable of dechlorinating TCE and cDCE to ethene.

Average results for microcosms with low levels of TCE and cDCE that were biostimulated under anaerobic conditions and were then switched to aerobic conditions (on day 136) are shown in Figures 2.2d, 2.2e, and 2.2f. The rate of reductive dechlorination for the HCH isomers, chlorinated benzenes and chlorinated ethenes was no faster or more complete with lactate added (day 47) compared to the as-is treatment (Figures 2.2a, 2.2b and 2.2c), indicating that the soil contained an adequate supply of electron donor. Nitrate and sulfate were measured on day 152 and none was detected, even though the amount of lactate added was less than the COD demand for nitrate and sulfate reduction. Ethene was the main dechlorination product from TCE and cDCE, although a low level of VC accumulated under anaerobic conditions (Figure 2.2f). After switching to aerobic conditions, lindane and α-HCH continued to decrease and reached the MCL for lindane (0.2 µg/L) by day 187 (Figure 2.2d). All of the monoaromatic compounds were removed during the aerobic phase (Figure 2.2e) and all of the ethene and low amount of VC were also biodegraded (Figure 2.2f), indicating that complete biodegradation was achieved without bioaugmentation (as was originally planned; Figure 2.1, Sequence I). Since bioaugmentation was unnecessary, the three bottles that were
designated for culture addition were only biostimulated, yielding a total of six bottles for the biostimulation treatment.

The trends observed in the microcosms with high initial concentrations of TCE and cDCE were similar to those with low initial concentrations. Results for the water controls and killed controls confirmed that percent decreases in live treatments were considerably higher in treatments that exhibited biodegradation activity (Table 2.2). For the killed controls, there was a notable decrease in α- and γ-HCH and CB, while there were no significant losses for the other compounds, suggesting that adsorption was not a major factor in the decreases observed in the live treatments.

2.4.2 Sequential Aerobic/Anaerobic Treatment in Soil Microcosms

In the as-is aerobic soil microcosms with low levels of TCE and cDCE present, 92-99% of the α- and γ-HCH were removed by the second sampling point on day 32 and decreased close to the MCL for γ-HCH by day 209 (Figure 2.3a). 1,2,4-TCB, DCB isomers, CB and benzene were completely degraded in the as-is aerobic soil microcosms, with the majority of the decrease occurring during the first 87 days of incubation (Figure 2.3b). Decreases in TCE and cDCE were observed under aerobic conditions in each of the triplicate microcosms (Figure 2.3c). In order to confirm the occurrence of aerobic biodegradation, a second addition of all contaminants was made on day 209. Continued biodegradation of all the added compounds was observed, with most of the decrease occurring within 20 days after the second addition. A majority of the decrease in TCE and cDCE occurred during the same time interval when the monoaromatic compounds were degraded.
Average results for microcosms with low levels of TCE and cDCE present that were incubated aerobically and then switched to anaerobic conditions are shown in Figure 2.3d, 2.3e, and 2.3f. These bottles were operated aerobically for the first 137 days and were then switched to anaerobic conditions by adding lactate. The majority of the biodegradation of α-HCH, γ-HCH and all of the monoaromatic compounds occurred during the aerobic phase and was similar to the observation in the as-is treatment (Figures 2.3a and 2.3b). After switching to anaerobic conditions by adding lactate, a minor decrease in α- and γ-HCH was observed, approaching the MCL for lindane by day 251. With this set of microcosms, TCE and cDCE decreased under aerobic conditions, but neither was completely consumed. After switching to anaerobic conditions by adding lactate, a rapid decrease occurred in the TCE and cDCE remaining from the aerobic phase, with an increase in VC, which was then dechlorinated to ethene (Figure 2.3f). This indicated that bioaugmentation was not required. Therefore, the triplicate microcosms intended for bioaugmentation were only biostimulated, and the results presented are the average of six microcosms (Figure 2.3d, 2.3e, and 2.3f).

The trends observed in the microcosms with high initial concentrations of TCE and cDCE were similar to those with low initial concentrations.

2.4.3 Sequential Anaerobic/Aerobic Treatment in Dolomite Microcosms

In the anaerobic as-is dolomite microcosms with low concentrations of TCE and cDCE, the concentrations of α- and γ-HCH decreased by 52% and 64% by day 3 (Figure 2.4a). The decrease in α- and γ-HCH continued under anaerobic conditions such that by day 162, nearly all was removed (Table 2.2). However, losses of both compounds from
the killed controls were comparable, indicating that the decreases in α- and γ-HCH were predominately abiotic. In spite of the significant decreases in α- and γ-HCH, their final concentrations were above the MCL for lindane.

There was also a large decline in the aromatic contaminants over the first 3 days of incubation (Figure 2.4b), but no apparent decreases in aromatic contaminants or daughter product formation beyond day 3. The behavior of killed controls was similar. Reductive dechlorination of TCE and cDCE was not observed over 809 days of incubation (Figure 2.4c). Biostimulation with lactate did not enhance anaerobic biodegradation of any of the contaminants in the presence of low concentrations of TCE and cDCE (Appendix 6.2.2, Figure 6.2-1). The biostimulated microcosms were switched to aerobic conditions on day 395 by adding pure oxygen to the headspace. No significant level of biodegradation occurred for the contaminants over the next 428 days of aerobic incubation.

Average results for the dolomite microcosms with low concentrations of TCE and cDCE that were incubated initially under anaerobic conditions and bioaugmented on two occasions are shown in Figures 2.4d, 2.4e and 2.4f. Similar to the as-is anaerobic dolomite microcosms and the killed controls, there was a rapid initial decrease in the HCH isomers and aromatic compounds. Average percent decreases are summarized in Table 2.2. Bioaugmentation (accompanied by addition of lactate) with soil slurry from as-is anaerobic soil microcosms (Figure 2.2) had no discernable effect on the aromatic compounds (Figures 2.4e). By contrast, there was a major improvement in reductive dechlorination of TCE and cDCE (Figure 2.4f). However, reduction stalled at VC, in
spite of a second addition of a chloroethene-respiring enrichment culture and more lactate. The bioaugmented microcosms were switched to aerobic conditions on day 393 by addition of pure oxygen to the headspace. In order to maintain aerobic conditions, it was necessary to make repeated additions of oxygen. This was due in part to the high oxygen demand created by the unused electron donor added during the anaerobic phase. A total of 189 mg of lactate was added (expressed as COD) during the anaerobic phase and approximately 160 mg of COD carried over to the aerobic phase (in the form of acetate and propionate; none of the lactate remained). At an average oxygen consumption rate of 0.27 mg/d, it would have taken nearly 600 days to satisfy the remaining oxygen demand. Aerobic conditions did not yield additional biodegradation of the aromatic compounds, TCE, cDCE and ethene, although a $78\pm9\%$ decrease in VC was observed between days 393 and 821 (Figures 2.4e and 2.4f). The HCH isomers were not evaluated under aerobic conditions because $\gamma$-HCH was already close to the MCL for lindane at the end of the anaerobic phase (Figure 2.4d).

2.4.4 Sequential Aerobic/Anaerobic Treatment in Dolomite Microcosms

In the aerobic as-is dolomite microcosms with low concentrations of TCE and cDCE, the concentration of $\alpha$- and $\gamma$-HCH decreased by $74\pm3\%$ and $73\pm2\%$ by day 3 (Figure 2.5a). Losses from the killed controls were also significant, but not as high ($55\%$ and $62\%$, respectively). In order to confirm the occurrence of aerobic biodegradation, a second addition of $\alpha$- and $\gamma$-HCH was made on day 59. Both decreased close to the MCL for lindane by day 73 (Figure 2.5a).
A similar pattern (i.e., a rapid initial decline over the first 3 days, followed by a sustained rate of decrease close to the detection limit by day 31) was observed with the aromatic contaminants (Figure 2.5b). Losses were significant for the killed controls over the same interval, although the percent decreases were higher in the live treatments (Table 2.2).

Percent decreases in TCE and cDCE exceeded those in the killed controls during first 59 days of aerobic incubation (Table 2.2). In order to confirm the occurrence of aerobic biodegradation of TCE and cDCE, two more additions were made on days 59 and 106. TCE and cDCE decreased by 44% and 23% between days 59 and 106, and by 46% and 21% between days 106 and 158 (Figure 2.5c). On day 158, more aromatic compounds were added but not TCE or cDCE. The decrease in TCE and cDCE observed on day 158 was due to the replacement of groundwater with saturated solutions of aromatic compounds. Between days 158 and 200, TCE and cDCE decreased by 38% and 23%, respectively; only a minor amount of additional losses occurred with further incubation through day 822 (Figure 2.5c). Most of the decreases in TCE and cDCE were observed during same interval when the aromatic compounds were degraded.

With the sequential aerobic/anaerobic dolomite microcosms, all of the monoaromatic compounds and most of the α- and γ-HCH decreased during the aerobic phase (Figures 2.5d and 2.5e), while TCE and cDCE decreased by 49±2% and 45±1% (Figure 2.5f). The switch to anaerobic conditions was made on day 101 by addition of lactate (Figures 2.5d, 2.5e and 2.5f). The establishment of low redox conditions was confirmed when the resazurin that was added to the groundwater turned from pink to
clear. Biostimulation alone did not yield further biodegradation of any of the contaminants (Appendix 6.2.2, Figure 6.2-2). With the bioaugmentation treatment, bioaugmentation with soil slurry (day 122) from the as-is anaerobic soil microcosms (Figure 2.2) had an immediate impact on reductive dechlorination of TCE and cDCE, with nearly stoichiometric accumulation of VC and ethene (Figure 2.5f). Reductive dechlorination stalled after approximately day 400, in spite of an ample supply of electron donor (2540 mg COD/L). A second bioaugmentation event on day 417 using a chloroethene-respiring enrichment culture did not yield any additional response through day 689.

2.4.5 Biodegradation Rates

Aerobic Conditions. For aerobic conditions, it was anticipated that the aerobic biodegradation rates for aerobic/anaerobic conditions (sequence II) would be higher than for anaerobic/aerobic conditions (sequence I). In the soil microcosms, this was indeed the case for benzene, CB and 1,4-DCB. Their first order aerobic biodegradation rates were statistically higher for the aerobic portion of the aerobic/anaerobic sequence compared to the aerobic portion of the anaerobic/aerobic sequence for both low and high initial concentrations of TCE and cDCE (Figure 2.6). 1,3-DCB was an exception. The aerobic biodegradation rate from the anaerobic/aerobic sequence was statistically greater for 1,3-DCB compared to the aerobic rate from aerobic/anaerobic sequence at low initial TCE and cDCE concentrations. However, at high initial TCE and cDCE concentrations, the aerobic biodegradation rate for 1,3-DCB was not statistically different between the two treatment strategies. A similar comparison was not possible for the other compounds
since they were completely removed under the anaerobic conditions of the anaerobic/aerobic sequence. In the dolomite microcosms, aerobic biodegradation rates were calculated for all of the compounds for the aerobic/anaerobic strategy (Figure 2.6). During the aerobic portion of the anaerobic/aerobic strategy, none of the aromatic compounds were biodegraded. No comparison can be made for α- and γ-HCH, since they were consumed anaerobically prior to the shift to aerobic conditions.

**Aerobic Versus Anaerobic.** For compounds that undergo biodegradation under aerobic and anaerobic conditions, often the rate under aerobic conditions is higher than under anaerobic conditions. This pattern was observed in the soil microcosms for 1,2-DCB and 1,4-DCB for both low concentrations of TCE and cDCE and high concentrations of TCE and cDCE (Figure 2.7a). Their highest rates for aerobic conditions were statistically greater than for anaerobic conditions. However, for γ-HCH the anaerobic biodegradation rates were higher in comparison to the aerobic rates at low concentrations of TCE and cDCE and high concentrations of TCE and cDCE. For α-HCH and 1,2,4-TCB, the highest anaerobic rates were statically greater than the highest aerobic rates at low concentrations of TCE and cDCE, although there was no significant difference in the rates at high concentrations of TCE and cDCE. For benzene, CB and 1,3-DCB, a comparison of aerobic and anaerobic rates is not possible since these compounds did not undergo anaerobic degradation.

In the dolomite microcosms, the highest aerobic rates of biodegradation for γ-HCH were significantly higher than the highest anaerobic rates at low concentrations of TCE and cDCE and high concentrations of TCE and cDCE (Figure 2.7b). All of the
other compounds underwent aerobic biodegradation in the aerobic/anaerobic treatments, while none underwent anaerobic biodegradation.

**Effect of TCE and cDCE Concentration.** In the anaerobic/aerobic sequence for the soil microcosms, the anaerobic biodegradation rates for 1,2-DCB, 1,4-DCB, 1,2,4-TCB, α-HCH, and γ-HCH in microcosms with a low initial concentration of TCE and cDCE were statistically greater than the anaerobic rates from microcosms with high initial concentrations of TCE and cDCE (Figure 2.8a). This suggests that high concentration of TCE and cDCE were inhibitory. No apparent anaerobic biodegradation of 1,3-DCB, CB and benzene was observed in anaerobic/aerobic sequence in both low and high initial concentration of TCE and cDCE. Under aerobic conditions for which rates are available, the rates with high levels of TCE and cDCE present were significantly lower for all of the compounds with two exceptions; for CB and γ-HCH, there was no statistically significant difference in rates for the aerobic/anaerobic sequence (Student’s t-test, α=0.05).

The higher level of TCE and cDCE in the dolomite microcosms had an inhibitory effect on biodegradation rates for some, but not all, of the contaminants (Figure 2.8b). The aerobic biodegradation rates for 1,3-DCB, 1,4-DCB, CB and benzene in the aerobic/anaerobic sequence with low initial concentrations of TCE and cDCE were statistically greater than the aerobic biodegradation rate with high concentration of TCE and cDCE.

**Soil Versus Dolomite.** Given the much higher organic content of the soil versus dolomite, it was anticipated that biodegradation rates would be higher in the soil
microcosms. This was the case for anaerobic biodegradation rates for $\gamma$-HCH in the anaerobic/aerobic sequence, with both low and high initial concentrations of TCE and cDCE (Figure 2.9a). However, in the aerobic/anaerobic sequence, the aerobic biodegradation rates in dolomite microcosms with low and high initial concentrations of TCE and cDCE were statistically greater than the aerobic biodegradation rates in the soil microcosms with low and high initial concentrations of TCE and cDCE for all of the contaminants (Figure 2.9b).

2.5 DISCUSSION

Conventional wisdom suggests that sequential anaerobic and aerobic conditions are desirable for bioremediating sites contaminated by mixtures of polychlorinated organic compounds (5, 12, 28). The results of this microcosm study indicate that, for the mix of contaminants investigated, a sequential aerobic and anaerobic approach may be more effective. Most of the contaminant mass at the site in question resides in the dolomite. In the dolomite microcosms incubated under aerobic conditions first, $\alpha$- and $\gamma$-HCH were biodegraded close to the MCL for lindane; all of the aromatic compounds were consumed; and there was partial removal of TCE and cDCE. The subsequent switch to anaerobic conditions yielded reductive dechlorination of the remaining TCE and a significant level of ethene was produced. However, not all of the cDCE and VC were reductively dechlorinated to ethene under anaerobic conditions. This suggests that a more active chlororespiring bioaugmentation culture would be needed than the ones used in this study. In contrast, sequential anaerobic and aerobic treatment for the dolomite microcosms was ineffective in completely removing the aromatic compounds, $\alpha$-HCH,
cDCE and VC. For the soil microcosms, both treatment sequences were effective, most likely reflecting a greater abundance of the necessary microbes and electron donor in this part of the site.

The reason cDCE and VC persisted during the anaerobic bioaugmentation phase of the aerobic/anaerobic dolomite microcosms is not yet known. An excess of electron donor was provided and the pH was maintained above 6.5. Bioaugmentation was first tested using indigenous microbes, i.e., from the anaerobic soil microcosms that had exhibited complete reduction of TCE and cDCE. The second bioaugmentation event involved an enrichment culture developed for the Savannah River Site (13). Apparently the geochemical conditions were not sufficient for either culture. It is also unclear why aerobic biodegradation of the aromatic compounds was incomplete in the sequence I dolomite microcosms (i.e., aerobic following anaerobic treatment; Figure 2.4e), while complete aerobic biodegradation occurred in the corresponding aerobic soil microcosms (Figure 2.2e). With additional time to acclimate a bioaugmentation culture to site conditions, complete reduction of the chlorinated ethenes and the polychlorinated benzenes should be achievable. Complete reduction of TCE to ethene has been demonstrated previously within fractured dolomite (37).

The sequential redox conditions imposed on the microcosms represents a “worst-case” scenario, from the perspective that the anaerobic population was exposed to several months of aerobic conditions, and vice versa. Nevertheless, in the soil microcosms, addition of lactate quickly established anaerobic conditions that resulted in reductive dechlorination of TCE and cDCE, without the need for bioaugmentation (Figure 2.3f).
Organic matter in the soil may have shielded the obligate anaerobes during aerobic incubation. Under in situ conditions, sequential treatment may be established spatially rather than temporally, thereby mitigating the impacts on obligate anaerobes and aerobes.

One of the main advantages to using an aerobic/anaerobic approach in the dolomite for the mix of contaminants in this study was the higher rate of biodegradation for all contaminants under aerobic conditions (Figure 2.7b). However, in the soil microcosms, the aerobic rates tended to be lower for the higher chlorinated contaminants (i.e., α- and γ-HCH and 1,2,4-TCB; Figure 2.7a) and higher for the lesser chlorinated contaminants (i.e., each of the DCB isomers, CB, and benzene), which is consistent with previous reports for biodegradation rates of aromatic compounds under aerobic and anaerobic conditions (14, 33).

First order biodegradation rates do not account for important experimental conditions, such as biomass concentrations and environmental influences. Taking note of these limitations, the first order aerobic biodegradation rates observed for benzene in this study (ranging from 5.5 to 66 yr⁻¹) fell within the range reported for 26 previous studies (34). For CB, the aerobic rates observed in this study were approximately three times lower than rates calculated from data presented for contaminated groundwater treated in activated sludge (18). Lapertotet et al. (20) reported aerobic biodegradation rates for CB that were three times higher than for 1,2-DCB, while in this study there was less difference in rates between these two chlorinated benzenes. Dermietze and Vieth (11) measured aerobic rates for CB and 1,4-DCB that were two times higher than 1,2-DCB, 1,3-DCB, and 1,2,4-TCB. In this study, the range in aerobic rates among the aromatic
compounds was also approximately two-fold for most of the treatments evaluated, although the ranking of rates varied depending on the presence of soil or dolomite, and the amount of TCE and cDCE initially present. The aerobic rate for 1,2,4-TCB estimated from data reported by Schroll et al. (33), i.e., 16 yr\(^{-1}\), was within the range observed for this study (6.6-37 yr\(^{-1}\)), although an order of magnitude higher rate was reported for *Burkholderia* sp. strain PS14 (31).

In the sequence II soil and dolomite microcosms (Figures 2.3c and 2.5c, respectively), aerobic transformation of TCE and cDCE was observed, in spite of the absence of primary substrates (e.g., methane, ethene, ammonia, toluene and phenol) that are known to support aerobic cometabolism of chlorinated ethenes (4). Decreases in TCE and cDCE under aerobic conditions tended to occur when the aromatics were being biodegraded, suggesting that oxygenase enzymes induced by the biodegradation of one or more aromatic compounds (21, 23-24, 30, 38) may be responsible for aerobic cometabolism of TCE and cDCE. Nevertheless, additional studies are needed to confirm that the oxygenases involved in biodegradation of chlorobenzenes are capable of cometabolizing TCE and cDCE. Aerobic biodegradation of HCH isomers also involves oxygenases (27), although the concentration of α- and γ-HCH in this study was considerably lower than TCE and cDCE.

In most previous studies, reductive dechlorination of 1,2,4-TCB proceeds through 1,4-DCB (3, 6-7, 25). In the soil microcosms for this study, the predominant product was 1,3-DCB. The first order rate coefficient for 1,2,4-TCB dechlorination to 1,3-DCB was approximately five times higher than in a column study with mixtures of chlorinated
benzenes (7), although it was an order of magnitude lower than the rate reported for an enrichment culture that received significantly lower concentrations (25). This reflects the diversity of cultures capable of dechlorinating 1,2,4-TCB, as well as the different experimental conditions used to estimate first order rate coefficients. The dolomite at the site evaluated in this study appears to lack the microbes necessary for reductively dechlorinating polychlorinated benzenes.

Of the three DCB isomers, 1,2-DCB was the most extensively dechlorinated to CB in the soil microcosms, which is consistent with observations made by Bosma et al. (7) in a column study. However, high rates of 1,4-DCB dechlorination have been reported with enrichment cultures (25), so it may be feasible to achieve dechlorination of all of the DCBs via bioaugmentation. CB has typically been considered the terminal product of reductive dechlorination of polychlorinated benzenes. However, Fung et al. (16) recently reported on an enrichment culture in which significant levels of DCB isomers and CB were completely reduced to benzene, using yeast extract as an electron donor. Nevertheless, neither CB nor benzene is an acceptable end point for remediation. We observed no significant anaerobic transformation of benzene or CB in any of the microcosm treatments, thereby confirming the need for an aerobic phase at some point in the treatment process. Although anaerobic biodegradation of benzene is occasionally reported (22), the majority of laboratory and field studies indicate it undergoes very low or no biodegradation (1, 7, 14, 34).

The high concentrations of TCE and cDCE evaluated in this study (8.4 and 16 mg/L, respectively) had an inhibitory effect on the rates of biodegradation for HCH
isomers and the polychlorinated benzenes, under anaerobic and aerobic conditions. What caused this effect under anaerobic conditions is not yet known, although one possibility is competition for electron donor. However, the inhibitory effect was present even in the biostimulated and bioaugmentation treatments, both of which received a considerable excess of electron donor. The mechanism of inhibition under aerobic conditions is also not known. In this case, since partial aerobic biodegradation of TCE and cDCE was observed, it seems possible that cometabolism of TCE and cDCE by aromatic and HCH oxygenases may have competitively inhibited their activity on the parent compounds. Competitive inhibition of primary substrates by chlorinated ethenes that undergo aerobic cometabolism is a well established phenomenon (4).

Although the results of this study indicate significant potential for using sequential aerobic and anaerobic conditions for bioremediation of HCH isomers, chlorinated benzenes, benzene and chlorinated ethenes, implementing this approach in the field presents some major challenges. This is especially true for the fractured dolomite, in which locally high groundwater velocities make it especially difficult to control redox conditions. For example, initial attempts to provide oxygen in situ have not been successful (data not shown). Thus, successful application is likely to hinge on the development of methods to effectively deliver electron donor, acceptor, and/or bioaugmentation cultures. Of course, the challenge of effectively delivering materials to groundwater in fractured rock applies to all forms of remediation, not just bioremediation.
2.6 REFERENCES


13. **Eaddy, A.** 2008. Scale-up and characterization of an enrichment culture for bioaugmentation of the P-Area chlorinated ethene plume at the savannah river site Clemson University, Clemson.


36. **USEPA.** 2008. Superfund site information system; sites contaminated with lindane. [http://cfpub.epa.gov/supercpad/cursites/srchrslt.cfm?start=1&CFID=1592952&CFTOKEN=20426970&jsessionid=b23046e3b9dbb49d9e505b7b4739765b3539TR](http://cfpub.epa.gov/supercpad/cursites/srchrslt.cfm?start=1&CFID=1592952&CFTOKEN=20426970&jsessionid=b23046e3b9dbb49d9e505b7b4739765b3539TR), Accessed on 07/14/08, 10:00 AM.


Table 2.1 Average Initial Contaminant Concentration for the Microcosms Study

<table>
<thead>
<tr>
<th>Contaminants</th>
<th>Concentration (mg/L)</th>
<th>Amount (µmol/bottle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-HCH&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>0.24±0.093</td>
<td>0.061±0.024</td>
</tr>
<tr>
<td>γ-HCH&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>0.38±0.14</td>
<td>0.099±0.037</td>
</tr>
<tr>
<td>1,2,4-TCB&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>6.2±4.3</td>
<td>2.7±1.8</td>
</tr>
<tr>
<td>1,2-DCB&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>3.2±1.4</td>
<td>1.7±0.77</td>
</tr>
<tr>
<td>1,3-DCB&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1.0±0.61</td>
<td>0.60±0.32</td>
</tr>
<tr>
<td>1,4-DCB&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>3.8±1.5</td>
<td>2.1±0.85</td>
</tr>
<tr>
<td>CB&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.57±0.23</td>
<td>0.43±0.18</td>
</tr>
<tr>
<td>Benzene&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.32±0.23</td>
<td>0.37±0.27</td>
</tr>
<tr>
<td>TCE (low)&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>0.66±0.17</td>
<td>0.51±0.13</td>
</tr>
<tr>
<td>cDCE (low)&lt;sup&gt;b,e&lt;/sup&gt;</td>
<td>0.97±0.45</td>
<td>0.86±0.40</td>
</tr>
<tr>
<td>TCE (high)&lt;sup&gt;b,e&lt;/sup&gt;</td>
<td>8.4±1.3</td>
<td>6.5±1.0</td>
</tr>
<tr>
<td>cDCE (high)&lt;sup&gt;b,e&lt;/sup&gt;</td>
<td>16±3.1</td>
<td>14±2.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> HCH aqueous phase concentrations were measured and converted to µmol/bottle by assuming all of the mass resided in the aqueous phase.

<sup>b</sup> The µmol/bottle was measured based on headspace analysis and then converted to aqueous phase concentrations based on Henry’s Law constants at 23 °C and the with headspace=70 mL for soil and 74 mL for dolomite, and liquid phase volume=75 mL.

<sup>c</sup> Averages of 46 bottles ± standard deviation.

<sup>d</sup> Averages of 36 bottles ± standard deviation.

<sup>e</sup> Averages of 25 bottles ± standard deviation.
Table 2.1 Incubation Times and Average Percent Removals for Killed Controls and As-Is Treatments

<table>
<thead>
<tr>
<th>Soil Type</th>
<th>Incubation time (d)</th>
<th>Percent decrease for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Killed Controls</td>
<td>Live, Anaerobic&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Incubation time (d)</td>
<td>248</td>
<td>172</td>
</tr>
<tr>
<td>a-HCH</td>
<td>38±21&lt;sup&gt;e&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>γ-HCH</td>
<td>61±33</td>
<td>100</td>
</tr>
<tr>
<td>Benzene</td>
<td>0</td>
<td>31±29</td>
</tr>
<tr>
<td>CB</td>
<td>21±14</td>
<td>0</td>
</tr>
<tr>
<td>1,2-DCB</td>
<td>0</td>
<td>99</td>
</tr>
<tr>
<td>1,3-DCB</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1,4-DCB</td>
<td>1.2±.07</td>
<td>46±7.8</td>
</tr>
<tr>
<td>1,2,4-TCB</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>cDCE</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>TCE</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on results from Figures 2.2a, b and c.
<sup>b</sup> Based on results from Figures 2.3a, b and c (first compound addition only).
<sup>c</sup> Based on results from Figures 2.4a, b and c.
<sup>d</sup> Based on results from Figures 2.5a, b and c (first compound addition only).
<sup>e</sup> ± represents the standard deviation for triplicate microcosms.
Figure 2.1 Experimental design for the treatments subjected to anaerobic/aerobic conditions (sequence I) and aerobic/anaerobic conditions (sequence II); AI=as-is; BL= biostimulation with lactate; BA= bioaugmentation; Low and High=relative concentrations of TCE and cDCE added.
Figure 2.2 Results for soil microcosms with low initial concentrations of TCE and cDCE under as-is anaerobic conditions (a, b, c; average of three bottles) and anaerobic biostimulation with lactate followed by a switch to aerobic conditions on day 136 (d, e, f; average of six bottles) ↓=lactate addition; O₂=oxygen addition.
Figure 2.3 Results for soil microcosms with low initial concentrations of TCE and cDCE under as-is aerobic conditions (a, b, c; average of three bottles) and aerobic conditions followed by anaerobic biostimulation with lactate on day 137 (d, e, f; average of six bottles) ↓=lactate addition.
Figure 2.4 Results for dolomite microcosms with low initial concentrations of TCE and cDCE under as-is anaerobic conditions (a, b, c; average of three bottles) and anaerobic bioaugmentation followed by a switch to aerobic conditions on day 393 (d, e, f; average of three bottles) ↓=lactate addition; ⭐ =bioaugmentation; O₂= oxygen addition.
Figure 2.5 Results for dolomite microcosms with low initial concentrations of TCE and cDCE under as-is aerobic conditions (a, b, c; average of three bottles) and aerobic conditions followed by anaerobic bioaugmentation on day 101 (d, e, f; average of three bottles); ↓=lactate addition; ★ = bioaugmentation.
Figure 2.6 First order biodegradation rate coefficients comparing aerobic conditions for sequence I (anaerobic/aerobic) versus sequence II (aerobic/anaerobic) at (a) low and (b) high levels of TCE and cDCE. No bar indicates the biodegradation rate was zero. Error bars represent standard errors based on regression fits to pooled data for 6-9 bottles. A horizontal line over adjacent treatments means they are statistically different (α=0.05); ▼ = rate could not be calculated (i.e., the concentration of the compound was too low by the time this condition was reached so that a rate could not be determined).
Figure 2.7  First order biodegradation rate coefficients comparing the highest aerobic rate versus the highest anaerobic rate at low and high levels of TCE for (a) soil and (b) dolomite microcosms. No bar indicates the biodegradation rate was zero. Error bars represent standard errors based on regression fits to pooled data for 9 bottles. A horizontal line over adjacent treatments means they are statistically different (α=0.05).
Figure 2.8 First order biodegradation rate coefficients comparing the effect of low and high levels of TCE and cDCE in (a) soil and (b) dolomite microcosms.
Figure 2.9  First order biodegradation rate coefficients comparing soil and dolomite microcosms under (a) anaerobic conditions from sequence I (anaerobic/aerobic) and (b) aerobic conditions from sequence II (aerobic/an aerobic). No bar indicates the biodegradation rate was zero. Error bars represent standard errors based on regression fits to pooled data for 9 bottles. A horizontal line over adjacent treatments means they are statistically different ($\alpha=0.05$).
3. USE OF γ-HEXACHLOROCYCLOHEXANE AS A TERMINAL ELECTRON ACCEPTOR BY ANAEROBIC ENRICHMENT CULTURES

3.1 ABSTRACT

In a previous microcosm study for an industrial site contaminated with hexachlorocyclohexane (HCH) isomers (predominantly γ-HCH), we observed rapid anaerobic biodegradation of γ-HCH to benzene and chlorobenzene. The pattern and rate of activity suggested that γ-HCH may be used as a terminal electron acceptor. Using inoculum from microcosms that exhibited high rates of γ-HCH reduction, enrichment cultures were developed in groundwater from the industrial site and subsequently transferred to an anaerobic mineral medium without loss of γ-HCH dechlorination. Analysis of the enrichment culture by denaturant gradient gel electrophoresis and sequencing revealed the presence of a Desulfomicrobium sp. and several uncultured bacteria. The absence of a Dehalobacter sp. (previously reported to anaerobically biodegrade β-HCH) was verified by amplification of its (16S rRNA gene with Dehalobacter specific primers. The culture was further enriched in a sulfate free media with two different types of buffers (bicarbonate and HEPES) and γ-HCH as the only terminal electron acceptor and hydrogen as electron donor. Electron balance calculations in the bicarbonate buffered enrichment cultures revealed that only a small fraction of the hydrogen was involved in γ-HCH dechlorination; most was consumed for acetogenesis. Based on the fraction of electron equivalents used for γ-HCH dechlorination in HEPES-
buffered enrichment culture and the ability to transfer this culture with γ-HCH as the sole terminal electron acceptor, this study is the first to demonstrate chlororespiration of γ-HCH. Molecular analysis of enrichment cultures (in bicarbonate and HEPES buffered medium) in this study did not provide sufficient information to associate a specific microbe with chlororespiration of γ-HCH. The development of γ-HCH dechlorinating culture in this study will improve our understanding of remediation of γ-HCH by natural attenuation and engineered approaches. However, γ-HCH dechlorination will have to be coupled with improvements in anaerobic bioremediation of the terminal dechlorination products, benzene and chlorobenzene or aerobic treatment of dechlorination products to ensure that the endpoint is environmentally acceptable.

3.2 INTRODUCTION

Use of lindane as an insecticide has resulted in global environmental release of 10 million tons and subsequent contamination of soil, groundwater and the atmosphere (25). Lindane consists of more than 90% of γ-hexachlorocyclohexane (HCH) plus lower levels of other HCH isomers (α, β and δ). HCH isomers differ in the orientation of their chlorine atoms (equatorial or axial) (35). The axially oriented chlorine atoms are known to be active sites for enzymatic degradation and therefore, γ-HCH and α-HCH, each with three axial chlorine atoms, are more easily biodegraded relative to other isomers, including β and δ (35). Only γ-HCH is known to exhibit insecticidal properties but both lindane and technical grade lindane (10-15% γ-HCH + 85-90% α-, β- and δ-HCH) have been used worldwide as an insecticide. Each of the major isomers in technical grade HCH exhibit different degrees of acute and chronic toxicity (46, 48). γ-HCH is also
known to cause renal, liver and reproductive problems in animals. According to the United States Environmental Protection Agency (43), technical grade HCH is a probable human carcinogen and the maximum contaminant level for lindane in drinking water is 0.2 µg/L.

Biodegradation of γ-HCH under anaerobic conditions has been widely reported (35) with pure cultures (6, 28, 34), enrichment cultures (6, 31, 44), and soil slurries (4). Benzene and chlorobenzene (CB) are the predominant end products of γ-HCH anaerobic dechlorination (5-6, 31), although other intermediates or products include tetrachlorobenzene, trichlorobenzenes, dichlorobenzenes, tetrachlorocyclohexadiene, and pentachlorocyclohexadiene (36). The earliest reported γ-HCH degrading anaerobic organism, *Clostridium sphenoides* was initially thought to use γ-HCH as a sole carbon and energy source (28). Later studies with $^{3}$H/$^{36}$Cl-labeled HCH demonstrated the release of $^{36}$Cl but not $^{3}$H, indicating that the cyclic structure was not cleaved to release hydrogen or carbon, suggesting the use of γ-HCH as an electron acceptor under anaerobic conditions rather than as a carbon source (21). All subsequent studies demonstrated the need for an exogenous electron donor for anaerobic γ-HCH dechlorination (6, 24, 34-36). Pure cultures of the sulfate reducing bacteria *Desulfovibrio gigas*, *D. africanus* and *Desulfococcus multivorans* are able to dechlorinate γ-HCH in the presence of sulfate (5-6). However, γ-HCH dechlorination was also observed with enrichment cultures in the absence of sulfate (6, 31), suggesting that sulfate reduction is not a requirement for anaerobic dechlorination of γ-HCH.
A co-culture of *Dehalobacter* and *Sedimentibacter* was shown to reductively dechlorinate β-HCH. The co-culture was maintained through successive transfers over three years with hydrogen as the electron donor and β-HCH as the only terminal electron acceptor, leading van Doesburg et al. (44) to conclude that β-HCH dechlorination occurs via chlororespiration. The same culture was also able to dechlorinate γ-HCH, although use of γ-HCH as a terminal electron acceptor was not demonstrated.

Numerous anaerobic bacteria have been identified that respire chlorinated aliphatic and aromatic compounds (13, 20, 29, 38). A variety of approaches have been used to demonstrate the occurrence of chlororespiration. For example, with *Dehalococcoides ethenogenes* strain 195, an increase in cell counts and protein occurred during chlororespiration of tetrachloroethene (30). With *Dehalococcoides* sp. strain BAV1, chlororespiration of vinyl chloride was demonstrated by an increase in 16S rRNA gene copies (17). In mixed cultures, chlororespiration of various compounds was demonstrated based on the fraction of electron equivalents from the electron donor used for reductive dechlorination ($f_e$); $f_e$ values of 0.6-0.7 were indicative of chlororespiration, while much lower values indicated that dechlorination was cometabolic (26). To our knowledge, reductive dechlorination of γ-HCH has not yet been linked to chlororespiration and microbes with such capability have not been identified. The objectives of this study were to demonstrate that γ-HCH can be used as a terminal electron acceptor via chlororespiration and to identify the microbes involved in this process.
3.3 MATERIAL and METHODS

3.3.1 Chemicals and media

The sources and purity of chemicals used were: γ-HCH (99%) from Sigma-Aldrich; CB (99.5%) from TCI America; benzene (99%) from Fisher Scientific; anhydrous sodium acetate (99%) from EM Science; sodium lactate from Mallinckrodt Baker, Inc. (60% w/w) syrup); yeast extract from Difco Laboratories; vancomycin (biotechnology grade) from Sigma-Aldrich; HEPES from VWR; hydrogen (99.99%) and methane (99%) from National Welders. All other chemicals used were reagent grade or equivalent in purity.

Three types of mineral salts medium (MSM) were used. MSM-1 was buffered at pH 6.5-7.2 with sodium bicarbonate in equilibrium with a headspace of 30% CO₂ and 70% N₂ and yeast extract was provided as a source of vitamins and growth factors (11). MSM-2 was the same as MSM-1 except that MgSO₄ was replaced with MgCl₂ (in order to remove all sulfate from the medium) and yeast extract was replaced with a defined vitamin mixture (11), to eliminate yeast extract as a potential electron donor; MSM-3 was the same as MSM-2 except it was buffered with 10 mM HEPES rather than bicarbonate (32) and the headspace was purged with high purity N₂ rather than the CO₂/N₂ gas mixture.

3.3.2 Analytical Methods

γ-HCH was analyzed by extracting aliquots of culture twice with hexane (23) and injecting 1 µL of hexane extract onto a Hewlett Packard 5890 Series II Plus gas chromatograph (GC) equipped with an HP 7673 autosampler, ZB 624 capillary column
(30-m x 0.53-mm x 3.0-µm film; Zebron) and electron capture detector. Before taking samples, microcosms were placed upright to allow the particulates to settle out and an aliquot of the clarified aqueous phase (200 µL) was transferred to a 2 mL glass vial. Hexane (500 µL) was added and the vial was closed with a screw cap and vortexed (Baxter Scientific Vortex Mixer) for 2 min. The hexane was then transferred to a clean 2 mL glass vial. Additional hexane (500 µL) was added to the first vial and the process was repeated to further extract γ-HCH from the sample. Hexachlorobenzene was added (0.56 ng/sample, dissolved in hexane) to the combined 1 mL of hexane extract, to serve as an internal standard. The injector, oven and detector temperatures were maintained at 220, 240 and 250°C, respectively. Helium (5.5 mL/min) was used as the carrier gas and nitrogen (30 mL/min) was the makeup gas. Elution times for hexachlorobenzene and γ-HCH were 14.4 and 18.4 min, respectively. The detection limit for γ-HCH was 0.2 µg/L.

Methane, benzene and CB were analyzed by injecting headspace samples (500 µL) onto a HP 5890 Series II Plus GC equipped with an RTX 5 column (30-m x 0.53-mm x 1.5-µm film; Restek Corp.) and flame ionization detector. The injector and detector temperatures were 250 and 325°C, respectively. The oven temperature program was 50°C for 4 min, increased at 10°C/min to 80°C, and hold 2 min. Helium (5 mL/min) was used as the carrier gas and nitrogen (30 mL/min) was the makeup gas. The GC response to a headspace sample was calibrated to give the total mass of compound (M) in that bottle (15). Assuming the headspace and aqueous phases were in equilibrium, the total mass present was converted to an aqueous phase concentration (Eq 3.1):

\[ C_l = \frac{M}{(V_l + H_c \cdot V_g)} \]  

(3.1)
where \( C_l \) = concentration in the aqueous phase (µM); \( M \) = total mass present (µmol/bottle); \( V_l \) = volume of the liquid in the bottle (L); \( V_g \) = volume of the headspace in the bottle (L); and \( H_c \) = Henry's constant ((mol·m\(^{-3}\) gas concentration)/(mol·m\(^{-3}\) aqueous concentration)) at 23°C (42). Aqueous phase detection limits were 1.0 µg/L for benzene and 2.0 µg/L for CB.

Hydrogen was analyzed by injecting headspace samples (100 µL) onto a HP 5890 Series II GC equipped with a thermal conductivity detector and Carboxive SII 100/120 column (1.0-m x 3.1-mm, Supelco). The injector, oven and detector temperatures were maintained at 200, 105 and 200°C, respectively. High purity nitrogen was used as the carrier gas (30 mL/min) and reference gas (30 mL/min).

Sulfate was quantified on a Dionex AS50 ion chromatography system equipped with a CD25 conductivity detector, Dionex guard column (AG9-HC, 4-mm x 50-mm) and IonPac\textsuperscript{®} AS9-HC anion-exchange column (4-mm x 250-mm) with 9 mM Na\(_2\)CO\(_3\) as the eluant (1.0 mL/min). Lactate and acetate were analyzed on a Waters 600E HPLC system composed of an autosampler (Waters 717 plus), pumping system (Waters 600), a UV/Vis detector (Model 490E) set at 210 nm, and an Aminex\textsuperscript{®} HPX-87H ion exclusion column (300-mm x 7.8-mm; BioRad) with 0.01 N H\(_2\)SO\(_4\) as eluant (0.6 mL/min).

3.3.3 Enrichment Cultures

Cultures were grown in 160 mL serum bottles with 100 mL of liquid. They were prepared in an anaerobic chamber containing an atmosphere of approximately 98% N\(_2\) and 2% H\(_2\). \( \gamma \)-HCH was added either dissolved in methanol or as neat crystals. The bottles were sealed with Teflon-faced red rubber septa and aluminum crimp caps.
Headspace were purged with either high purity nitrogen or 30% CO₂ and 70% N₂. Bottles were incubated in the anaerobic chamber under quiescent conditions, in an inverted position.

Four types of enrichment cultures were developed. Enrichment I was started with groundwater from an industrial site contaminated with α- and γ-HCH, benzene, and chlorinated benzenes. We previously performed a microcosm study with groundwater and soil or crushed dolomite from this site, to compare sequential anaerobic/aerobic versus aerobic/anaerobic bioremediation (12). Several of the anaerobic soil microcosms exhibited high rates of HCH biodegradation; these were used as the source of inoculum for enrichment culture I in this study. γ-HCH was delivered dissolved in methanol (0.38 g/L) to provide an initial aqueous concentration of 10 mg/L (3.3 µmol/bottle), which is approximately the saturation level (35). Lactate was also added (500-600 mg/L). The total initial amount of electron donor exceeded the amount needed for complete dechlorination of the γ-HCH by a factor of 375. Samples of the liquid were removed periodically to measure γ-HCH; when the concentration decreased below 1.0 mg/L, more was added.

Enrichment II was initiated by making a 1% transfer (v/v) from enrichment I to MSM-1. In the course of making eight transfers in MSM-1, use of lactate was stopped, leaving methanol as the only available electron donor; it was also used to deliver the γ-HCH. Samples of the liquid were removed periodically to measure γ-HCH; when the concentration decreased below approximately 1.0 mg/L, more was added. At least 40 mg/L of cumulative γ-HCH was consumed before making a transfer to fresh MSM-1.
Enrichment III was prepared by making a 1% transfer (v/v) from enrichment II to MSM-2. At this stage, hydrogen was the only electron donor added; γ-HCH was added as neat crystals rather than dissolved in methanol. Hydrogen (5.0 mL/bottle) was added as a neat gas. Initially, 10 mg of γ-HCH was added; when measurement of benzene + CB indicated that 60-80% of the γ-HCH had been consumed, the bottles were opened in the anaerobic chamber, 30-50 mg of additional γ-HCH was added, the headspaces were purged with the N₂/CO₂ gas, and the bottles were resealed. Enrichment III was transferred to fresh medium four times (1% v/v). At least 4 mg/L of benzene and 14 mg/L of CB were formed as γ-HCH dechlorination products before making a transfer to fresh MSM-2. Some of the treatments evaluated with enrichment III included addition of acetate (i.e., 500-600 mg/L) without hydrogen, to determine if acetate could serve as an electron donor; addition of acetate (50-100 mg/L) along with hydrogen, to determine if acetate was needed as a carbon source; and the effect of vancomycin (100 mg/L, based on a previous study by DiStefano et al. (9)) on the rate of γ-HCH dechlorination.

Enrichment IV was prepared by making a 1% transfer (v/v) from enrichment III to MSM-3. This enrichment was transferred to fresh medium (1% v/v) three times. It was maintained in the same manner as enrichment III; i.e., γ-HCH was added as neat crystals; hydrogen was the only electron donor added; and some of the treatments received acetate as a potential carbon and energy source. At least 8 mg/L of benzene and 34 mg/L of CB were formed as γ-HCH dechlorination products before making a transfer to fresh MSM-3.
3.3.4 Electron equivalents for $\gamma$-HCH dechlorination

For enrichments III and IV, the fraction of hydrogen consumed for $\gamma$-HCH dechlorination was calculated on an electron equivalent (eq) basis, as described by Löffler et al. (26). The net cumulative amount of hydrogen consumed (i.e., total minus hydrogen loss from autoclaved controls) was plotted against the cumulative amount of benzene + CB formed, using the conversions: 2 µeq/µmol H$_2$; 4 µeq/µmol CB; and 6 µeq/µmol benzene. The slope of the linear regression provided the fraction of hydrogen used for $\gamma$-HCH dechlorination.

3.3.5 DNA Extraction, PCR and DGGE

Samples of enrichment cultures III (20-30 mL) and IV (60-80 mL) were pelleted at 7500 rpm and the genomic DNA was extracted using the UltraClean Soil DNA isolation kit (MO BIO Laboratories, Inc.) following the manufacturer’s alternative protocol for maximum yield (16). PCR reactions (50 µL) were performed to amplify the 16S rRNA gene on a Mini Cycler (MJ Research Inc., MA) using 30-50 ng of enrichment culture genomic DNA, 10 µM universal primers 1055 F (5’-ATG GCT GTC GTC AGC T-3’) and 1406 R (5’- CGC CCG CCG CGC CCC GCG CCC GGC CCG CCC CCG CCC CAC GGG CGG TGT GTA C-3’) and Promega Master Mix. PCR cycling routines consisted of 94°C for 5 min, and 25 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 1.5 min and a final extension at 72°C for 10 min (16). PCR products were separated by DGGE with a 10-50% gradient (33). Several bands obtained from the DGGE analysis were excised and amplified using the same universal bacterial primers that were initially used to amplify the genomic DNA. The amplified product was cleaned
using an Ultra Clean PCR Purification kit (MO BIO Laboratories, Inc.) following the manufacturer’s protocol and sequenced using the 1055 F universal primer at the Clemson University Genomics Institute. The closest sequence match was identified using GenBank’s BLASTN utility (NCBI BLAST database).

Genomic DNA from the bicarbonate enrichment cultures with methanol as the electron donor (i.e., enrichment III) was also amplified with universal primers 1055F and 1406 R and *Dehalobacter* specific forward primer (5’-CCT CTC CTG TCC TCA AGC CAT A-3’) and reverse primer (5’-GTT AGG GAA GAA CGG CAT CTG T-3’). The genomic DNA amplification with *Dehalobacter* specific primer was verified with a *Dehalobacter* positive control (8). Genomic DNA was extracted as described above. PCR reactions (50 µL) were performed on an Eppendorf PCR Thermocycler using 20-50 ng of genomic DNA with 10 µM primers. The PCR cycling routine consisted of ten cycles of 95°C for 2 min, 50°C for 1 min, 72°C for 1 min, 20 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension for 5 min at 72°C. PCR products that were amplified using universal primers were separated by DGGE using a 0-100% gradient and sequenced as described above (33).

### 3.3.6 Cloning and Identification

Genomic DNA from several of the enrichment culture III and IV treatments were extracted as described above and amplified using universal primers 8F (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 1541 R (5’-AAG GAG GTG ATC CAN CCR CA-3’) with PCR cycles of 94°C for 5 min, 25 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 1.5 min, and a final extension at 72°C for 10 min (16). The PCR product was cloned
with the TOPO-TA cloning kit (Invitrogen, Inc, CA) using the manufacturer’s protocol. White colonies from the clone library were verified for the presence of an insert by alkaline mini prep and digestion with EcoR1 and analysis by agarose gel electrophoresis. Clones were sequenced with primers M13F and M13R at the Clemson University Genomics Institute and the closest sequence matches were identified using GenBank’S BLASTN utility (NCBI BLAST database).

3.4 RESULTS

3.4.1 Enrichment in Groundwater and MSM-1

Following a lag of several weeks, γ-HCH dechlorination started in enrichment culture I (i.e., in groundwater) and rates increased with subsequent additions of γ-HCH (Fig. 3.1). Decreases in γ-HCH coincided with increases in benzene and CB, the final products of HCH dihaloelimination and dehydrohalogenation, respectively. Concurrent with dechlorination of γ-HCH was a significant level of methanogenesis. Approximately 10% of the electron equivalents of methanol and lactate added was used for methane formation, versus only 0.06% for γ-HCH dechlorination.

Following transfer (1% v/v) to MSM-1, a robust level of γ-HCH dechlorination continued in enrichment culture II (i.e., in MSM-1). When methanol was added as the electron donor without lactate, there was no noticeable decrease in the rate of γ-HCH dechlorination; subsequently, we stopped adding lactate as an electron donor. During successive transfers, enrichment culture I dechlorinated a total of 80-90 mg/L of γ-HCH at the rate of 1.0-1.5 mg/L/d. Benzene and CB were the only volatile daughter products detected. Methanogenesis continued to be a prominent activity, with greater than 90% of
the methanol added as electron equivalents recovered as methane. The sulfate present in MSM-1 (0.50 mM) was also consumed, indicating the occurrence of sulfidogenesis.

3.4.2 γ-HCH Dechlorination in Bicarbonate Buffered MSM-2

γ-HCH was rapidly dechlorinated to benzene and CB by enrichment culture III (i.e., in MSM-2) (Fig. 3.2a). In this version of the media, vitamins replaced the yeast extract, there was no sulfate present and γ-HCH was delivered as neat compound. Benzene and CB were the only volatile daughter products detected, accounting for 87±8.6% of the γ-HCH consumed (25% benzene + 62% CB). The addition of acetate along with hydrogen did not increase the rate of benzene and CB accumulation. Addition of vancomycin strongly inhibited dechlorination of γ-HCH (Fig. 3.2a). Hydrogen consumption was greatest in the treatments without γ-HCH present, indicating that another use for hydrogen was significant. Acetate accumulated as hydrogen was consumed in all of the treatments without vancomycin. Based on the amount of acetate formed, acetogenesis was the dominant process in enrichment culture III (Table 3.1). Hydrogen consumption was higher in the treatments that did not receive γ-HCH, suggesting that γ-HCH was at least partially inhibitory to acetogenesis (Fig. 3.2b; Table 3.1). Vancomycin significantly inhibited hydrogen consumption, in addition to inhibiting γ-HCH dechlorination; however, the treatments with vancomycin produced methane and consumed acetate, presumptively for methanogenesis (Table 3.1). The fraction of hydrogen electron equivalents consumed relative to the equivalents needed to form benzene and CB was 0.052 for the treatment with H₂ as electron donor and 0.056 for the treatment with H₂ and acetate added (Fig. 3.2c); the majority of hydrogen consumption in
enrichment culture III was associated with acetogenesis and methanogenesis. Dechlorination of \(\gamma\)-HCH did not occur in uninoculated MSM-3 controls, based on a lack of any accumulation of benzene or CB.

3.4.3 \(\gamma\)-HCH Dechlorination in MSM-3

In order to prevent hydrogen use for acetogenesis, enrichment culture III in MSM-2 (bicarbonate buffered) was used to initiate enrichment culture IV in MSM-3 (HEPES buffered). Following a lag period, \(\gamma\)-HCH dechlorination to benzene + CB occurred at a higher overall rate in the treatments with hydrogen available (Fig. 3.3a) versus the companion enrichment III treatments in MSM-2 (Fig. 3.2a). Accumulation of benzene and CB reached 17 and 51 \(\mu\)mol/bottle (Table 3.1), or 26 mg/L and 76 mg/L, respectively, when taking partitioning between the headspace and liquid into account. In spite of these high concentrations, there was no apparent inhibition of \(\gamma\)-HCH dechlorination. Without hydrogen added, little or no \(\gamma\)-HCH dechlorination to benzene and CB occurred (Fig. 3.3a). None of the treatments in MSM-3 yielded any accumulation of acetate or methane (Table 3.1). Enrichment culture IV with hydrogen as the electron donor (with and without acetate added) was successfully maintained for one year through three transfers (1% v/v), suggesting that \(\gamma\)-HCH dechlorination is linked to the growth of microbes mediating this reaction.

Consistent with the extent of benzene and CB accumulation, the highest level of hydrogen consumption occurred in the MSM-3 treatments with \(\gamma\)-HCH added (Fig. 3.3b). A low level of hydrogen consumption occurred in the inoculated treatments without \(\gamma\)-HCH added, which was only slightly greater than the hydrogen consumption in
uninoculated sterile controls. Hydrogen consumption in the latter was likely due to diffusive losses.

The fraction of hydrogen electron equivalents consumed relative to the equivalents needed to form benzene and CB was 0.82 and for the treatment with H$_2$ as electron donor and 0.88 for the treatments with H$_2$ + acetate added (Fig. 3.3c). These values are considerably higher than for enrichment culture III in MSM-2 (Fig. 3.2c), consistent with the lack of acetogenesis and methanogenesis in HEPES-buffered MSM-3 (Table 3.1).

A transfer was made from the MSM-3 enrichment IV that received γ-HCH + hydrogen (with and without acetate) back to MSM-2. The rate of benzene and CB formation was very similar to the rate shown in Figure 3.2a (Appendix 6.3.1); however, the amount of hydrogen consumed decreased by one order of magnitude to approximately 140 µmol/bottle (versus 1654-1786 in enrichments always maintained in MSM-2; Table 3.1), and no methane was produced (versus 51-58 µmol/bottle; Table 3.1). Thus, it was possible to achieve γ-HCH dechlorination in MSM-2 at much lower levels of acetogenesis and no methanogenesis.

3.4.4 DGGE and Cloning Analysis

DGGE analysis of PCR-amplified DNA from the MSM-1 enrichment cultures with and without γ-HCH added did not reveal significant differences, with methanol serving as the sole electron donor in both treatments (Appendix 6.3.2). Sequencing of bands excised from the DGGE gel revealed the presence of a Desulfomicrobium sp. and several uncultured bacteria. In the same enrichment cultures, the absence of a
*Dehalobacter* sp. was indicated by a lack of DNA amplification using *Dehalobacter* specific primers. *Dehalobacter* sp. have previously been shown to anaerobically dechlorinate β-HCH in other enrichment cultures (44).

DGGE analysis of selected treatments from enrichments III and IV is shown in Figure 3.4; sequencing results for 12 of the prominent bands excised from the gel are shown in Table 3.2. The enrichment III cultures that were evaluated included two that received γ-HCH (Fig. 3.4, lanes C and D) and two that did not (lanes E and F). In spite of this difference, there were no apparent differences in the predominant bands in these lanes. Bands 4, 6, 7, 9, 10 and 12 were common to the six treatments evaluated (Fig. 3.4) and most closely matched an uncultured *Pseudomonas* (Table 3.2). Bands 8 and 11 were present in the two enrichment III cultures that received H₂ and acetate and most closely matched *Sporomusa* sp. Less prominent bands at the same position were also observed in the enrichment III treatments that received only H₂ (lanes D and F), although these were not sequenced. Bands 1, 2, 3 and 5 were present in the enrichment IV culture with γ-HCH, H₂ and acetate (lane A) and included two iron-reducing bacteria, another uncultured *Pseudomonas*, and an uncultured β-Proteobacterium (Table 3.2). Bands at the same position as 1 and 2 were also evident in lane F.

Clone libraries were developed for three of the treatments. Of the six clones sequenced for the enrichment IV culture with γ-HCH and H₂ (corresponding to Fig. 3.4, lane A), three most closely matched an uncultured *Pseudomonas* sp., two matched an uncultured bacterium and one matched an iron reducing bacterium. Of the eight clones sequenced for the enrichment III culture with γ-HCH and H₂ (corresponding to Fig. 3.4,
lane D), four matched an uncultured *Pseudomonas* sp., three clones matched a *Sporomusa* sp. and one matched an uncultured bacterium. Of the eight clones sequenced for the enrichment III culture with only H₂ (corresponding to Fig. 3.4, lane F), five matched an uncultured *Pseudomonas* sp., two matched an uncultured bacterium and one matched a *Desulfovibrio* sp. Accession numbers for each clone sequence are provided in Appendix 6.3.3.

3.5 DISCUSSION

Based on the fraction of electron equivalents used for γ-HCH dechlorination in HEPES-buffered enrichment culture IV and the ability to transfer this culture with γ-HCH as the sole terminal electron acceptor, this study is the first to demonstrate chlororespiration of γ-HCH. The fraction of electron equivalents used for γ-HCH dechlorination to a mixture of benzene and CB ranged from 82-88%, confirming that γ-HCH dechlorination was the major sink for H₂ consumption. This range for fₐ values is consistent with ones reported for other chlororespiratory processes (26, 37). The HEPES-buffered enrichment culture was maintained on H₂ as the sole electron donor and γ-HCH as the sole electron acceptor through three transfers (1% vol/vol) to fresh MSM-3 over a one year period, without loss of dechlorination activity. These results are consistent with other reports of growth associated with the use of chlorinated compounds as the terminal electron acceptor (14, 44).

Attempts were made to quantify growth in enrichment cultures III and IV based on protein measurements (Appendix 6.3.4). However, due to the relatively slow growth rate with γ-HCH as the electron acceptor (i.e., compared to other chlorinated organic compounds), the apparently low yield, and the difficulty of
measuring protein in a medium containing iron sulfide precipitates, protein measurements were not conclusive. Similar problems in quantifying growth by protein levels were encountered with enrichment cultures that reductively dechlorinate β-HCH (1, 44) and chlorinated benzenes (1, 44).

The source of carbon used by the HEPES-buffered enrichment culture IV during chlororespiration of γ-HCH is not yet clear. Acetate is a common carbon source for many chlororespiring cultures that use hydrogen as an electron donor. In the treatments that received acetate along with hydrogen and γ-HCH, the rate of γ-HCH dechlorination (based on the rate of benzene and CB accumulation) become higher beginning on day 60 (Fig. 3.3a). However, there was no detectable decrease in acetate (Table 3.1 and Appendix 6.3.5), although the amount of acetate used for biomass synthesis may have been within the error of the HPLC method used. Since we were able to make several transfers of the treatment that received only hydrogen and γ-HCH, the possibility of HEPES serving as the carbon source cannot be ruled out. Enrichment cultures that reductively dechlorinate trichlorobenzene to dichlorobenzene with only H₂ additions has been reported, although activity was eventually lost over successive transfers (2). Additional studies are needed to conclusively identify the carbon source(s) used during chlororespiration of γ-HCH.

γ-HCH dechlorination rates were notably higher in the HEPES-buffered enrichment culture (Fig. 3.3a) compared to the bicarbonate-buffered enrichment culture (Fig. 3.2a). Competing demands for hydrogen is a likely explanation. In the bicarbonate-buffered enrichment culture, acetogenesis and methanogenesis were the dominant uses
for hydrogen (Table 3.1), while both of these processes were absent in the HEPES-buffered enrichment culture. The effect of these competing hydrogenotrophic processes on chlororespiration is widely reported (10, 19, 49). By eliminating sulfate from MSM-2 and MSM-3, it was possible to rule out cometabolic reductive dechlorination of γ-HCH by sulfate reducing microbes, as described previously (5-6). Although acetate was added in considerable excess to both HEPES and bicarbonate-buffered treatments, the results indicate that acetate was not used as an electron donor. This was most evident in the HEPES-buffered treatment that received acetate without hydrogen; only a minor amount of γ-HCH dechlorination occurred (Fig. 3.3a). Among chlororespiring mixed and pure cultures, use of hydrogen as an electron donor is more common than acetate (3, 26, 44).

Addition of vancomycin (100 mg/L) to the enrichment culture III treatment with H₂ as the electron donor strongly inhibited γ-HCH dechlorination. Similar results were reported with an enrichment culture that reductively dechlorinates β-HCH (31, 50) and another that dechlorinates 4,5,6,7-tetrachlorophthalide (31, 50); both enrichments contained Dehalobacter spp. In other cases, addition of vancomycin (100-500 mg/L) has not inhibited reductive dechlorination. For example, 100 mg/L of vancomycin did not impact reductive dechlorination of tetrachloroethene to ethene by an enrichment culture later shown to contain Dehalococcoides ethenogens (2, 9); and 500 mg/L did not inhibit chlororespiration of polychlorinated benzenes, phenols and dibenzodioxins (2, 9). The effect of vancomycin on γ-HCH dechlorination in this study suggests that the microbe responsible is most likely non-spore forming and gram positive.
Molecular analysis of enrichment cultures III and IV in this study did not provide sufficient information to associate a specific microbe with chlororespiration of γ-HCH. None of the 16S rRNA sequences identified by DGGE or in the clone libraries correspond to known dechlorinators, including *Dehalobacter* spp. Inhibition of γ-HCH dechlorination and acetogenesis by vancomycin suggests that the microbe(s) responsible for γ-HCH dechlorination share some degree of similarity to acetogens, many of which are sensitive to vancomycin. Acetogens are common in dechlorinating enrichment cultures and play a key role in fermentation of organic substrates. However, they can also compete with dechlorinating microbes for hydrogen; their direct role in reductive dechlorination has usually been restricted to cometabolism (10, 19, 26, 41, 45, 47). In the enrichment cultures developed in this study, dechlorination of γ-HCH was a respiratory process and it is conceivable that an acetogen was responsible.

Each type of MSM used in this study to enrich for γ-HCH dechlorination was strongly reduced with iron sulfides. The occurrence of acetogenesis and methanogenesis in the bicarbonate-buffered enrichment cultures (Table 3.1) confirmed the establishment of low redox conditions. For this reason, the occurrence of *Pseudomonas* spp. in all of the treatments evaluated (Table 3.2) was unexpected. *Pseudomonas* spp. are most commonly associated with aerobic or nitrate reducing conditions. It is unlikely that they played a direct role in γ-HCH dechlorination, since they are not impacted by vancomycin. The presence of a *Sporomusa* sp. in the bicarbonate-buffered medium (Table 3.2) was consistent with the significant level of acetogenesis from H$_2$ + CO$_2$ in enrichment culture III (Table 3.1) (22). Several types of iron reducing bacteria are known to chlororespire
(18, 39-40); two strains were identified in enrichment culture IV by DGGE (Table 3.2) and one in the clone library, although none were recovered in bicarbonate-buffered enrichment culture III. Additional studies are needed to identify which type of microbe is responsible for chlororespiration of $\gamma$-HCH.

Advances in understanding the physiology and phylogeny of chlororespiration of chlorinated organic compounds have facilitated improvements in anaerobic bioremediation of chlorinated organic compounds. The development of $\gamma$-HCH dechlorinating enrichment cultures in this study creates an opportunity to improve anaerobic bioremediation of $\gamma$-HCH by natural attenuation and engineered approaches. Chlororespiring organisms efficiently couple chlorinated contaminant reduction to energy metabolism. Nevertheless, the main products of $\gamma$-HCH dechlorination, benzene and CB, are not environmentally acceptable endpoints. In order for complete anaerobic bioremediation of $\gamma$-HCH to become feasible, improvements are also needed in anaerobic biodegradation of benzene and CB. Reductive dechlorination of CB to benzene has recently been demonstrated (14) and cultures that anaerobically oxidize benzene have been reported (7, 27), although further advances will be needed before a fully anaerobic approach is feasible in situ. An alternative approach is to follow reductive dechlorination of $\gamma$-HCH with aerobic biodegradation of benzene and chlorobenzene.
3.6 REFERENCES


Table 3.1 Mass Balance between Hydrogen Consumed and Benzene and CB Produced from γ–HCH Dechlorination

<table>
<thead>
<tr>
<th>Enrichment culture (medium)</th>
<th>Treatment(^a)</th>
<th>(\text{H}_2) consumed (µmol/btl)(^b)</th>
<th>Benzene formed (µmol/btl)</th>
<th>CB formed (µmol/btl)</th>
<th>CH(_4) formed (µmol/btl)</th>
<th>Acetate (µmol/btl)(^c)</th>
<th>eq Balance (%)(^d)</th>
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</thead>
<tbody>
<tr>
<td>III (MSM-2)</td>
<td>(\text{H}_2+\text{Ac}+\gamma\text{-HCH})</td>
<td>1654±29(^e)</td>
<td>11±0.90</td>
<td>28±4.5</td>
<td>51±21</td>
<td>325±34</td>
<td>97</td>
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<tr>
<td>III (MSM-2)</td>
<td>(\text{H}_2+\text{Ac}+\gamma\text{-HCH}+\text{Vcm})</td>
<td>936±80</td>
<td>3±0.61</td>
<td>6±1.4</td>
<td>125±36</td>
<td>-54±26</td>
<td>33</td>
</tr>
<tr>
<td>IV (MSM-3)</td>
<td>(\text{H}_2+\text{Ac}+\gamma\text{-HCH})</td>
<td>192±11</td>
<td>17±0.32</td>
<td>51±2.6</td>
<td>0</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>III (MSM-2)</td>
<td>(\text{H}_2+\gamma\text{-HCH})</td>
<td>1786±28</td>
<td>11±3.3</td>
<td>30±5.6</td>
<td>58±81</td>
<td>458±52</td>
<td>123</td>
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<td>III (MSM-2)</td>
<td>(\text{H}_2+\gamma\text{-HCH}+\text{Vcm})</td>
<td>940±17</td>
<td>5±1.3</td>
<td>9±2.1</td>
<td>98±33</td>
<td>46±16</td>
<td>67</td>
</tr>
<tr>
<td>IV (MSM-3)</td>
<td>(\text{H}_2+\gamma\text{-HCH})</td>
<td>178±7.9</td>
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<td>40±4.1</td>
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<td>0</td>
<td>67</td>
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<td>III (MSM-2)</td>
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<td>NA</td>
<td>NM(^g)</td>
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<td>III (MSM-2)</td>
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<td>NA</td>
<td>NM</td>
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<td>NA</td>
<td>NM</td>
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<tr>
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<td>(\text{H}_2)</td>
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<td>NA</td>
<td>NA</td>
<td>NM</td>
<td>0</td>
<td>-</td>
</tr>
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</table>

\(^a\) Ac = acetate; Vcm = vancomycin.
\(^b\) \(\text{H}_2\) consumed in each treatment after subtracting for \(\text{H}_2\) consumed in media controls.
\(^c\) A positive number indicates acetate accumulation, a negative number indicates net consumption; btl = bottle.
\(^d\) eq balance = [( benzene*6 + CB*4 + CH\(_4\)*8 + acetate formed*8) / (\(\text{H}_2\)*2)]*100.
\(^e\) Average and standard deviation of triplicate bottles.
\(^f\) NA = not applicable, since \(\gamma\)-HCH was not added.
\(^g\) NM = not measured; headspace samples were evaluated for hydrogen; however, methane was not quantified.
Table 3.2 16S rRNA Sequencing Results for Bands Excised from the DGGE Gel Shown in Figure 3.4

<table>
<thead>
<tr>
<th>Lane in Fig. 4</th>
<th>Band in Fig. 4</th>
<th>Enrichment (medium)</th>
<th>Treatment</th>
<th>Closest Match</th>
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<td>H₂+γ-HCH</td>
<td>Iron reducing bacterium</td>
<td>GU565223</td>
<td>98</td>
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<tr>
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<td>H₂+γ-HCH</td>
<td>Uncultured <em>Pseudomonas</em></td>
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Figure 3.1 $\gamma$-HCH dechlorination by enrichment culture I with methanol and lactate as electron donors; each data point represents the average of triplicate bottles; Ben = Benzene; btl = bottle.
Figure 3.2 γ-HCH dechlorination by enrichment culture III; cumulative benzene and CB formation (a); cumulative H₂ consumption (b); and determination of $f_e$ (c); Ac = acetate; Ben = Benzene; btl = bottle; Vcm = vancomycin; each data point represents the average of triplicate bottles.
Figure 3.3 γ-HCH dechlorination by enrichment culture IV; cumulative benzene and CB formation (a); cumulative H₂ consumption (b); and determination of \( f_c \) (c); Ac = acetate; Ben = Benzene; btl = bottle; Inc = inoculated with enrichment culture IV; each data point represents the average of triplicate microcosms.
Figure 3.4 DGGE profile of enrichment cultures in HEPES and bicarbonate buffered media; the description of each lane is given in the matrix. Numbered bands were excised and sequenced (Table 3.2).
4. AEROBIC COMETABOLISM OF TRICHLOROETHENE AND 
cis-DICHLOROETHENE WITH BENZENE AND CHLORINATED 
BENZENES AS GROWTH SUBSTRATES

4.1 ABSTRACT

Using inoculum from a previous microcosms study that exhibited aerobic 
transformation of cis-dichloroethene (cDCE) and trichloroethene (TCE) commensurate 
with biodegradation of the monoaromatic compounds, enrichment cultures were 
developed in groundwater by providing benzene, chlorobenzene, dichlorobenzene 
isomers and 1,2,4-trichlorobenzene as carbon and energy sources. These enrichments 
were subsequently transferred to a mineral salt medium and were grown on each of the 
monoaromatic compounds separately and were successfully maintained through several 
transfers. Isolates growing on benzene, chlorobenzene (CB), 1,2-dichlorobenzene (DCB) 
and 1,3-DCB were identified as Rhodococcus, Ralstonia, Variovorax and Ralstonia, 
respectively, by 16S rRNA gene sequence. The yield measured on the isolates growing 
on corresponding substrates ranged from 0.36-0.45 mg biomass/mg substrate, with 
highest yield on benzene and lowest yield on 1,3-DCB. Cometabolic transformation of 
cDCE and TCE evaluated based on pseudo-first order cometabolic degradation rate 
constant, transformation capacity and transformation yield for resting cells were observed 
to be on the low end of the reported values compared to phenol and toluene-grown 
isolates. Cometabolic transformation of cDCE and TCE was also evaluated with growing 
cells of each isolate and were observed to be on low end of reported values for TCE,
however, this is the first study to report for cDCE. In general, the cometabolic transformation parameters observed for cDCE were greater than TCE. The results of this study confirm the potential for cometabolism of cDCE and TCE during aerobic growth on benzene, CB, 1,2-DCB and 1,3-DCB. This is especially relevant for natural attenuation scenarios when these compounds occur as co-contaminants and may be transported from an anaerobic to an aerobic environment. Although it may seem reasonable to assume that all aerobically biodegradable monoaromatic compounds may serve as primary substrates for cDCE and TCE cometabolism, the wide range of dioxygenases involved indicates this is not necessarily the case.

4.2 INTRODUCTION

Trichloroethene (TCE) is mainly used as a solvent for degreasing metals and also as a component in adhesives, paints and spot removers. Improper storage and disposal has resulted in widespread contamination of groundwater and soil. cis-1,2-Dichloroethene (cDCE) does not have significant industrial application but occurs as a contaminant in groundwater and soil from incomplete anaerobic reductive dechlorination of tetrachloroethene and TCE. Both TCE and cDCE undergo aerobic transformation by cometabolic reactions catalyzed by oxygenases (3, 22, 28), a process that does not yield the energy or carbon needed for cell growth (22). In spite of intensive efforts to find a microbe that can grow aerobically using TCE as a sole carbon and energy source, none has been identified. Several reports have shown that cDCE can be used as a growth substrate under aerobic conditions (4, 7, 19, 29); two of these studies have identified
isolates, including *Polaromonas vacuolata* (7), *Bacillus, Burkholderia, Corynebacterium, Micrococcus* and *Pseudomonas* (29) that can grown on cDCE.

Studies with numerous pure and mixed cultures have demonstrated aerobic cometabolism of TCE and cDCE using a wide range of growth substrates, including toluene (39), phenol (18), methane (41), propane (11), propene (10), ammonia (38) and butane (2-3). Of these growth substrates, toluene and methane are among the most intensively evaluated (2). Cometabolic transformation of TCE with methane as the growth substrate is catalyzed by soluble and particulate methane monooxygenase (23, 35). *Methylosinus trichosporium* OB3b is perhaps the best characterized methanotroph that supports aerobic cometabolism of TCE (30). Aerobic metabolism of toluene and cometabolic transformation of TCE is supported by toluene 2-monooxygenase in *Burkholderia cepacia* G4 (12), toluene 4-monooxygenase in *Pseudomonas mendocina* KR1 (20), toluene 3-monooxygenase in *Ralstonia pickettii* PKO1 (20), and toluene-dioxygenase in *Pseudomonas putida* F1 (39). Isolates growing on toluene (16) and benzene (21) were able to cometabolize TCE in the presence of mixtures of toluene and monoaromatic hydrocarbons such as benzene, chlorobenzene (CB), and dichlorobenzene (DCB) isomers. However, not all toluene degrading microbes are capable of aerobic cometabolic transformation of TCE and significant variations have been observed in biodegradation of aromatic mixtures, suggesting that oxygenase activity varies depending on the substrate and that different types of oxygenases can be induced in the presence of mixtures of monoaromatic compounds (13, 16, 20, 26). Monoaromatics such as benzene and chlorinated benzenes occasionally occur as co-contaminants with chlorinated ethenes.
It seems likely that most monoaromatic compounds are good candidates to serve as primary substrates for cometabolism of chlorinated ethenes. However, considerably less is known about cometabolism associated with monoaromatic compounds other than toluene and phenol.

During a microcosm study with groundwater, soil, and crushed dolomite from an industrial site contaminated with TCE, cDCE, and several monoaromatic compounds, we observed aerobic biodegradation of TCE and cDCE. The monoaromatics included benzene, CB, 1,2-DCB, 1,3-DCB, 1,4-DCB and 1,2,4-trichlorobenzene (TCB). Decreases in TCE and cDCE occurred at the same time as the aromatic compounds were consumed and stopped when the aromatics were depleted. Beyond this correlation, however, we had no direct evidence to indicate that metabolism of the monoaromatics was responsible for the decreases in cDCE and TCE.

Under aerobic conditions, benzene, CB, DCB isomers and 1,2,4-TCB are converted to catechols by dioxygenases and dehydrogenases (15, 33, 37, 40, 42), which is similar to aerobic mineralization of toluene catalyzed by toluene 1,2-dioxygenase in *Pseudomonas putida* (39). The involvement of several dioxygenase enzymes in the biodegradation of benzene, CB, DCB isomers and 1,2,4-TCB, suggests that one or more of these can catalyze aerobic cometabolism of cDCE and TCE. However, no information was found in the literature that specifically tested these compounds as growth substrates for aerobic cometabolism of chlorinated ethenes. Also, earlier studies have demonstrated significant differences in cDCE and TCE aerobic cometabolic transformation depending on the type of organism, growth substrate, and type of oxygenases (2, 20, 26).
The effectiveness of a compound to serve as growth substrate for cometabolic transformation of chlorinated ethenes is commonly evaluated based on its transformation capacity \( T_c \) and transformation yield \( T_y \) \( (1) \). \( T_c \) is defined as the mass ratio of non-growth substrate removed (e.g., TCE and cDCE) to mass of resting cells grown on the primary substrate (e.g., monoaromatic compounds), while \( T_y \) is the mass ratio of non-growth substrate removed to growth substrate consumed \( (1) \). The objectives of this study were (i) to isolate and identify microbes that grow on benzene, CB, DCB isomers and 1,2,4-TCB, using as inoculum the aerobic microcosms described above; and (ii) to evaluate the ability of the isolates to cometabolize cDCE and TCE, with both resting cells grown on the various monoaromatic substrates and with cells that were actively consuming the monoaromatic compounds.

4.3 MATERIAL and METHODS

4.3.1 Chemicals and Media

The purity and sources of chemicals used were: 1,2,4-TCB (99%), 1,3-DCB (99%), 1,4-DCB (99%), CB (99.5%), cDCE (99%) from TCI America; benzene (99%), 1,2-DCB (99.5%), and TCE (99%) from Fisher Scientific. All other chemicals were reagent grade or equivalent. The mineral salts medium (MSM) described by Spain and Nishino \( (33) \) was used for enrichment and isolation of aromatic degraders.

4.3.2 Analytical Methods

Benzene, CB, 1,2-DCB, 1,3-DCB, 1,4-DCB, 1,2,4-TCB, TCE and cDCE were analyzed by injecting headspace samples (500 µL) onto a HP 5890 Series II Plus Gas Chromatograph (GC) equipped with an RTX 5 column (30-m × 0.53-mm × 1.5-µm film;
Restek Corp.) and flame ionization detector. The injector and detector temperatures were 250 and 325°C, respectively. The oven temperature program was 50°C for 4 min, increased at 10°C/min to 80°C, hold 10 min, increased at 10°C/min to 150°C, and hold for 1 min. Helium (5 mL/min) was used as the carrier gas and nitrogen (30 mL/min) was the makeup gas. The elution times were 2.8 min for cDCE, 3.3 min for benzene, 4.2 min for TCE, 7.9 min for CB, 14.8 min for 1,3-DCB, 15.3 min for 1,4-DCB, 17.1 min for 1,2-DCB, and 22.6 min for 1,2,4-TCB.

The GC response to a headspace sample was calibrated to give the total mass of compound \((M)\) in that bottle (14). Assuming the headspace and aqueous phases were in equilibrium, the total mass present was converted to an aqueous phase concentration (Eq. 1):

\[
C_l = \frac{M}{(V_l + H_c \cdot V_g)}
\]  

(4.1)

where \(C_l\) = concentration in the aqueous phase (µM); \(M\) = total mass present (µmol/bottle); \(V_l\) = volume of the liquid in the bottle (L); \(V_g\) = volume of the headspace in the bottle (L); and \(H_c\) = Henry's constant \(((\text{mol} \cdot \text{m}^{-3} \text{ gas concentration})/(\text{mol} \cdot \text{m}^{-3} \text{ aqueous concentration}))\) at 23°C (36). Aqueous phase detection limits were 1.0 µg/L for benzene, 2.0 µg/L for CB, 4.0 µg/L for 1,2-DCB, 3.0 µg/L for 1,3-DCB, 3.0 µg/L for 1,4-DCB, and 10 µg/L for 1,2,4-TCB.

Oxygen was analyzed by injecting a headspace sample (100 µL) onto a HP 5890 Series II Plus GC equipped with thermal conductivity detector and molecular sieve 5A 60/80 column (1.8-m × 3.1-mm; Alltech). The detector, oven, and injector temperatures
were 120, 70 and 120°C, respectively. Helium (30 mL/min) was used as the reference gas and carrier gas. The elution time for oxygen was 0.79 min.

Protein was measured with the BCA protein assay reagent (Pierce Chemical Company). Cells were lysed by adding 0.1 mL 10 M NaOH to 0.9 mL culture and heating at 90°C for 10 min (7). The lysed cells were cooled to room temperature and 50 µL was used for protein measurement following the manufacturer’s protocol. Protein was converted to biomass based on the assumption that protein accounts for approximately 50% of biomass (24).

4.3.3 Enrichment and Isolation

Enrichment cultures were developed on each monoaromatic substrate (i.e., benzene, CB, 1,2-DCB, 1,3-DCB, 1,4-DCB, and 1,2,4-TCB) as the sole carbon and energy source. The source of inoculum was the aerobic microcosms developed by Elango et al. (9) to compare sequential anaerobic/aerobic versus aerobic/anaerobic bioremediation of the industrial site mentioned above. Enrichment cultures were prepared in 160 mL serum bottles with 74 mL of groundwater and 1 mL of soil slurry from the microcosms, leaving 85 mL of headspace. Benzene and CB were added as water saturated solutions (since the volume needed was <1% v/v of the total aqueous volume) and 1,2-DCB, 1,3-DCB, 1,4-DCB and 1,2,4-TCB were added as groundwater saturated solutions (since the total needed was >1% of the total aqueous volume) to achieve 0.5 to 5.0 mg/L of each contaminant. TCE and cDCE were also added (0.5-1.0 mg/L) using water saturated solutions with the intent of favoring the growth of microbes that can grow in the presence of these co-contaminants. These concentrations mimic the
field conditions reported in our microcosm study (9). The bottles were sealed with Teflon-faced red rubber septa and crimp caps and incubated at room temperature (22-24°C) under quiescent condition in an inverted position. When three additions of monoaromatic compounds were consumed, 1.3% (v/v) transfers were made to MSM. Oxygen in the headspace was monitored periodically and pure oxygen was added to maintain 5-21%. The pH of the MSM was approximately neutral but decreased with increasing amounts of biodegradation; 10 M NaOH was added periodically to maintain the pH between 6.5 and 7.2.

After a total of 320-400 mg/L of the monoaromatics was consumed in the MSM enrichment cultures, serial dilutions were streaked on tripticase soy agar and incubated at room temperature in the dark for approximately 24 hr. Well isolated individual colonies were transferred to 160 mL serum bottles with MSM (100 mL) and the corresponding monoaromatic compound. Selected bottles that exhibited growth were streaked again on triplicate plates containing tripticase soy agar. Following approximately 24 hr of incubation, well isolated colonies were transferred to new serum bottles with MSM and the corresponding monoaromatic compound. One of these bottles was subsequently used as inoculum to grow each culture in a larger volume bottle (4.7 L). After consuming 1.7-2.7 g of the monoaromatic compounds, the pure cultures were frozen (-80°C), placed in a wide mouth filter seal flask attached to a freeze dryer (VirTis Benchtop 6K freeze drier model NEWBT6KES-EIV2), and a vacuum was applied (approximately 54 mTorr for 60 h) until lyophilization was complete. The dried product was stored at 4°C.
4.3.4 Identification

Isolates were identified based on the sequence of their 16S rRNA gene. DNA from the pure cultures was extracted with the UltraClean Microbial DNA isolation kit (MO BIO Laboratories, Inc), using the manufacturer’s protocol. PCR reactions (50 µL) were performed on an Eppendorf PCR Thermocycler using 20-50 ng of genomic DNA with 10 µM of universal primers 8F and 1492R. The PCR cycling routine consisted of ten cycles of 95°C for 2 min, 50°C for 1 min, 72°C for 1 min, 20 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension for 5 min at 72°C. The PCR product was cloned using the TOPO-TA cloning kit (Invitrogen, Inc, CA) and a clone library was created. White colonies from the library were verified for the presence of the insert by alkaline mini prep and digestion with EcoR1 and analysis by agarose gel electrophoresis. Clones positives for the insert were sequenced at the Clemson University Genomics institute and the sequences were analyzed using BLAST to search GenBank.

4.3.5 Growth Yields

Experiments to measure yields were started by adding 10 mg of lyophilized cells in MSM (containing approximately 1.6 mg protein) to 100 mL MSM, along with the growth substrate. When the growth substrate was depleted, more was added as neat compound (5-10 µL). With each addition of substrate, 1 mL of liquid was withdrawn for protein measurement. The bottles were maintained in this manner until 35-75 mg of substrate was consumed. Yields were calculated by linear regression of protein formed
versus the amount of substrate consumed. Oxygen in the headspace was monitored periodically; pure oxygen was added to maintain 5-21%.

4.3.6 cDCE and TCE Transformation by Resting Cells

Cells were first grown in MSM on their respective substrate in the absence of non-growth substrate, i.e., TCE or cDCE. Lyophilized cells in MSM (10 mg, containing approximately 1.6 mg protein) were transferred to 160 mL serum bottles containing 100 mL MSM. The bottles were sealed with septa and crimp caps and 1.0-2.0 mg/L of substrate was added (using water saturated solutions). When the substrate was consumed, more was added as neat compound (5-10 µL), resulting in concentrations of 50-150 mg/L. With CB, 1,2-DCB, and 1,3-DCB, the onset of consumption lagged significantly in one or more of the replicates. To initiate activity, a second addition of lyophilized cells was made. If that was not effective, then 10-50 mL of the contents from a serum bottle that was active was transferred to the bottles that were still lagging behind (after removing an equivalent volume); the volume removed from the active bottles was replaced with fresh MSM. With 1,3-DCB, none of the bottles showed significant activity even after a second addition of lyophilized cells. To initiate activity in these bottles, 30 mL was added from a bottle that had never undergone lyophilization. Oxygen in the headspace was monitored periodically; pure oxygen was added to maintain 5-21%.

When the protein concentration reached 50-100 mg/L (approximately 100-200 mg cells/L), the contents of each bottle was then centrifuged (7500 rpm for 5 min; Fisher Centrifuge centrifuge), the supernatant was discarded, and the resulting pellet was washed three times in MSM to remove residual growth substrate. The pellet was resuspended in
100 mL of MSM, providing a resting cell concentration of 100-200 mg/L. cDCE (700-1000 µg/L) or TCE (110-190 µg/L) was then added. These concentrations were selected based on preliminary experiments with benzene-grown cells; the intent was to provide enough cDCE and TCE to avoid complete degradation, but a low enough amount to avoid significant competitive inhibition (1). Triplicate water controls were prepared with 100 mL of distilled deionized water and the same concentrations of cDCE or TCE used in the live treatments.

The bottles were incubated at room temperature in an inverted position on a rotary shaker table (150 rpm). The cDCE and TCE remaining was monitored by analysis of headspace samples. Based on the total amount of cDCE and TCE degraded and the mass of resting cells added, the cometabolic transformation parameters $T_c$ and $T_y$ were calculated as follows (1):

$$T_c = \frac{X_c}{X_m}$$  \hspace{1cm} (4.2)

$$T_y = T_c \cdot Y$$  \hspace{1cm} (4.3)

where $X_c$ = concentration of cDCE or TCE consumed (µg/L); $X_m$ = concentration of resting cells initially added (mg/L); and $Y$ = yield (mg cells/mg substrate).

Pseudo-first order transformation rates for cDCE or TCE were calculated by linear regression of batch depletion data for cDCE and TCE over time, pooled from triplicate bottles. The pseudo-first order cometabolic degradation rate constant ($k_1$) was calculated by dividing the pseudo-first order transformation rate by the biomass concentration at the end of the experiment (2).
4.3.7 **cDCE and TCE Transformation by Growing Cells**

Serum bottles were prepared in the same manner as described above except that cDCE (270-280 µg/L) or TCE (30-60 µg/L) was added along with the growth substrate (1.2-7.8 mg/L). The lower initial concentration of TCE and cDCE was intended to compensate for the lower initial concentration of cells compared to the resting cell assay, yet the concentrations were still environmentally relevant. Bottles were incubated and monitored as described above. When growth substrate was depleted, more was added as neat compound (5-10 µL). Oxygen in the headspace was monitored periodically; pure oxygen was added to maintain 5-21%. After the last measurement of cDCE or TCE and growth substrate, the protein produced was measured and converted to cell mass. Based on the mass of cDCE or TCE transformed, the cumulative mass of growth substrate consumed, and the increase in cell mass, $T_c'$ and $T_y'$ (6) were calculated:

$$T_c' = \frac{X_c'}{X_m'}$$  \hspace{1cm} (4.4)

$$T_y' = \frac{X_c'}{S}$$ \hspace{1cm} (4.5)

where $X_m'$ = biomass concentration at the end of the incubation period minus the initial biomass (mg/L); $S$ = cumulative concentration of primary substrate consumed (mg/L) and $X_c'$ = cumulative amount of cDCE or TCE consumed (µg/L) between the last data point and the first data point at which the decrease observed in live bottles was greater than the decrease observed in water controls. The prime after $T_c$ and $T_y$ is intended to distinguish these measurements of the transformation capacity and yield from the ones made with resting cells (i.e., equations 4.2 and 4.3). Yields were also calculated from these
experiments based on the ratio of the net biomass accumulated on the last day of analysis and cumulative growth substrate consumed.

4.4 RESULTS

4.4.1 Identification of Isolates and Growth Yields

Isolates were obtained for all of the monoaromatic substrates. Based on the sequence of their 16S rRNA gene, the isolates growing on benzene, CB, 1,2-DCB and 1,3-DCB were identified as *Rhodococcus* sp., *Ralstonia* sp., *Variovorax* sp., and *Ralstonia* sp., respectively (Table 4.1). With 1,4-DCB, growth was extremely slow in MSM, partly due to its poor solubility. Attempts to grow the isolate with 1,4-DCB crystals in the MSM (and therefore a saturation level of the compound) were ineffective. Consequently, insufficient biomass was obtained to allow for identification. Yields are reported in Table 4.1 for benzene, CB, 1,2-DCB and 1,3-DCB. The 1,2,4-TCB isolate grew too slowly when starting with the lyophilized inoculum in order to measure a yield. Although the yields for 1,2- and 1,3-DCB were lower on a mass basis, they were actually higher on the basis of chemical oxygen demand equivalents (Table 4.1).

4.4.2 Cometabolism of TCE and cDCE by Resting Cells

Aerobic cometabolism of cDCE by resting cells grown on benzene, CB, 1,2-DCB and 1,3-DCB is shown in Figure 4.1, panels a, b, c and d, respectively. Cells grown on benzene had the highest cDCE transformation capacity, followed by CB and 1,3-DCB (Table 4.2). There was no statistically significant consumption of cDCE by the *Variovorax* cells grown on 1,2-DCB. Transformation yields for cDCE followed the same
trend (Table 4.2). Results for the water controls indicated little or no loss of cDCE during the 1.3-5 days of incubation.

Aerobic cometabolism of TCE by resting cells grown on benzene, CB, 1,2-DCB and 1,3-DCB are shown in Figure 1, panels e, f, g and h, respectively. The highest \( T_c \) value for TCE was observed with resting cells grown on CB, followed by benzene and 1,3-DCB (Table 4.2). As with cDCE, there was no statistically significant cometabolism of TCE by resting cells grown on 1,2-DCB. Results for the TCE water controls indicated little or no loss of TCE during the 3-5 days of incubation.

The pseudo-first order cometabolic degradation rate constants \((k_1)\) for cDCE and TCE followed the same trends as \( T_c \) and \( T_y \) (Table 4.1). The rates were higher for cDCE compared to TCE in the three isolates that demonstrated transformation. The highest \( k_1 \) value for cDCE transformation was observed with the benzene-grown isolate, followed by CB and 1,3-DCB. With TCE, the highest \( k_1 \) was observed with the CB-grown isolate, followed by the benzene and 1,3-DCB grown isolates.

4.4.3 Cometabolism of TCE and cDCE by Growing Cells

Aerobic cometabolism of cDCE during growth of the isolates on benzene, CB, 1,2-DCB and 1,3-DCB is shown in Figure 4.2, panels a, b, c and d, respectively. The highest rates of cDCE consumption occurred when high rates of primary substrate were consumed. Losses of cDCE from the water controls were comparatively minor. The \textit{Rhodococcus} sp. growing on benzene was the most effective, both in terms of \( T_c' \) and \( T_y' \), followed by 1,3-DCB, CB, and 1,2-DCB (Table 4.3). For all isolates and substrates, the
$T_c\text{'}$ and $T_y\text{'}$ values for cDCE exceeded the corresponding $T_c$ and $T_y$ values for resting cells (Table 4.2).

Aerobic cometabolism of TCE during growth of the isolates on benzene, CB, 1,2-DCB and 1,3-DCB is shown in Figure 4.2, panels e, f, g and h, respectively. As with cDCE, the highest rates of TCE consumption occurred when the primary substrates were being consumed at high rates. Lag periods prior to the onset of high rates of primary substrate utilization were similar in the presence of cDCE and TCE, with one exception: with CB, the lag period was considerably longer in the presence of cDCE (Fig. 4.2b) versus TCE (Fig. 4.2f). However, the CB-grown cells used in the resting cell experiment (Fig. 4.1b) also experienced a long lag period (Appendix 6.4.1, Figure 6.4-1); the reason for this is not known. Consequently, the extended lag period for CB during the experiment with growing cells (Fig. 4.2b) does not appear to be related to cDCE. Lag periods were generally similar when growing each culture in the absence of TCE and cDCE (Appendix 6.4.1, Figures 6.4-2 and 6.4-3), suggesting that TCE and cDCE did not exert a noticeably inhibitory effect at the concentrations evaluated. Losses of TCE from the water controls was comparatively minor (Figure 4.2, panels e, f, g and h). CB and 1,3-DCB were better substrates than benzene and 1,2-DCB in terms of $T_c\text{'}$ and $T_y\text{'}$. Unlike cDCE, $T_c\text{'}$ and $T_y\text{'}$ values for TCE were lower than the corresponding $T_c$ and $T_y$ values for resting cells grown on benzene, 1,2-DCB, and 1,3-DCB (Table 4.3). However, for 1,2-DCB, cometabolism of TCE only occurred in the presence of growing *Variovorax* cells, not with the resting cells (Table 4.2). The yields obtained during growth in the presence of cDCE or TCE were not statistically different (Student’s $t$ test, $\alpha=0.05$) from
the yields obtained in the absence of cDCE or TCE, for all the growth substrates (Appendix 6.4.2).

4.5 DISCUSSION

The results of this study confirm the ability of microbes that grow aerobically on benzene, CB, 1,2-DCB and 1,3-DCB to cometabolize TCE and cDCE, as was suggested in previous microcosm study (9). In one respect this outcome was expected, since a variety of dioxygenases are involved in aerobic metabolism of these aromatic compounds (Table 4.4). However, not all dioxygenases are reactive towards TCE and cDCE. For example, *Pseudomonas putida* F1 grows on toluene and cometabolizes TCE. Both toluene dioxygenase and catechol 2,3-dioxygenase are involved in the pathway and both were considered candidates for mediating cometabolism of TCE. To address this, Nelson et al. (27) constructed a mutant of F1 that was defective in toluene dioxygenase; it could no longer cometabolize TCE even though the catechol 2,3-dioxygenase was still functioning, indicating that toluene dioxygenase was responsible for TCE oxidation and not catechol 2,3-dioxygenase. The differences in transformation capacities and yields for the different substrates evaluated in this study (Tables 4.2 and 4.3) suggest that different dioxygenases were involved. The catabolic pathways for benzene, CB, and 1,3-DCB involve at least one dioxygenase that is unique to each substrate (i.e., benzene 1,2-dioxygenase for benzene; 2,3-dihydroxybiphenyl 1,2-dioxygenase for CB; and chlorocatechol 3,5-dioxygenase for 1,3-DCB; Table 4.4). Catechol 1,2-dioxygenase is common to the pathways for all of the substrates evaluated in this study, as well as for phenol (25), suggesting it is not a major contributor to cometabolism. Based on the work
by Nelson et al. (27), it appears unlikely that catechol 2,3-dioxygenase was responsible for the oxidation of TCE or cDCE observed in this study. Additional research is needed to identify which of the remaining dioxygenases were responsible.

No prior studies were found that reported $T_c$ and $T_y$ values for TCE and cDCE using benzene, CB, 1,2-DCB and 1,3-DCB as substrates, so direct comparisons to the results from this study are not possible. Alvarez-Cohen and Speitel (2) summarized 11 previously reported $T_c$, $T_y$ and $k_1$ values for TCE cometabolism by resting cells of toluene and phenol-grown microbes in pure and mixed cultures. $T_c$ averaged 86 µg TCE/mg biomass and ranged from 3.1 to >510; $T_y$ averaged 64 µg TCE/mg substrate and ranged from 1.9 to 222; and $k_1$ averaged 0.56 L/mg-day and ranged from 0.012 to 3.8. Pure cultures tended to have lower $T_c$, $T_y$, and $k_1$ values than the mixed cultures. The $T_c$ values observed in this study were at the lower end of the ranges listed above and all of the $T_y$ values were lower. The $k_1$ values were an order of magnitude lower than the lowest reported value (Table 4.2). Nevertheless, the results of this study are for pure cultures and for substrates that would never be used for active remediation of an aquifer in the manner that toluene and phenol have been considered.

For cDCE, Alvarez-Cohen and Speitel (2) summarized only two studies that reported $T_c$ and one study that reported $k_1$ values by resting cells of phenol-grown microbes in mixed cultures, and none for toluene or any other aromatic substrate. $T_c$ ranged from 138 to >260 µg cDCE/mg biomass, which are two orders of magnitude higher than the results for the resting cells and substrates used in this study (Table 4.2). Previously reported $k_1$ values ranged from 0.09 to 8.8 L/mg-day. The highest $k_1$ in this
study for cDCE, for the benzene-grown isolate, was on the same order of magnitude as the lower end of this range; \( k_1 \) values for cDCE by cultures grown on CB and 1,3-DCB were an order of magnitude lower. No prior results were reported for \( T_y \). Although the isolates from this study were less efficient in their extent of cDCE cometabolism, the relationship between the \( T_c \) values for TCE and cDCE were the same as in other studies, i.e., \( T_c \) values were consistently higher for cDCE compared to TCE.

\( T_c \) is measured in the absence of growth substrate, a condition that often does not occur in situ. \( T_y \) is then calculated from \( T_c \) based on the yield for the growth substrate (also in the absence of non-growth substrate). In separate experiments, we measured \( T_c \) and \( T_y \) when substrate and non-growth substrate were present together, i.e., \( T_c' \) and \( T_y' \). The parameter \( T_y' \) was first introduced by Chang and Criddle (6). In spite of the utility of \( T_y' \), we found only a few studies that reported it for an aromatic growth substrate. Morono et al. (26) measured \( T_y' \) values ranging from 0.19 to >5.2 µg TCE/mg toluene. The \( T_y' \) values we measured are on the lower end of this range. During a pilot study of in-situ aerobic TCE cometabolism through the injection of phenol and oxygen into a confined aquifer at Moffett Field, Hopkins et al. (17, 26) measured a \( T_y' \) value of 62 µg TCE/mg phenol, approximately one half the value measured in the laboratory with resting cells.

No previous studies were found that report \( T_y' \) values for cDCE. The considerably higher \( T_y' \) values for cDCE versus TCE in this study (Table 4.3) suggests that TCE is more toxic during growth on the primary substrate. In the case of 1,2-DCB, cometabolism of cDCE and TCE only occurred during active consumption of the growth
substrate (Fig. 4.1c versus 4.2c and Fig. 4.1g versus 4.2 g) at levels comparable to the other substrates.

We found no previous descriptions of $T_c'$ in the literature, yet we believe it has similar merit to $T_y'$, i.e., it reflects the extent of cometabolism that is achievable in the presence of growth substrate. We measured $T_c'$ based on the amount of TCE and cDCE consumed and divided that by the amount of cells present at the end of the incubation period (Table 4.3). An alternative approach is to calculate $T_c'$ by dividing $T_y'$ by the yield, i.e., analogous to how $T_y$ is calculated from $T_c$ with resting cells using Y (equation 4.3). The $T_c'$ values obtained using this method are similar to the measured values (Appendix 6.4.3). This outcome is consistent with the fact that the yields measured for isolates grown on benzene, CB, 1,2-DCB and 1,3-DCB were the same in the presence or absence of cDCE and TCE. At higher ratios of non-growth substrate to substrate, it is likely that yields would be reduced.

The results of this study confirm the potential for cometabolism of TCE and cDCE during aerobic growth on benzene, CB, 1,2-DCB and 1,3-DCB. This is especially relevant for natural attenuation scenarios when these compounds occur as co-contaminants and may be transported from an anaerobic to an aerobic environment. Although it may seem reasonable to assume that all aerobically biodegradable monoaromatic compounds may serve as primary substrates for TCE and cDCE cometabolism, the wide range of dioxygenases involved indicates this is not necessarily the case. Additional studies on the specific dioxygenases involved in cometabolism will assist in making more informed predictions.
4.6 REFERENCES


degradation of 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzene of


of halogenated aliphatic compounds by the ammonia- oxidizing bacterium


toluene dioxygenase in whole-cell studies with _Pseudomonas putida_ F1. Appl.

40. **Werlen, C., H. P. E. Kohler, and J. R. vanderMeer.** 1996. The broad substrate
chlorobenzene dioxygenase and _cis_-chlorobenzene dihydrodiol dehydrogenase of

_Pseudomonas_ sp. strain P51 are linked evolutionarily to the enzymes for benzene


42. **Zamanian, M., and J. R. Mason.** 1987. Benzene dioxygenase in _Pseudomonas
_putida_. Biochem. J. **244**:611-616.
<table>
<thead>
<tr>
<th>Growth Substrate</th>
<th>Identification</th>
<th>Accession Number</th>
<th>Yield (mg biomass/mg substrate)</th>
<th>Yield (mg biomass COD/mg substrate COD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td><em>Rhodococcus sp.</em></td>
<td>GU565219</td>
<td>0.45±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td>CB</td>
<td><em>Ralstonia sp.</em></td>
<td>GU565220</td>
<td>0.41±0.01</td>
<td>0.29±0.01</td>
</tr>
<tr>
<td>1,2-DCB</td>
<td><em>Variovorax sp.</em></td>
<td>GU565221</td>
<td>0.37±0.06</td>
<td>0.37±0.06</td>
</tr>
<tr>
<td>1,3-DCB</td>
<td><em>Ralstonia sp.</em></td>
<td>GU565222</td>
<td>0.36±0.05</td>
<td>0.36±0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup> COD = chemical oxygen demand; 1 mg biomass = 1.42 mg biomass COD; 1 mg benzene = 3.08 mg COD; 1 mg CB = 1.99 mg COD; 1 mg DCB = 1.41 mg COD.

<sup>b</sup> ± represents the standard deviation for triplicate microcosms.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Growth substrate</th>
<th>cDCE</th>
<th>TCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$T_c$</td>
<td>$T_y$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(µg cDCE/ mg biomass)</td>
<td>(µg cDCE/ mg substrate)</td>
</tr>
<tr>
<td><em>Rhodococcus</em></td>
<td>Benzene</td>
<td>5.3±0.58</td>
<td>2.1±0.26</td>
</tr>
<tr>
<td><em>Ralstonia</em></td>
<td>CB</td>
<td>3.2±0.42</td>
<td>0.94±0.05</td>
</tr>
<tr>
<td><em>Variovorax</em></td>
<td>1,2-DCB</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Ralstonia</em></td>
<td>1,3-DCB</td>
<td>1.3±0.59</td>
<td>0.46±0.19</td>
</tr>
</tbody>
</table>

$^a$ ± represents the standard deviation for triplicate microcosms.
Table 4.3 Cometabolic Transformation of cDCE and TCE by Growing Cells

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Growth Substrate</th>
<th>cDCE $T_c'$ (µg cDCE/mg biomass)</th>
<th>cDCE $T_y'$ (µg cDCE/mg substrate)</th>
<th>TCE $T_c'$ (µg TCE/mg biomass)</th>
<th>TCE $T_y'$ (µg TCE/mg substrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhodococcus</em></td>
<td>Benzene</td>
<td>11±1.7$^a$</td>
<td>4.6±1.1</td>
<td>0.18±0.02</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td><em>Ralstonia</em> CB</td>
<td></td>
<td>5.6±0.18</td>
<td>2.1±0.15</td>
<td>0.30±0.12</td>
<td>0.11±0.04</td>
</tr>
<tr>
<td><em>Variovorax</em> 1,2-DCB</td>
<td></td>
<td>4.9±1.3</td>
<td>1.5±0.39</td>
<td>0.15±0.03</td>
<td>0.06±0.02</td>
</tr>
<tr>
<td><em>Ralstonia</em> 1,3-DCB</td>
<td></td>
<td>7.2±1.4</td>
<td>1.7±0.12</td>
<td>0.33±0.08</td>
<td>0.08±0.02</td>
</tr>
</tbody>
</table>

$^a$ ± represents the standard deviation for triplicate microcosms.
### Table 4.4 Dioxygenases Involved in the Metabolism of Substrates Evaluated in this Study

<table>
<thead>
<tr>
<th>Dioxygenase</th>
<th>Benzene</th>
<th>CB</th>
<th>1,2-DCB</th>
<th>1,3-DCB</th>
<th>1,4-DCB</th>
<th>1,2,4-TCB</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzene 1,2-</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(42)</td>
</tr>
<tr>
<td>catechol 1,2-</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>(8, 15, 31, 33, 37, 42)</td>
</tr>
<tr>
<td>catechol 2,3-</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>(5, 8, 31, 33)</td>
</tr>
<tr>
<td>catechol 3,5-</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td>(8, 31)</td>
</tr>
<tr>
<td>chlorobenzene</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td>(15, 37, 40)</td>
</tr>
<tr>
<td>2,3-dihydroxybiphenyl 1,2-</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(32)</td>
</tr>
<tr>
<td>chlorocatechol 1,2-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td>(34)</td>
</tr>
<tr>
<td>chlorocatechol 3,5-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td>(8)</td>
</tr>
</tbody>
</table>
Figure 4.1 Cometabolism of cDCE and TCE by resting cells grown on benzene (150 mg cells/L; a, e); CB (130-180 mg cells/L; b, f); 1,2-DCB (130-140 mg cells/L; c, g); and 1,3-DCB (120-130 mg cells/L; d, h); data points represent the average of triplicate bottles and error bars indicate one standard deviation.
Figure 4.2 Cometabolism of cDCE and TCE by cells actively growing on benzene (a, e); CB (b, f); 1,2-DCB (c, g); and 1,3-DCB (d, h); data points represent the average of triplicate bottles and error bar indicate one standard deviation. Data for the substrates represent the cumulative amounts consumed based on repeated additions.
5. CONCLUSIONS AND RECOMMENDATION FOR FUTURE RESEARCH

5.1 CONCLUSIONS

This dissertation evaluated three aspects of bioremediation for treatment of complex mixtures of contaminants: First, a microcosm study was conducted to compare conventional anaerobic/aerobic treatment with a less well-practiced approach, aerobic/anaerobic treatment. Second, using inoculum from one of the anaerobic microcosms, enrichment cultures were developed and used to evaluate the potential for use of γ-HCH as a terminal electron acceptor via chlororespiration. Third, using inoculum from one of the aerobic microcosms, pure cultures were obtained that grow on benzene and several chlorinated benzenes; these isolates were then tested for their ability to cometabolize TCE and cDCE.

The results from the microcosm study to evaluate sequential treatment strategy (Chapter 2) indicate that, for the mix of contaminants investigated, a sequential aerobic and anaerobic approach may be more effective than the more conventionally applied anaerobic and aerobic sequence. Based on the fraction of electron equivalents used for γ-HCH dechlorination in HEPES-buffered enrichment culture IV and the ability to transfer this culture with γ-HCH as the sole terminal electron acceptor, this study is the first to demonstrate chlororespiration of γ-HCH (Chapter 3). The research on the evaluation of cometabolic transformation of cDCE and TCE (Chapter 4) demonstrated the potential of
microbes that grow aerobically on benzene, CB, 1,2-DCB and 1,3-DCB to cometabolize TCE and cDCE.

The specific conclusions from this dissertation based on the microcosm study presented in Chapter 2 are:

1. The soil from an industrial site in the northeastern US, which is contaminated with hexachlorocyclohexane isomers, chlorinated ethenes, chlorinated benzenes and benzene, contained an indigenous microbial population capable of biodegrading all the contaminants and also contained sufficient organic substrates to serves as electron donor.

2. Both sequential anaerobic/aerobic and aerobic/anaerobic treatments were effective in soil, which suggests that the necessary microbes and electron donor were abundant in this part of the site.

3. The contaminated dolomite obtained from the same site contained an indigenous microbial population capable of aerobic biodegradation of hexachlorocyclohexane isomers, all of the aromatic contaminants, and partial aerobic transformation of chlorinated ethenes.

4. Anaerobic biodegradation of hexachlorocyclohexane isomers was observed with the indigenous population in dolomite but bioaugmentation with biostimulation was necessary for anaerobic reduction of chlorinated ethenes and no anaerobic biodegradation of aromatic contaminants was observed, even with biostimulation and bioaugmentation.
5. The novel aerobic/anaerobic treatment strategy is preferable over the conventional anaerobic/aerobic treatment sequence for bioremediation of the dolomite; for soil, both sequential treatment strategies were effective.

   The specific conclusions from this dissertation based on the study presented in Chapter 3 are:

   1. The use of γ-HCH as terminal electron acceptor via chlororespiration was demonstrated with an enrichment culture developed from the anaerobic soil and groundwater microcosms for the industrial site.

   2. The microorganism(s) responsible for reducing γ-HCH by chlororespiration is sensitive to vancomycin, suggesting that it likely to be gram positive.

   3. The microbial community profile analysis of the γ-HCH dechlorinating enrichment culture, including sequencing of DGGE bands and clone libraries, did not reveal any of the known chlororespiring microorganisms.

   4. The γ-HCH dechlorinating enrichment culture can also use CO₂ as an electron acceptor in addition to γ-HCH when methanol, H₂ and lactate served as electron donors.

   The specific conclusions from this dissertation based on the study presented in Chapter 4 are:

   1. Pure cultures that can use benzene, CB, 1,2-DCB and 1,3-DCB as sole sources of carbon and energy under aerobic conditions were isolated from the aerobic soil and groundwater microcosms used for the evaluation of the industrial site. They consisted of a *Rhodococcus* sp, a *Variovorax* sp., and two strains of *Ralstonia* spp.
2. Resting cells of isolates growing on benzene, CB and 1,3-DCB were able to transform cDCE and TCE by cometabolism under aerobic conditions, whereas the resting cells growing on 1,2-DCB were not able to catalyze aerobic cometabolic transformation of cDCE and TCE.

3. Growing cells of the four pure cultures were able to catalyze aerobic cometabolic transformation of cDCE and TCE in the presence of their respective aromatic growth substrate.

5.2 **RECOMMENDATIONS for FUTURE RESEARCH**

Based on the outcome from research presented in Chapter 3, the following suggestions are made for future research:

1. Isolation and identification of γ-HCH chlororespiring microbe.
3. Characterization of the γ-HCH dechlorinating isolate for different electron donors, effects of co-contaminants, etc.
4. Pilot study to evaluate survival of an enrichment culture or isolate under field conditions and bioaugmentation strategies for field applications.

Based on the outcome from research presented in Chapter 4, the following suggestions are made for future research:

1. Enzyme assay with different dioxygenase enzyme induced during biodegradation of monoaromatic contaminants.
2. Development of dioxygenase enzyme based probe to quantify the activity of dioxygenase enzyme as a function of cometabolic transformation of cDCE and TCE.

3. Use of an enzyme based probe to understand the differences in cometabolic transformation capacity observed between different isolates.

4. Evaluate cometabolic transformation in each isolate with monoaromatic contaminants studied in Chapter 4, phenol, toluene and methane and compare with reported values.
APPENDICES
Appendix 6.1: Preparation of Calibration Standards and Analytical Results

The purpose of this section of the Appendix is to present representative calibration curves for each of the contaminants monitored during this research.
Figure 6.1-1  Representative calibration plot for benzene.

Figure 6.1-2  Representative calibration plot for chlorobenzene benzene.
Figure 6.1-3  Representative calibration plot for 1,2-dichlorobenzene.

Figure 6.1-4  Representative calibration plot for 1,3-dichlorobenzene.
Figure 6.1-5  Representative calibration plot for 1,4-dichlorobenzene.

Figure 6.1-6  Representative calibration plot for 1,2,4-TCB.
Figure 6.1-7  Representative calibration plot for cDCE.

Figure 6.1-8  Representative calibration plot for TCE.
Figure 6.1-9  Representative calibration plot for α-HCH.

Figure 6.1-10  Representative calibration plot for γ-HCH.
Appendix 6.2: Supplementary Results for Chapter 2

The purpose of this section of the Appendix is to present additional results that support the data and conclusions reached based on the study reported in Chapter 2.
6.2.1 Assessment of HCH Adsorption

The method used to quantify HCH isomers in the microcosm study involved the use of settled liquid rather than slurry samples. This approach minimized interferences from the soil and dolomite. However, there was concern that a significant percentage of the mass of HCH may be adsorbed to the solids. To evaluate this concern, samples of groundwater were analyzed at the same time as soil slurry. Results are presented in Table 6.2-1. Although some adsorption was evident, the extent (10-12%) was considered to be small enough to justify the use of clarified liquid rather than the soil slurry.

Table 6.2-1 Adsorption of HCH Isomers on Microcosm Sediments

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Concentration (µg/L)</th>
<th>Concentration (µg/L)</th>
<th>Concentration (µg/L)</th>
<th>Concentration (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Groundwater</td>
<td>Groundwater</td>
<td>Soil</td>
<td>Settled liquid</td>
</tr>
<tr>
<td>α-HCH, run 1</td>
<td>56.70</td>
<td>65.15</td>
<td>42.85</td>
<td>17.41</td>
</tr>
<tr>
<td>α-HCH, run 2</td>
<td>53.66</td>
<td></td>
<td>48.26</td>
<td>42.43</td>
</tr>
<tr>
<td>α-HCH, ave</td>
<td></td>
<td></td>
<td>48.26</td>
<td>42.43</td>
</tr>
<tr>
<td>% difference between soil slurry and settled liquid</td>
<td></td>
<td></td>
<td></td>
<td>12%</td>
</tr>
<tr>
<td>γ-HCH, run 1</td>
<td>59.39</td>
<td>70.97</td>
<td>48.91</td>
<td>22.05</td>
</tr>
<tr>
<td>γ-HCH, run 2</td>
<td>62.96</td>
<td></td>
<td>55.94</td>
<td>50.10</td>
</tr>
<tr>
<td>γ-HCH, ave</td>
<td></td>
<td></td>
<td>55.94</td>
<td>50.10</td>
</tr>
<tr>
<td>% difference between soil slurry and settled liquid</td>
<td></td>
<td></td>
<td></td>
<td>10%</td>
</tr>
</tbody>
</table>
6.2.2 Additional Microcosm Data

In section 2.4, average results are presented for the microcosms that demonstrated biodegradation activity. The purpose of this section of the Appendix is to provide the results for bottles that did not show significant levels of biodegradation activity and also the observations in the microcosms with high levels of cDCE and TCE.
Figure 6.2-1 Results for dolomite microcosms with low initial concentrations of TCE and cDCE under anaerobic biostimulation followed by a switch to aerobic conditions on day 393.
Figure 6.2-2 Results for dolomite microcosms with low initial concentrations of TCE and cDCE under aerobic conditions followed by a switch to anaerobic biostimulation on day 101.
Figure 6.2-3 Results for Soil killed control microcosms.
Figure 6.2-4 Results for water control microcosms.
Figure 6.2-5  Results for dolomite killed control microcosms.
Figure 6.2-6 Results for soil microcosms with high initial concentrations of TCE and cDCE under as-is anaerobic conditions (a, b, c; average of three bottles) and anaerobic biostimulation with lactate followed by a switch to aerobic conditions on day 251 (d, e, f; average of six bottles); ↓=lactate addition; O₂=oxygen addition.
Figure 6.2-7 Results for soil microcosms with high initial concentrations of TCE and cDCE under as-is aerobic conditions (a, b, c; average of three bottles) and aerobic conditions followed by anaerobic biostimulation with lactate on day 201 (d, e, f; average of six bottles); ↓=lactate addition.
Figure 6.2-8  Results for dolomite microcosms with high initial concentrations of TCE and cDCE under as-is anaerobic conditions (a, b, c; average of three bottles) and anaerobic bioaugmentation with lactate followed by a switch to aerobic conditions on day 251 (d, e, f; average of three bottles); ↓=lactate addition; O$_2$=oxygen addition.
Figure 6.2-9  Results for dolomite microcosms with high initial concentrations of TCE and cDCE under as-is aerobic conditions (a, b, c; average of three bottles) and aerobic conditions followed by anaerobic bioaugmentation with lactate on day 137 (d, e, f; average of three bottles); ↓=lactate addition.
Figure 6.2-10 Results for dolomite microcosms with high initial concentrations of TCE and cDCE under anaerobic biostimulation on days 30, 99, 156, 217 and 247.
Figure 6.2-11 Results for dolomite microcosms with high initial concentrations of TCE and cDCE under aerobic conditions followed by anaerobic biostimulation on days 101, 122, 156, 217 and 247.
6.2.3 Data Used to Determine Pseudo First Order Rate Constants

Figures 2.7-2.10 present pseudo first order rate constants for various treatments. The purpose of this section of the Appendix is to present example data and plots that were used to arrive at these rate constants. Sample data are presented in Tables 6.2-2 and 6.2-3; the corresponding plots are shown in Figures 6.2-12 and 6.2-13.

Table 6.2-2 Sample data set for regression analysis to determine pseudo first order aerobic benzene biodegradation rate in soil microcosms with low initial concentration of cDCE and TCE, S1 = anaerobic/aerobic sequence, BL = biostimulation microcosms, BA = bioaugmentation microcosms.

<table>
<thead>
<tr>
<th>Microcosm ID</th>
<th>Time (Days)</th>
<th>Normalized Time (days)</th>
<th>ln (C/Co)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1BLLow1</td>
<td>136</td>
<td>0</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>138</td>
<td>2</td>
<td>0.0602</td>
</tr>
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Figure 6.2-12 Sample regression analysis result to determine the pseudo first order aerobic benzene biodegradation rate in soil microcosms with a low initial concentration of cDCE and TCE; the corresponding regressed data is shown in Table 6.2-2; the slope of the regression line, $y$, is the pseudo first order rate with units of day$^{-1}$, which is equal to 13.0 year$^{-1}$, with a standard error of 0.64.
Table 6.2-3  Sample data set for regression analysis to determine pseudo first order anaerobic 1,2-dichlorobenzene biodegradation rate in soil microcosms with low initial concentration of cDCE and TCE, S1 = anaerobic/aerobic sequence, AI = as-is microcosms, BL = biostimulation microcosms, BA = bioaugmentation microcosms

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Figure 6.2-13  Sample regression analysis result to determine the pseudo first order anaerobic 1,2-dichlorobenzene biodegradation rate in soil microcosms with a low initial concentration of cDCE and TCE; the corresponding regressed data is shown in Table 6.2-3; slope of the regression line, $y$, is the pseudo first order rate with units of day$^{-1}$, which is equal to 7.1 year$^{-1}$ with a standard error of 0.37.
Appendix 6.3: Supplementary Results for Chapter 3

The purpose of this section of the Appendix is to present additional results that support the data and conclusions reached based on the study reported in Chapter 3.
6.3.1 \( \gamma \)-HCH Dechlorination when Transferred from MSM-3 and MSM-2

Section 3.4.3 presents the results for \( \gamma \)-HCH dechlorination activity in the MSM-3 culture. The purpose of this section of the Appendix is to present data on \( \gamma \)-HCH dechlorination when enrichment culture IV (MSM-3) was transferred back to MSM-2. Figure 6.3-1 demonstrates that enrichment culture IV can maintain dechlorination activity when transferred back to MSM-2.

![Graph showing dechlorination activity](image)

Figure 6.3-1 Appearance of \( \gamma \)-HCH dechlorination products in enrichments grown in HEPES media (MSM 3) and transferred to bicarbonate media (MSM 2); \( \text{H}_2 \) = hydrogen, \( \text{Ac} \) = acetate; each data point represents average of triplicate bottles.
6.3.2 DGGE Results for MSM-1 Enrichment Culture and Test of Dehalobacter sp.

Section 3.4.4 presents the DGGE results for \(\gamma\)-HCH dechlorinating enrichment cultures. The purpose of this section of the Appendix is to present the DGGE results for enrichment culture MSM-1 and the PCR results with Dehalobacter specific primers. The panel PCR Dehalobacter sp. demonstrates the absence of a band in the enrichment culture and the presence of a band in the lane corresponding to the positive control (Figure 6.3-2). The panel titled DGGE demonstrates the same pattern of bands in the enrichment cultures with and without \(\gamma\)-HCH.

![PCR and DGGE analysis of MSM-1 enrichment cultures](image)

L: Ladder  N: Negative Control  D: Dehalobacter sp.

1: Methanol + \(\gamma\)-HCH,  2: Methanol only

Figure 6.3-2 PCR and DGGE analysis of MSM-1 enrichment cultures; \(\rightarrow\) = dominant bands in panel C
6.3.3 *Sequences and Accession Numbers from Cloning Study*

Section 3.4.4 presents the DGGE and cloning results for \( \gamma \)-HCH dechlorinating enrichment cultures. The purpose of this section of the Appendix is to present the sequence identification and the accession numbers for the clones that were evaluated (Table 6.3-1).
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6.3.4 Phase Contrast Microscopic Observations

Section 3.5 discusses the problems encountered in measuring protein in enrichment cultures III and IV, due in part to the low biomass yield. The purpose of this section of the Appendix is to present photomicrographs of the cultures. To demonstrate the occurrence of a live microbial population, phase contrast microbial observations were made with enrichment culture IV, with and without γ-HCH. Two types of staining, DAPI (4’,6-diamidino-2-phenylindole) and propidium iodide were done to evaluate the presence of active and dead cells in the enrichment cultures. The DAPI stain fluoresces with both live and dead cells, whereas the propidium iodide stain fluoresces only with dead cells. The fluorescence results are shown in Figure 6.3-3 (panels A, B, C and D). In panel A (DAPI), the fluorescence was much higher than in panel B (propidium iodide), which suggests the occurrence of more live cells in the enrichment culture IV with γ-HCH. The fluorescence observed in panel C (DAPI) for the culture without γ-HCH is significantly lower than the fluorescence in panel A, which suggests the occurrence of more cells in the presence of γ-HCH. Comparison between panels C and D reveals that the enrichment culture without γ-HCH does not have any dead cells, as we do not see fluorescence in panel D. It was not possible to make a clear distinction between the number of live and dead cells because of the low growth yield and the presence of precipitates in the media; therefore cell counts were not done.
Figure 6.3-3 Phase-contrast light microscopic observation of enrichment culture IV (MSM-3), (A) H₂ + γHCH culture observed with DAPI staining, (B) H₂ + γHCH culture observed with propidium iodide staining, (C) H₂ without γHCH culture observed with DAPI staining, (D) H₂ without γHCH culture observed with propidium iodide staining.
Similar to enrichment culture IV, phase contrast microbial observation was done with enrichment culture III (Figure 6.3-4). The fluorescence observed in panels A, B, C and D demonstrates the distribution of both live and dead cells. As observed in Figure 6.3-4, it was not possible to make a clear distinction between the number of live and dead cells due to the low growth yield and the presence of precipitates in the media; therefore, a cell count was not done. However, the occurrence of fluorescence in panels A, B, C and D demonstrates that microbes were present in these enrichments.

Figure 6.3-4 Phase-contrast light microscopic observation of enrichment culture III (MSM-2), (A) H2 + γHCH culture observed with DAPI staining, (B) H2 + γHCH culture observed with propidium iodide staining, (C) H2 without γHCH culture observed with DAPI staining, (D) H2 without γHCH culture observed with propidium iodide staining.
6.3.5 *Acetate Measurement in Enrichment Culture IV*

Section 3.5 discusses the results for γ-HCH dechlorination in enrichment culture IV and the absence of acetate consumption in these bottles. The purpose of this section of the Appendix is to present the results for acetate measurements in enrichment culture IV bottles. As shown in the Table 6.3-2, no significant consumption of acetate occurred over 163 days of incubation.

Table 6.3-2 Acetate Measurements in Enrichment Culture IV (MSM-3)

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<td>47</td>
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<tr>
<td>163</td>
<td>108±15</td>
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<sup>a</sup> Average and standard deviation of triplicate bottles.
6.3.6 $\gamma$-HCH Dechlorination in MSM-1 Enrichments with Methanol and Lactate

Section 3.4.1 presents the average results for $\gamma$-HCH dechlorination in the groundwater enrichment culture. The purpose of this section of the Appendix is to present results for the enrichment culture in MSM-1 when both methanol and lactate served as electron donors. As observed in Figures 6.3-5 and 6.3-6, $\gamma$-HCH dechlorination was maintained over two generations without loss of activity and benzene and CB accumulated. Conversion of methanol to methane was also observed in these enrichments and is shown in the plots.
Figure 6.3-5 γ-HCH enrichment in media with lactate and methanol as donor, generation 1.
Figure 6.3-6 γ-HCH enrichment in media with lactate and methanol as donor, generation 2.
6.3.7 \(\gamma\)-HCH Dechlorination in MSM-1 Enrichments with Methanol

Section 3.4.1 presents the results for \(\gamma\)-HCH dechlorination in the enrichment culture when methanol served as the only electron donor. The purpose of this section of the Appendix is to present the results for \(\gamma\)-HCH dechlorination in MSM-1 with methanol as the electron donor for eight generations. As observed from Figures 6.3-7 to 6.3-14, \(\gamma\)-HCH dechlorination was accompanied by accumulation of benzene and CB. The majority of the methanol added as electron donor was converted to methane; only a small fraction was used for \(\gamma\)-HCH dechlorination.
Figure 6.3-7 γ-HCH enrichment in media with methanol as donor, generation 1.
Figure 6.3-8  γ-HCH enrichment in media with methanol as donor, generation 2.
Figure 6.3-9 \( \gamma \)-HCH enrichment in media with methanol as donor, generation 3.
Figure 6.3-10  γ-HCH enrichment in media with methanol as donor, generation 4.
Figure 6.3-11 γ-HCH enrichment in media with methanol as donor, generation 5.
Figure 6.3-12 γ-HCH enrichment in media with methanol as donor, generation 6.
Figure 6.3-13  γ-HCH enrichment in media with methanol as donor, generation 7.
Figure 6.3-14 γ-HCH enrichment in media with methanol as donor, generation 8.
6.3.8 Evaluation of Methanogenic and Sulfidogenic Inhibitors on γ-HCH

Dechlorination

Section 3.4.1 presents the results for γ-HCH dechlorination with lactate and methanol as electron donors. The purpose of this section of the Appendix is to present results for evaluation of the effect of electron donors (lactate+methanol and methanol alone) and also the effects of methanogenic and sulfidogenic inhibitors. Significant levels of methane production were observed in the enrichment cultures grown with methanol as electron donor. Also, the enrichment culture media (MSM-1) contained sulfate, which was observed to be reduced. Therefore, the role of methanogens and sulfate reducing bacteria in γ-HCH was evaluated with the methanogenic inhibitor bromoethanesulfonate (BES) and the sulfidogenic inhibitor molybdate (Figure 6.3-15).

Two important observations were made from Figure 6.3-15. First, it was apparent that the γ-HCH dechlorination rate was same for both electron donor treatments (methanol only versus methanol+lactate). Based on this observation, subsequent experiments were conducted only with methanol as the electron donor.

The second observation was a decrease in the γ-HCH dechlorination rate in the presence of BES and molybdate. Based on this plot, it was observed that molybdate and BES inhibited γ-HCH dechlorination but did not completely stop dechlorination activity, which suggested that sulfate reducing microbes and methanogens may play an indirect role in γ-HCH dechlorination. These results led to an an evaluation of the role of sulfate in the media in relation to γ-HCH dechlorination. Subsequent experiments revealed that
\( \gamma \)-HCH dechlorination activity was independent of the occurrence of sulfate reduction and therefore, sulfate was removed from the media.

Figure 6.3-15 Role of BES and molybdate in \( \gamma \)-HCH dechlorination; MeOH = methanol, Lac = lactate, BES = bromoethane sulfonate, Molyb = molybdate. Note: (i) All treatments have \( \gamma \)-HCH, (ii) MeOH+Lac is the second generation in MSM-1, (iii) MeOH is the first generation without lactate, (iv) MSM-1, enrichment culture 1 served as inoculum for all these microcosms except for BES+Molyb+MeOH for which MeOH enrichment 1 served as inoculum.
Section 3.4.1 presents the results for the enrichment culture with methanol and no
γ-HCH. The purpose of this section of the Appendix is to present the results for the
enrichment culture grown only with methanol and no γ-HCH over seven generations.
The objective of developing an enrichment culture without γ-HCH was to compare the
enrichment culture with and without γ-HCH by molecular analysis and understand the
differences in the microbial community profile. The enrichment culture without γ-HCH
converted approximately 90% of the added methanol to methane (Figures 6.3-16 to 6.3-
22). The microbial community analysis by DGGE did not reveal significant difference
over several transfers.
Figure 6.3-16 Methanol only enrichment in MSM-1, generation 1.
Figure 6.3-17 Methanol only enrichment in MSM-1, generation 2.
Figure 6.3-18  Methanol only enrichment in MSM-1, generation 3.
Figure 6.3-19 Methanol only enrichment in MSM-1, generation 4.
Figure 6.3-20 Methanol only enrichment in MSM-1, generation 5.
Figure 6.3-21 Methanol only enrichment in MSM-1, generation 6.
Figure 6.3-22 Methanol only enrichment in MSM-1, generation 7.
6.3.10 Enrichment Culture with Methanol and no γ-HCH followed by Return to γ-HCH

Section 3.4.1 and Appendix 6.3-9 present results for the enrichment culture grown only on methanol. The purpose of this section of the Appendix is to present the results for the methanol-only enrichment culture when exposed to γ-HCH. The enrichment cultures grown for three generations on methanol were exposed to γ-HCH to check if γ-HCH dechlorination activity can be recovered after enrichment without γ-HCH (Figure 6.3-23). As can be observed from the plot, γ-HCH dechlorination activity was recovered in the enrichment culture grown on only methanol. The organisms responsible for γ-HCH dechlorination were able to survive in the methanol only enrichment and then, when exposed to γ-HCH, were able to begin dechlorination activity again. It is not clear what would have been the possible growth mechanism for the γ-HCH dechlorinating microbe in the methanol only enrichment culture.
Figure 6.3-23  γ-HCH dechlorination in enrichments grown only on methanol for three generations and then exposed to γ-HCH.
6.3.11 Effect of sulfate concentration on γ-HCH Dechlorination

The purpose of this section of the Appendix is to present the results for the study done with two different concentrations of sulfate in the media. Two sets of triplicate microcosms; (i) bottles with 14.8 mM SO$_4^{2-}$ (equivalent to 110% of the electron equivalence of donor (methanol) added) and (ii) bottles with 1.3 mM SO$_4^{2-}$ (same concentration as in methanol enrichments, Appendix 6.3.7). The expectation was that in the bottles with high sulfate concentration (14.8 mM), sulfate would compete for the electron donor and exert competitive inhibition on γ-HCH dechlorination. Also, if a sulfate reducing microbe is responsible for γ-HCH dechlorination, then sulfate reduction will be the dominant process resulting in the use of all the added electron donor (methanol) for sulfate reduction. The presence of a high sulfate concentration initially had an inhibitory effect on γ-HCH dechlorination (Figure 6.3-24). However, γ-HCH dechlorination continued at a decreased rate of dechlorination even in the presence of a sulfate concentration greater than the equivalence of the donor added. Competitive inhibition for electron donor would be the likely reason for the decrease in γ-HCH dechlorination in the presence of a high sulfate concentration. In subsequent studies (Figure 6.3-26), robust dechlorination of γ-HCH occurred in the absence of sulfate, which suggests that the presence of sulfate is not a requirement for γ-HCH dechlorination.
Figure 6.3-24  Effect of sulfate concentration on $\gamma$-HCH dechlorination.
6.3.12 Evaluation of Neat $\gamma$-HCH Addition in MSM-2

Section 3.4.2 presents the results for enrichment culture III in MSM-2. The purpose of this section of the Appendix is to present results for preliminary experiments conducted to evaluate the addition of neat compound. The results for the first generation of enrichment cultures evaluated when $\gamma$-HCH was delivered as a neat compound are shown in Figures 6.3-25, 6.3-26 and 6.3-27. The electron donor conditions included methanol, hydrogen+acetate and hydrogen only. No hydrogen production was observed in the microcosms with methanol as the electron donor. In the microcosms with hydrogen as the electron donor, extensive consumption of hydrogen was observed, suggesting that hydrogen can serve as an electron donor for $\gamma$-HCH dechlorination. No $\gamma$-HCH dechlorination was observed in the microcosms with acetate as the only donor and in the no substrate controls (Figure 6.3-26). In the microcosms with hydrogen as the electron donor, extensive $\gamma$-HCH dechlorination was observed (Figures 6.3-26 and 6.3-27).
Figure 6.3-25  Hydrogen consumption in enrichments when γ-HCH was delivered as neat compound, the inoculum was from generation 7 enrichment culture with methanol and γ-HCH, H2 = hydrogen, Ac = acetate.
Figure 6.3-26 Appearance of γ-HCH dechlorination products in enrichment III (MSM-2) generation 1 H$_2$ = hydrogen, Ac = acetate, No Sub = no added electron donor.
Figure 6.3-27 Appearance of γ-HCH dechlorination products in enrichment culture III (MSM-2), bicarbonate generation 2 H2 = hydrogen, Ac = acetate, No Sub = no added electron donor.
6.3.13 *Hydrogen Consumption by the MSM-2 culture with and without γ-HCH*

The treatments that did not receive γ-HCH consumed the most hydrogen relative to the microcosms that received γ-HCH (Figure 6.3-28). Additional analysis of these microcosms revealed that the majority of the added hydrogen was being consumed for acetate production by the acetogens (Table 3.1).

![Graph showing hydrogen consumption](image)

**Figure 6.3-28** Hydrogen consumption in enrichment culture III (MSM-2), bicarbonate generation 2 H₂ = hydrogen, Ac = acetate.
6.3.14 Evaluation of Neat $\gamma$-HCH Addition in MSM-3 Culture

Section 3.3.4 presents the results for $\gamma$-HCH dechlorination in the MSM-3 enrichment cultures. The purpose of this section of the Appendix is to present the results for the preliminary work done to evaluate hydrogen and acetate as electron donors and the effects of adding $\gamma$-HCH as a neat compound. The different electron donor conditions were hydrogen+acetate, hydrogen, acetate and no electron donor. No $\gamma$-HCH dechlorination was observed in the microcosms with acetate as the only donor and in the no substrate controls (Figure 6.3-29). In the bottles with hydrogen as the electron donor, extensive $\gamma$-HCH dechlorination was observed. The mass of dechlorination product observed in enrichment III cultures (Figure 6.3-28) was nearly 50% lower than the observation in enrichment IV cultures (MSM-3).

![Graph showing benzene and chlorobenzene concentration over time with different electron donor conditions](image)

Figure 6.3-29 Appearance of $\gamma$-HCH dechlorination products in enrichment IV (MSM-3) generation 2. H2 = hydrogen, Ac = acetate, No Sub = no added electron donor.
The no γ-HCH control was used to demonstrate that microbes will not be able to survive or consume the electron donor (hydrogen) in the absence of terminal electron acceptor γ-HCH. As can be seen from Figure 6.3-30, there was some initial consumption of electron donor during the first 50 days in the no γ-HCH controls; however, beyond day 50, no significant hydrogen consumption was observed. In the microcosms with γ-HCH, hydrogen consumption continued as long as γ-HCH was provided. The initial hydrogen consumption in the no γ-HCH control is likely due to abiotic losses. However, the no γ-HCH control continued to consume small amounts of hydrogen in subsequent transfers (Table 3.1).

Figure 6.3-30 Hydrogen consumption in enrichment IV (MSM-3) generation 2 H2 = hydrogen, Ac = acetate.
6.3.15 Transfer from Enrichment Culture IV to Enrichment Culture III

Sections 3.4.2 and 3.4.3 present results for enrichment cultures III and IV. Based on earlier observations (Figure 6.3-25 and 6.3-30), the hydrogen consumption in enrichment culture IV in MSM-3 was significantly lower than in enrichment culture III in MSM-2 and also there was a higher rate of \( \gamma \)-HCH dechlorination in enrichment culture IV. Therefore, enrichment culture IV was transferred back to MSM-2 to evaluate the rate of hydrogen consumption. The hydrogen consumption in the culture transferred from MSM-3 to MSM-2 was lower than in the enrichment cultures that were always enriched in MSM-2 but higher than the cultures that were always enriched in MSM-3 (Figure 6.3-31). The higher consumption in MSM-2 cultures was mainly due to the formation of acetate by acetogenesis.

![Graph showing hydrogen consumption in enrichments grown in HEPES media (MSM 3) and then transferred to bicarbonate media (MSM 2).](image)

**Figure 6.3-31** Hydrogen consumption in enrichments grown in HEPES media (MSM 3) and then transferred to bicarbonate media (MSM 2). H2 = hydrogen, Ac = acetate.
6.3.16 Recovery of γ-HCH Dechlorination in MSM-2 Culture Enriched without γ-HCH

Section 3.4.2 presents results for hydrogen consumption in MSM-2 culture without γ-HCH, where acetogenesis was the dominant process. The purpose this section of the Appendix is to demonstrate the recovery of γ-HCH dechlorination in enrichment culture III grown without γ-HCH. As can be seen from Figures 6.3-32 and 6.33, γ-HCH dechlorination was recovered immediately and hydrogen consumption was maintained. In the enrichment cultures maintained with γ-HCH, acetogenesis was the dominant process. It is possible that an acetogen is responsible for γ-HCH dechlorination, which can grow even in the absence of γ-HCH by acetogenesis. Alternatively, the γ-HCH dechlorinating microbes were able to survive long periods of time without γ-HCH and recover dechlorination activity when exposed to γ-HCH.

![Graph](image)

**Figure 6.3-32** Appearance of γ-HCH dechlorination products in enrichments grown in bicarbonate media (MSM 2) without γ-HCH and then exposed to γ-HCH, H2 = hydrogen, Ac = acetate.
Figure 6.3-33 Hydrogen consumption in enrichments grown in bicarbonate media (MSM 2) without γ-HCH and then exposed to γ-HCH, H2 = hydrogen, Ac = acetate.
6.3.17 Recovery of γ-HCH Dechlorination in MSM-3 Culture Enriched without γ-HCH

Similar to the observations in Appendix 6.3.15, enrichment culture IV grown without γ-HCH was exposed to γ-HCH to evaluate the recovery of γ-HCH dechlorination. As can be seen in Figures 6.3-34 and 6.3-35, γ-HCH dechlorination was recovered immediately and hydrogen consumption rates were greater than the rate observed in the absence of γ-HCH (Table 3.1). It was not clear how the γ-HCH dechlorinating microbes were able to survive extended periods of time without γ-HCH and be able to recover dechlorination activity immediately when exposed to γ-HCH.

![Graph showing benzene + chlorobenzene concentrations over time](image)

Figure 6.3-34 Appearance of γ-HCH dechlorination products in enrichments grown in HEPES media (MSM 3) without γ-HCH and then exposed to γ-HCH. H2 = hydrogen, Ac = acetate.
Figure 6.3-35  Hydrogen consumption in enrichments grown in HEPES media (MSM 3) without γ-HCH and then exposed to γ-HCH  H2 = hydrogen, Ac = acetate.
6.3.18 α-HCH Dechlorination in Groundwater Enrichment

An attempt was made to grow an enrichment culture that dechlorinated α-HCH. The purpose this section of the Appendix is to present the results for an α-HCH enrichment culture in groundwater with methanol and lactate provided as the electron donors. The degradation of α-HCH and accumulation of dechlorination of products benzene and CB were observed in the α-HCH enrichment culture (Figure 6.3-36). However, the rate of α-HCH dechlorination was considerably lower than the dechlorination rate observed with γ-HCH (Figure 3.1). Therefore, it was decided to concentrate our efforts on the γ-HCH dechlorinating enrichment. Also, γ-HCH accounts for 90% of lindane and therefore, an enrichment culture that dechlorinates γ-HCH would be more relevant for bioremediation of sites contaminated with lindane.
Figure 6.3-36 α-HCH dechlorination and daughter products accumulation in MSM-1
Appendix 6.4: Supplementary Results for Chapter 4

The purpose of this section of the Appendix is to present additional results that support the data and conclusions reached based on the study reported in Chapter 4.
6.4.1 Lag Time Observed in Biodegradation of CB, 1,2-DCB and 1,3-DCB

Sections 4.3.2 and 4.3.3 present the results for cometabolic transformation of cDCE and TCE by resting cells and growing cells. The purpose this section of the Appendix is to demonstrate that even in the absence of cDCE or TCE, significant lag times were observed with the isolates growing on CB, 1,2-DCB and 1,3-DCB (Figures 6.4-1, 6.4-2 and 6.4-3).

![Graph showing lag phase in CB grown cells used for the resting cell experiment.](image)

Figure 6.4-1 Representative plot showing the lag phase in CB grown cells used for the resting cell experiment.
Figure 6.4-2 Representative plot showing the lag phase in 1,2-dichlorobenene grown cells used for the resting cell experiment.

Figure 6.4-3 Representative plot showing the lag phase in 1,3-dichlorobenene grown cells used for the resting cell experiment.
6.4.2 Effect of cDCE and TCE on Biomass Yield

Section 4.4.1 and Table 4.1 present the results for yields determined by growth on monoaromatics in the absence of cDCE or TCE. The purpose of this section of the Appendix is to compare the yields observed in the presence and absence of cDCE or TCE. As shown in Table 6.4-1, no significant differences were observed in growth yields both in the presence and absence of cDCE and TCE.

Table 6.4-1 Biomass of yield for growth substrates in the presence and absence of cDCE or TCE

<table>
<thead>
<tr>
<th>Growth Substrate</th>
<th>In the absence of cDCE or TCE</th>
<th>In the presence of cDCE</th>
<th>In the presence of TCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>0.45±0.02</td>
<td>0.48±0.08</td>
<td>0.47±0.03</td>
</tr>
<tr>
<td>CB</td>
<td>0.41±0.01</td>
<td>0.40±0.03</td>
<td>0.40±0.01</td>
</tr>
<tr>
<td>1,2-DCB</td>
<td>0.37±0.06</td>
<td>0.31±0.01</td>
<td>0.33±0.01</td>
</tr>
<tr>
<td>1,3-DCB</td>
<td>0.36±0.05</td>
<td>0.36±0.01</td>
<td>0.28±0.03</td>
</tr>
</tbody>
</table>
6.4.3 Measured and Calculated Transformation Capacities in the Presence of cDCE and TCE

Section 4.4.3 presents the results for $T_c'$ based on equation 4.3 in the presence of cDCE or TCE. The purpose of this section of the Appendix is to compare the measured $T_c'$ (section 4.5) with a calculated $T_c'$. As shown in Table 6.4-2, there was no appreciable difference between the measured and calculated valued of $T_c'$.

Table 6.4-2 Measured and Calculated Values for $T_c'$

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Growth substrate</th>
<th>cDCE $T_c'$ (µg cDCE/mg biomass)</th>
<th>cDCE $T_c$ $^a$ (µg cDCE/mg biomass)</th>
<th>TCE $T_c'$ (µg TCE/mg biomass)</th>
<th>TCE $T_c$ $^a$ (µg TCE/mg biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodococcus</td>
<td>Benzene</td>
<td>11±2.0</td>
<td>9.6±1.0</td>
<td>0.18±0.02</td>
<td>0.15±0.03</td>
</tr>
<tr>
<td>Ralstonia</td>
<td>CB</td>
<td>5.6±0.2</td>
<td>5.3±0.1</td>
<td>0.30±0.1</td>
<td>0.28±0.04</td>
</tr>
<tr>
<td>Variovorax</td>
<td>1,2-DCB</td>
<td>4.9±1.0</td>
<td>4.8±0.4</td>
<td>0.15±0.03</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>Ralstonia</td>
<td>1,3-DCB</td>
<td>7.2±1.0</td>
<td>4.7±0.1</td>
<td>0.33±0.08</td>
<td>0.28±0.04</td>
</tr>
</tbody>
</table>

$^a$ Calculated based on the yield value obtained in the presence of cometabolic substrate (Table 6.4-1).
6.4.4 Enrichment and Isolation of Benzene Degrading Isolate

Section 4.4.1 presents the results for identification of the benzene-grown isolate and the growth yield measurements. The purpose of this section of the Appendix is to present results for enrichment of the benzene isolate and growth of the subsequent lyophilized culture (Figures 6.4-4 to 6.4-7). The first generation enrichment culture (Figure 6.4-4) in groundwater and soil slurry from aerobic microcosms (Chapter 2) degraded all aromatic contaminants, cDCE and TCE. The majority of decreases in cDCE and TCE were observed when aromatic contaminants were being degraded. The second generation enrichment culture in mineral salts medium (MSM) with benzene, cDCE and TCE are shown in Figure 6.4-5. In the second generation enrichment culture, benzene degradation was observed with a short lag time but transformation of cDCE and TCE was observed only from day 150 after significant levels of benzene consumption. When benzene not added to these bottles, cDCE and TCE transformation stalled (days 180-195). The second generation enrichment culture was streaked on a tripticase soy agar and well isolated colonies were transferred back to MSM and evaluated for biodegradation of benzene, cDCE and TCE (Figure 6.4-6). Benzene degradation was observed with a short lag time in bottles with colony in MSM; cDCE and TCE degradation was observed only after significant levels of benzene consumption. The process of streaking and transferring back to MSM to evaluate biodegradation of benzene, cDCE and TCE was done three times. The MSM culture was streaked on tripticase soy agar plates. Their purity was verified by microscopic observation. The
isolate was then used for growing in a large volume (Figure 6.4-7). The culture grown in large volume was later lyophilized and used for all the experiments.

![Graph showing biodegradation](image)

**Figure 6.4-4** First generation enrichment culture with soil slurry and groundwater from contaminated site. Biodegradation of aromatic contaminants (A), biodegradation of cDCE and TCE (B), each data point represents average of triplicate microcosms.
Figure 6.4-5 Second generation enrichment culture with benzene as growth substrate. Benzene biodegradation (A), biodegradation of benzene, cDCE and TCE from day 150 to 200 (B), no apparent biodegradation of cDCE and TCE was observed during first 150 days, each data point represents average of triplicate microcosms.
Figure 6.4-6 Representative figure for biodegradation of benzene, cDCE and TCE by a colony transferred from a TSA plate to MSM.
Figure 6.4-7  Biodegradation of benzene in 4.7 L culture, which was used for lyophilization.
6.4.5 Enrichment and Isolation of CB Degrading Isolate

Section 4.4.1 presents the results for identification of the CB grown isolate and the growth yield measurement. The purpose of this section of the Appendix is to present results for enrichment of the CB isolate and subsequent growth of the lyophilized culture (Figures 6.4-8 to 6.4-10). CB degrading organism was isolated by following the protocol described for the isolating the benzene degrading organism (Appendix 6.4.4).

![Graph A](image1)

**Figure 6.4-8** Second generation enrichment culture with chlorobenzene as growth substrate; chlorobenzene biodegradation (A), biodegradation of cDCE and TCE (B); each data point represents the average of triplicate microcosms.
Figure 6.4-9  Representative results for biodegradation of CB by a colony transferred from a TSA plate to MSM.
Figure 6.4-10  Biodegradation of chlorobenzene in 4.7 L culture, which was used for lyophilization.
6.4.6 Enrichment and Isolation of 1,2-DCB Degrading Isolate

Section 4.4.1 presents the results for identification of the 1,2-DCB grown isolate and the growth yield measurement. The purpose of this section of the Appendix is to present the results for enrichment of the 1,2-DCB isolate and growth of the lyophilized pure culture (Figure 6.4-11 to 6.4-13). The 1,2-DCB degrading organism was isolated by following the protocol described for the isolating the benzene degrading organism (Appendix 6.4.4).

![Graph](image)

Figure 6.4-11 Second generation enrichment culture with 1,2-dichlorobenzene as growth substrate. 1,2-DCB biodegradation (A), biodegradation of cDCE and TCE (B), each data point represents average of triplicate microcosms.
Figure 6.4-12  Representative result for biodegradation of 1,2-DCB by a colony transferred from a TSA plate to MSM.
Figure 6.4-13  Biodegradation of 1,2-DCB in 4.7 L culture, which was used for lyophilization.
6.4.7  Enrichment and Isolation of 1,3-DCB Degrading Isolate

Section 4.4.1 presents the results for identification of 1,3-DCB grown isolate and the growth yield measurement. The purpose of this section of the Appendix is to present results for enrichment of the 1,3-DCB isolate and subsequent growth of the lyophilized culture (Figures 6.4-14 to 6.4-16). The 1,3-DCB degrading organism was isolated by following the protocol described for isolating the benzene degrading organism (Appendix 6.4.4).

![Graph A: 1,3-Dichlorobenzene (mg/L)]

![Graph B: Ethenes (mg/L)]

Figure 6.4-14  Second generation enrichment culture with 1,3-dichlorobenzene as growth substrate. 1,3-DCB biodegradation (A), biodegradation of cDCE and TCE (B), each data point represents average of triplicate microcosms.
Figure 6.4-15 Representative result for biodegradation of 1,3-DCB by a colony transferred from a TSA plate to MSM.
Figure 6.4-16  Biodegradation of 1,3-DCB in a 4.7 L culture, which was used for lyophilization.