MICROBIAL COMMUNITY STRUCTURE DYNAMICS IN OHIO RIVER SEDIMENTS DURING REDUCTIVE DECHLORINATION OF PCBS

Andres Enrique Nunez

University of Kentucky

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ABSTRACT OF DISSERTATION

Andres Enrique Nunez

The Graduate School
University of Kentucky
2008
MICROBIAL COMMUNITY STRUCTURE DYNAMICS IN OHIO RIVER SEDIMENTS DURING REDUCTIVE DECHLORINATION OF PCBS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
Andres Enrique Nunez

Director: Dr. Elisa M. D’Angelo

Lexington, KY

2008

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ABSTRACT OF DISSERTATION

MICROBIAL COMMUNITY STRUCTURE DYNAMICS IN OHIO RIVER SEDIMENTS DURING REDUCTIVE DECHLORINATION OF PCBS

The entire stretch of the Ohio River is under fish consumption advisories due to contamination with polychlorinated biphenyls (PCBs). In this study, natural attenuation and biostimulation of PCBs and microbial communities responsible for PCB transformations were investigated in Ohio River sediments.

Natural attenuation of PCBs was negligible in sediments, which was likely attributed to low temperature conditions during most of the year, as well as low amounts of available nitrogen, phosphorus, and organic carbon. Moreover, surface sediments were relatively oxidized, as indicated by the prevalence of aerobic bacteria such as beta-Proteobacteria, alpha-Proteobacteria, Sphingobacteria, and Nitrospira in 16S rRNA sediment clone libraries. On the other hand, several reductive dechlorinators were detected in sediments, including *Dehalococcoides*, *Desulfitobacterium* spp. which suggested that reductive dechlorination might be possible in sediments under certain biogeochemical conditions.

Considerable amounts of PCBs were transformed by reductive dechlorination (80% in 177 days by pattern $\text{N}$) when sediments were maintained under anaerobic conditions, amended with nutrients and organic carbon, and incubated at 25 °C in lab microcosms. Analysis of 16S rRNA clone libraries from these treatments revealed that Bacteroidetes, Chloroflexi and Firmicutes were enriched and Proteobacteria were depleted compared to clone libraries from treatment without organic amendments. Reductive dechlorination was decreased in sediments incubated at 10 and 40 °C, and was not affected by FeSO$_4$ amendments compared to unamended sediments incubated at 25 °C.

Transformations of PCB-153 were investigated in sediments under anaerobic, aerobic and sequential anaerobic and aerobic conditions. Transformations were only observed in treatments with an anaerobic phase, which occurred by reductive dechlorination by pattern $\text{N}$. Neither PCB-153 nor dechlorination products PCB-99 or PCB-47 were transformed under aerobic conditions. Analysis of 16S rRNA clone libraries revealed that Bacteroidetes, Chloroflexi, and Firmicutes were enriched under anaerobic conditions and Proteobacteria were enriched under aerobic conditions.
Results from this study revealed that natural attenuation and biostimulation were not effective at removing PCBs from Ohio River sediments. Hence, other remediation methods will need to be employed to decrease PCB levels in this ecosystem.

Keywords: Polychlorinated biphenyls, Reductive Dechlorination, Phylogenetic Analysis, Natural attenuation, Biostimulation
MICROBIAL COMMUNITY STRUCTURE DYNAMICS IN OHIO RIVER SEDIMENTS DURING REDUCTIVE DECHLORINATION OF PCBS

By

Andres Enrique Nunez

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Director of Dissertation

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Director of Graduate Studies
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To Lilia, Nicole and Samuel
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Overview of the problem

Polychlorinated biphenyls (PCBs) are synthetic compounds that were synthesized in the US and other countries between 1929 and 1977 for a wide range of industrial uses. Since their introduction, several hundred million pounds have been released to the environment, which has caused adverse effects to humans and biota due to their toxicity and tendency to bioaccumulate in the food chain. Although most PCBs are chemically and thermally inert, several aerobic bacterial strains such as *Pseudomonas*, *Burkholderia* and *Rhodococcus* spp. can transform the biphenyl ring of PCBs to relatively innocuous products, including benzoate, CO₂ and H₂O. Unfortunately, aerobic biotransformation of PCBs and other pollutants are limited in environments devoid of oxygen (e.g. anaerobic sediments from aquatic environments). Aerobic biotransformation of PCBs are further restricted to PCBs with a low number of chlorines on the biphenyl ring (e.g. <5). In many cases, PCBs have been released as complex mixtures (referred to as Aroclors and many other names) with between 10% (Aroclor 1242) and 100% (Aroclor 1260) of congeners having >5 chlorine atoms per biphenyl ring (Erickson, 1997). A prerequisite for the removal of these congeners from the environment is the removal of several chlorine atoms from the biphenyl ring (Abramowicz, 1995; Mohn and Tiedje, 1992).

Experiments with anaerobic sediments have shown that chlorines can be removed from the biphenyl ring by microbial communities by a process referred to as reductive dechlorination. Polychlorinated biphenyls tend to be dechlorinated preferentially at the *meta* and *para* positions of the biphenyl ring, leading to the accumulation of ortho-
chlorinated congeners in environments with active dechlorination activity. To date, very few bacteria with the ability to dechlorinate PCBs have been characterized, which include *Dehalococcoides* spp. (Fennel et al., 2004), ortho dechlorinator o-17 (Cutter et al., 2001) and double-flanked dechlorinator DF-1 (Wu et al., 2002). The capacity of these bacteria to dechlorinate PCBs and other naturally-occurring and synthetic chlorinated compounds, is attributed to the presence of one or more reductive dehalogenase genes, which couple the oxidation of simple electron donor compounds (e.g. H₂, acetate) to the reduction of the PCBs in an energy-yielding process (Smidt and de Vos, 2004).

Reductive dechlorination is a highly syntrophic process in which dechlorination populations depend on electron donor substrates provided by other fermenting, and non-dechlorinating populations such as sulfate reducers. For this reason, it has been difficult to isolate and characterize reductively dechlorinating bacteria from anaerobic environments, determine their relationships to other microbial groups, or elucidate the environmental factors that affect dechlorinating microbial consortia and dechlorination activity. The main motivation of the research in this dissertation was to address questions such as how temperature, and electron donor/acceptor availability affect the microbial community composition in Ohio River sediments during reductive dechlorination of PCBs.
Nomenclature, production, and uses of PCBs

Polychlorinated biphenyls (PCBs) are synthetic compounds that were manufactured in large quantities (1.4 billion lbs) from 1929 to 1977 in the United States for a multitude of industrial uses. They include 209 congeners that differ in the number of chlorines attached to the biphenyl ring (Figure 1.1). Congeners with the same number of chlorine atoms are called homologs, so that the pentachlorobiphenyls, for example, contain 46 congeners (Table 1.1). They have been commercialized as complex mixtures with different degrees of chlorination, under various trade names including Aroclor (Monsanto, US and UK), Clophen (Bayer, Germany), Kanechlor (Kanegafuchi, Japan), Phenoclor (Prodelec, France), and Fenclor (Caffaro, Italy). The more common products are Aroclors 1242–1260, Clophens A30–A60 and Kanechlorls 300–600. For Aroclors, the first two digits of the numerical designation indicate the number of carbon atoms in the biphenyl ring system and the second two digits indicate the percent chlorine mass in the product. Monsanto manufactured Aroclor mixtures until 1970 in Anniston, AL, and until 1977 in Sauget, IL. Initially, these products were used as coolant/dielectric fluids in transformers and capacitors, as heat transfer fluids, and as flame resistant wood coatings. Due to their inertness, their use was later expanded to paints, inks, pesticides, and other uses (Table 1.2).

The extensive use of PCBs over several decades has resulted in widespread contamination of the environment. It has been estimated that several hundred million pounds have been released to the environment (Bedard, 2003). Unfortunately, many of the same chemical properties that make PCBs excellent choices for so many industrial
Figure 1.1 Structure of polychlorinated biphenyls

\[\begin{align*}
&\text{ortho} = C2, C6 \\
&\text{meta} = C3, C5 \\
&\text{para} = C4
\end{align*}\]
Table 1.1 Selected Properties of Polychlorinated Biphenyls (Erickson, 1997)

<table>
<thead>
<tr>
<th>Isomer group</th>
<th>Molecular formula</th>
<th>Molecular Weight</th>
<th>No. of compounds</th>
<th>Vapor Pressure (Pa)</th>
<th>Water solubility (mg L⁻¹)</th>
<th>Log Kₐw</th>
<th>Log Kₒc&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BAF in fish</th>
<th>E at 25 ºC (g m⁻² h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono-CB</td>
<td>C₁₂H₉Cl</td>
<td>188.7</td>
<td>3</td>
<td>1.1</td>
<td>4.0</td>
<td>4.7</td>
<td>4.0</td>
<td>2.5×10⁻³</td>
<td>0.25</td>
</tr>
<tr>
<td>Di-CB</td>
<td>C₁₂H₈Cl₂</td>
<td>223.1</td>
<td>12</td>
<td>0.24</td>
<td>1.6</td>
<td>5.1</td>
<td>4.5</td>
<td>6.3×10⁻³</td>
<td>0.065</td>
</tr>
<tr>
<td>Tri-CB</td>
<td>C₁₂H₇Cl₃</td>
<td>257.5</td>
<td>24</td>
<td>0.054</td>
<td>0.65</td>
<td>5.5</td>
<td>4.9</td>
<td>1.6×10⁻⁴</td>
<td>0.017</td>
</tr>
<tr>
<td>Tetra-CB</td>
<td>C₁₂H₆Cl₄</td>
<td>292.0</td>
<td>42</td>
<td>0.012</td>
<td>0.25</td>
<td>5.9</td>
<td>5.3</td>
<td>4.0×10⁻⁴</td>
<td>4.2×10⁻³</td>
</tr>
<tr>
<td>Penta-CB</td>
<td>C₁₂H₅Cl₅</td>
<td>326.4</td>
<td>46</td>
<td>2.6×10⁻³</td>
<td>0.099</td>
<td>6.3</td>
<td>5.8</td>
<td>1.0×10⁻⁵</td>
<td>1.0×10⁻³</td>
</tr>
<tr>
<td>Hexa-CB</td>
<td>C₁₂H₄Cl₆</td>
<td>360.9</td>
<td>42</td>
<td>5.4×10⁻⁴</td>
<td>0.038</td>
<td>6.7</td>
<td>6.2</td>
<td>2.5×10⁻⁵</td>
<td>2.5×10⁻⁴</td>
</tr>
<tr>
<td>Hepta-CB</td>
<td>C₁₂H₃Cl₇</td>
<td>395.3</td>
<td>24</td>
<td>1.3×10⁻⁴</td>
<td>0.014</td>
<td>7.1</td>
<td>6.6</td>
<td>6.3×10⁻⁵</td>
<td>6.2×10⁻⁵</td>
</tr>
<tr>
<td>Octa-CB</td>
<td>C₁₂H₂Cl₈</td>
<td>429.8</td>
<td>12</td>
<td>2.8×10⁻⁵</td>
<td>5.5×10⁻³</td>
<td>7.5</td>
<td>7.0</td>
<td>1.6×10⁻⁶</td>
<td>1.5×10⁻⁵</td>
</tr>
<tr>
<td>Nona-CB</td>
<td>C₁₂H₁Cl₉</td>
<td>464.2</td>
<td>3</td>
<td>6.3×10⁻⁶</td>
<td>2.0×10⁻³</td>
<td>7.9</td>
<td>7.5</td>
<td>4.0×10⁻⁶</td>
<td>3.5×10⁻⁶</td>
</tr>
<tr>
<td>Deca-CB</td>
<td>C₁₂Cl₁₀</td>
<td>498.7</td>
<td>1</td>
<td>1.4×10⁻⁶</td>
<td>7.6×10⁻⁴</td>
<td>8.3</td>
<td>7.9</td>
<td>1.0×10⁻⁷</td>
<td>8.5×10⁻⁷</td>
</tr>
</tbody>
</table>

Note: Most of the values presented are averages from congeners in group. BAF, bioaccumulation factor; E, evaporation rate

<sup>a</sup>Log Kₒc values estimated according to Girvin and Scott, 1997.
<table>
<thead>
<tr>
<th>Category</th>
<th>Type of product</th>
<th>Before 1971</th>
<th>After 1971</th>
</tr>
</thead>
<tbody>
<tr>
<td>Closed systems</td>
<td>Transformer, capacitors, other electrical insulating/cooling applications</td>
<td>61</td>
<td>100</td>
</tr>
<tr>
<td>Nominally closed systems</td>
<td>Hydraulic fluids, heat transfer fluids, lubricants</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Open-end applications</td>
<td>Plasticizers, surface coatings, ink and dye carriers, adhesives, pesticide extenders, carbonless copy paper, dyes.</td>
<td>26</td>
<td>0</td>
</tr>
</tbody>
</table>
uses also make them some of the worst materials that can be released to the environment.

Even by 1937, their toxic effect was evident in exposed workers, and by 1970, production was banned and the use of PCBs was restricted to “closed systems” such as those shown in Table 1.2 (Becton et al., 1979). Currently, PCBs are classified as the twelfth most persistent organic pollutant in the world, according to the United Nations Environment Programme Stockholm Convention (UNEP, http://www.pops.int/).

**Toxicology of PCBs**

Polychlorinated biphenyls are classified as probable carcinogens to humans (ATSDR, 2001). Due to the extensive contamination of aquatic systems with PCBs, fish consumption represents the largest PCB exposure route to humans. As of 2004, the EPA issued 873 fish consumption advisories due to PCBs in 39 US states. According to the Code of Federal Regulations, the tolerance for PCBs in fish is about 2 ppm (21CFR109.30) and the maximum contaminant level for PCBs in drinking water to reduce cancer risk is 0.5 ppb (40CFR141.61). The Occupational Safety and Health Administration (29CFR1910.1000) has also established permissible inhalation exposure levels of 0.5-1.0 mg/m³ in air for 8-h work periods.

The toxicity of commercial mixtures of PCBs depends on several factors, such as chlorine content and purity of the mixture, animal species, age, gender, and route and duration of exposure. Symptoms of PCB exposure include elevated serum lipid levels, serum enzymes, chloracne, dermal lesions, hepatic responses, respiratory problems, eye irritation, decreased birth weight, and variable effects on cancer formation. The acute,
subacute, and subchronic toxic responses to commercial PCBs are characterized by lethal dose (LD₅₀) values that are usually > 1000 mg/kg. In chronic feeding studies, most of the above toxic responses were observed, with liver being the primary target site (Safe, 1993). The University of California in Berkeley has developed the Carcinogenic Potency Database (CPDB, http://potency.berkeley.edu/) providing tumor dose (TD₅₀) values for several chemicals including PCBs. This value indicates the required dose in mg kg⁻¹ d⁻¹ for the appearance of tumors in 50% of the test animals when the control animals do not exhibit any tumors. Although individual PCB congeners were not tested, the TD₅₀ values for Aroclor mixtures range from 53.9 in Aroclor 1016 to 2.81 mg kg⁻¹ d⁻¹ in Aroclor 1260, indicating a higher potency of higher chlorinated mixtures as carcinogenic.

Commercial PCB mixtures produce a wide range of biochemical and toxic responses and most of these are similar to those caused by dioxins (Safe, 1994). In particular, three PCB congeners, PCB-77 (3,3’,4,4’-tetraCB), PCB-126 (3,3’,4,4’,5-pentaCB), and PCB-169 (3,3’,4,4’,5,5’-hexaCB) have the same spectrum of toxic and biochemical responses observed for dioxins, and therefore are the most toxic among all congeners (Safe, 1994). Maximum dioxin-like activities are associated with presence of two para chlorines, two or more meta chlorines, and no ortho chlorines (Safe, 1993) which allows for coplanarity of the biphenyl rings, thus resembling dioxin structures.

In eukaryotes, the metabolism and toxicity of PCBs is associated with activation of the Aryl Hydrocarbon Receptor (AhR), which is a ligand-dependent transcription factor that regulates transcription of certain key enzymes involved in the metabolism of xenobiotics (Hankinson, 1995; Denison and Nagy, 2003; Marlowe and Puga, 2005). Although dioxins are the most potent ligands (Hankinson, 1995, Mimura and Fujii-
Kuriyama, 2003), PCBs can bind to the AhR, resulting in a complex that is transported to the nucleus where it binds to specific genomic sequences to induce gene transcription. Enzymes activated by this mechanism, are also known as Phase I-Phase II enzyme system (Rose and Hodgson, 2004). As xenobiotics are usually lipophilic and tend to accumulate in the lipid membranes and be transported with lipoproteins in the blood, Phase I enzymes introduce polar reactive groups into the molecule to increase water solubility and to make it a more favorable substrate for Phase II enzymes. During Phase II, conjugation reactions introduce bulky groups such as sulfates, sugars or amino acids that make the xenobiotics much more soluble and easily excreted from the organism (Fig. 1.2). Phase I enzymes usually involve cytochrome P450 (CYPs) and flavin-containing (FMOs) monooxygenases, and several other dehydrogenases, oxidases, cyclooxygenases, reductases, and hydroxylases, while some Phase II enzymes include glucuronidases, sulfotransferases, methyltransferases, glutathione transferases, and acetyl transferases (Rose and Hodgson, 2004).

The Phase I-Phase II enzyme system metabolizes a wide range of foreign substances in the organism, however, Phase I enzymes and especially CYPs can produce reactive intermediates such as arene oxides which are powerful electrophiles that can associate with nucleic acids and cause mutations leading to the activation of protooncogenes or inactivation of tumor suppressor genes (Hankinson, 1995).
Figure 1.2 Metabolism of PCBs (adapted from Safe, 1994)
PCB contamination in the Ohio River

Many aquatic ecosystems near industrial areas are contaminated with PCBs. The most commonly studied systems include the Hudson River in New York, the Housatonic River in Massachusetts, and the Fox River in Wisconsin. Although less studied, the Ohio River is also extensively contaminated with PCBs, which impacts the health of wildlife and millions of people that utilize the resource (ORSANCO, 2002).

The Ohio River starts at the confluence of the Allegheny and Monongahela Rivers in Pittsburg, Pennsylvania, and travels 981 miles until it joins the Mississippi River in Cairo, Illinois. Six states border the Ohio River (Illinois, Indiana, Kentucky, Ohio, Pennsylvania, and West Virginia) and several other states (New York, North Carolina, Maryland, Tennessee, and Virginia) are located in the Ohio River basin, which covers about 190,000 square miles and is inhabited by more than 25 million people (Fig. 1.3). The Ohio River has four designated uses, including warm water aquatic habitat, public water supply, contact recreation, and fish consumption. Unfortunately, the river does not fully support the fish consumption use, due to contamination with PCBs, dioxin, and mercury (ORSANCO, 2006) (Table 1.3).

Several non-point and point sources are believed to contribute PCBs to the Ohio River. According to an ORSANCO report (2002) the main non-point sources of PCBs to the river are atmospheric deposition, resuspension of contaminated sediments, overland runoff, and inflow of groundwater. On the other hand, the main point sources of PCBs identified so far are wastewater treatment plants, but several other industries are listed as potential PCB sources including several power generation and manufacturing industries.
Figure 1.3 Ohio River and its basin. Fish consumption advisories are indicated according to Table 1.3. Arrows indicate extension of advisory.
<table>
<thead>
<tr>
<th>State</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pennsylvania</td>
<td>From RM 0 to RM 31.7 (#2024)</td>
</tr>
<tr>
<td></td>
<td>From RM 31.7 to RM 40.0 (#4830)</td>
</tr>
<tr>
<td>West Virginia</td>
<td>Entire length in West Virginia (#876)</td>
</tr>
<tr>
<td>Ohio</td>
<td>Entire Length in Ohio (#2015)</td>
</tr>
<tr>
<td>Kentucky</td>
<td>From RM 317.1 to RM 436.2 (#2094)</td>
</tr>
<tr>
<td></td>
<td>From RM 436.2 to RM 605.0 (#104031)</td>
</tr>
<tr>
<td></td>
<td>From RM 605.0 to RM 981.0 (#104032)</td>
</tr>
<tr>
<td>Indiana</td>
<td>Entire length in Indiana</td>
</tr>
<tr>
<td>Illinois</td>
<td>Entire length in Illinois (#4839)</td>
</tr>
</tbody>
</table>
Polychlorinated biphenyls in Ohio River waters range from 0.54 to 4.55 ng/L, but in sediments this range goes up to 8 ppm, with hot spots located near zones with high industrial activity. For example, 8 ppm of PCBs were found in sediments at river miles 71.4, and 122.9, locations associated with steel and aluminum manufacturing industries, and wastewater treatment plants.

**Fate processes of PCBs in aquatic environments**

Once PCBs are released to aquatic environments, they may undergo numerous fate processes that depend largely on chemical properties of the PCB congeners and the ecosystem. Some of the most important fate processes of PCBs are depicted in Fig. 1.4, and key chemical properties of PCBs that govern the extent of processes are provided in Table 1.1.

The extent of PCB volatilization into the atmosphere depends largely on the vapor pressure of individual PCBs, which range from $10^{-6}$ Pa for highly chlorinated congeners to 1 Pa for monochlorinated congeners (Table 1.1). Thus, the less chlorinated congeners would be expected to volatilize to a greater extent and be more widely distributed in the environment than highly chlorinated congeners. On the other hand, the highly chlorinated congeners would be expected to be relatively enriched in contaminated environments.

Once PCBs enter the atmosphere, they can remain in the vapor phase or associate with particulate matter. Reactions with OH radicals are the main atmospheric degradation pathway (Anderson and Hites, 1996) although photolysis with solar radiation or UV rays is also possible (Manzano et al., 2004). PCBs are eventually removed from the
Figure 1.4 Environmental fate of PCBs (from Safe et al., 1985).
atmosphere either by dry deposition (in which particulate materials settle down to the ground or water bodies), or by wet deposition (in which they are removed from the atmosphere by rain, snow, etc).

The concentration of dissolved PCBs in the water column of lakes and rivers depends largely on the extent to which PCBs partition in the suspended and deposited sediments. The extent that chemicals partition between the sediment and water is described by the equilibrium sorption coefficient ($K_d$), or by the soil organic carbon content normalized adsorption coefficient ($K_{OC}$). The $K_{OC}$ is defined as the sorption coefficient divided by the organic carbon content in soil or sediment. The $K_{OC}$ values can be estimated from the $K_{OW}$ values, according to the following expression: $\log(K_{OC}) = 1.07 \times \log(K_{OW}) - 0.98$ (Girvin and Scott, 1997). Once the $K_{OC}$ is estimated, $K_d$ can be determined by the following relationship: $K_d = K_{OC} \times f_{OC}$, where $f_{OC}$ is the fraction of organic carbon in the soil or sediment. As shown in Table 1.1, log $K_{OC}$ values for PCBs range from 4.0, for less chlorinated PCBs, to 7.9 for highly chlorinated PCBs. Based on log $K_{OC}$ values, lower chlorinated PCBs would be expected to occur at higher concentrations in the water column than higher chlorinated congeners. This is important because the amount of PCBs in the water column largely determines PCB mobility, bioavailability, and volatilization processes. In addition, PCBs with high $K_{OC}$ values would tend to accumulate in sediments, which could act as PCB sources to other parts of the aquatic system via resuspension/desorption events.

Polychlorinated biphenyls may also accumulate in aquatic biota, particularly in the fatty tissue due to the hydrophobic characteristics of PCBs. For hydrophobic contaminants, the concentration of a chemical in biota is commonly described by the
equilibrium octanol-water coefficient ($K_{OW}$) of the compound. The $K_{OW}$ is defined as the concentration of a chemical in octanol divided by the concentration of a chemical in water at equilibrium, with units of L water/L octanol. Octanol is commonly used because it has the same carbon:oxygen ratio as lipids, and $K_{OW}$ values have a good correlation with lipid-water partition coefficients (Shea, 2004). As shown in Table 1.1, the log $K_{OW}$ of PCBs ranges between 4.9 for low chlorinated PCBs to 8.3 for highly chlorinated PCBs. Thus, the highly chlorinated PCBs would tend to accumulate to a greater extent in aquatic biota than lower chlorinated PCBs in contaminated environments.

Several terms are commonly used to describe the extent and processes by which contaminants accumulate in biota, including bioconcentration, biomagnification, and bioaccumulation (IUPAC, 1993; Leblanc, 2004). Bioconcentration is defined as the ratio of the chemical concentration in an organism and the chemical concentration in surrounding media. Biomagnification refers to the increase in chemical concentration with each progressive link in the food chain. Bioaccumulation is defined as the process by which organisms accumulate chemicals from surrounding media and dietary sources. An example of how a chemical with a bioaccumulation factor (BAF) of 2 increases in the food chain is depicted in Figure 1.5. According to the example in the figure, the chemical concentration in the water has an arbitrary value of one. Assuming that the first trophic level accumulates the chemical only from the surrounding media, the concentration in the organism is two. The second trophic level can accumulate chemicals from both the surrounding media (two units) and the first trophic level (four units), yielding an overall concentration of chemical in the organism of six. BAF values are related to the $K_{OW}$ values as follows: $BAF = 0.048 \times K_{OW}$ (Mackay, 1982).
**Figure 1.5** Bioaccumulation of a chemical along a generic food chain. Assumptions in this model include water concentration of 1, and bioaccumulation factor of 2 either from water or from one trophic level to another. Circled numbers represent the concentration in the respective compartment. Arrow numbers represent the concentration of chemical transferred from one compartment to another (from Leblanc, 2004).
Aerobic biotransformations

Polychlorinated biphenyls can be transformed by several bacteria under aerobic conditions, including gram-negative genera *Achromobacter, Acinetobacter, Alcaligenes, Burkholderia, Comamonas, Moraxella, Pseudomonas, Ralstonia, and Sphingomonas*, as well as gram-positive genera such as *Arthrobacter, Bacillus, Corynebacterium, and Rhodococcus*, (Abraham et al., 2002; Field and Sierra-Alvarez, 2008). In most cases, these organisms transform PCBs cometabolically, i.e. they do not gain carbon or energy from the reaction. For this reason, biphenyl or monochlorobiphenyls are commonly used to trigger the production of biphenyl dioxygenase enzymes that can also attack PCBs (Borja et al., 2005). Bacteria capable of aerobic PCB degradation have been isolated from most of contaminated site samples (Field and Sierra-Alvarez, 2008) indicating the ubiquitous presence of aerobic PCB-degrading bacteria. Unfortunately, cometabolic reactions often lead to the production of dead-end and toxic products, such as chlorobenzoates, that can accumulate unless other microorganisms in the environment can mineralize these compounds (Pieper, 2005).

A common pathway by which aerobic bacteria transform PCBs is the 2,3-dioxygenase pathway or “upper pathway” that refers to the conversion of PCBs to chlorobenzoic acids (Borja et al., 2005, Pieper, 2005) (Figure 1.6). In this pathway, the PCB is hydroxylated at positions 2 and 3 of the aromatic ring, the aromatic ring is cleaved, and chlorobenzoate intermediates are generated. For this reaction to occur, no chlorine substituents can occur at positions 2 or 3 of the aromatic rings. Several species transform PCBs using this pathway, including gram negative strains of *Pseudomonas,*
Figure 1.6 Aerobic degradation of PCBs in the “Upper Pathway”
Alcaligenes, Achromobacter, Acinetobacter, and Moraxella and gram positive strains of Arthrobacter and Rhodococcus (Furukawa et al., 1989).

Some bacterial strains, such as Ralstonia eutrophus H850 (Bedard et al., 1987; Field and Sierra-Alvarez, 2008) and Pseudomonas sp. LB400 (Haddock et al., 1995) also possess 3,4-dioxygenase enzymes that can transform PCBs through 3,4-dioxygenation (Fig. 1.7). Positions 2 and 3 are the preferred for attack in the upper pathway. However, if the ortho positions are blocked, the 3,4-dioxygenases provide the flexibility of attacking at positions 3 and 4 if no chlorines are present on those positions (Bedard et al., 1987). This increases the substrate range for these genera and thus the aerobic degradation potential of these compounds. Both the 2,3- and 3,4-dioxygenation pathways are mostly limited to lower chlorinated congeners with no chlorine atoms in the indicated positions due to steric hindrance and low water solubility of highly chlorinated congeners.

**Anaerobic biotransformations**

Polychlorinated biphenyls can also be transformed by bacteria under anaerobic conditions by a process called reductive dechlorination. In this process, two-electrons and a proton are transferred to the PCB, which results in the release of a chloride ion and a less chlorinated PCB congener (Fetzner and Lingens, 1994) (Fig. 1.8). The redox reaction is exergonic, and many organisms gain energy from the reaction by a process called dehalorespiration (Löffler et al., 2003).

Another benefit of reductive dechlorination is that it converts highly chlorinated congeners to less chlorinated congeners, which are often not as toxic and are more amenable to aerobic biotransformations. A number of other halogenated chemicals
Figure 1.7 3,4-dioxygenation of polychlorinated biphenyls (Haddock et al., 1995)
**Figure 1.8** Reductive dehalogenation of chloroorganic compounds

\[ R-\text{Cl} + e^- + n\text{ ATP} \rightarrow R-H + H^+ + Cl^- \]
Besides PCBs can be reductively dechlorinated, including hexachlorobenzene, tetrachloroethylene, chlorobenzoates, chlorophenols, chlorobenzenes, chloromethanes, chloroethanes, and chloroethenes (Holliger et al., 2003). Early evidence of the importance of reductive dechlorination of PCBs in the environment was alterations in congener profiles in PCB contaminated aquatic sediments compared to those of the original commercial mixtures (Brown et al., 1987; Abramowicz, 1995; Wu et al., 1998). Typically, less chlorinated, ortho-chlorinated congeners tend to accumulate and highly chlorinated meta- and para-chlorinated congeners tend to be depleted in these environments (Quensen et al., 1988). More recently, several other PCB reductive dechlorination patterns have been described, which are summarized in Table 1.4 (Bedard, 2003), and it is believed that different bacteria are responsible for the various pathways.

Several bacterial species with reductive dechlorination abilities have been identified (Fig. 1.9). However, only three microorganisms are recognized to reductively dechlorinate PCBs, which include o-17 (Cutter et al., 2001), DF-1 (Wu et al., 2002), and Dehalococcoides spp. (Cutter et al., 2001). Reductive dechlorination is catalyzed by reductive dehalogenase enzymes (RD), such as trichloroethylene RD and vinyl chloride RD from Dehalococcoides ethenogenes (Magnuson et al., 2000) chlorophenol RD from Desulfitobacterium dehalogenans (van de Pas et al., 1999), and tetrachloroethylene RD (pceA) from Desulfitobacterium hafniense (Maillard et al., 2004). Although no enzymes have been identified to specifically dechlorinate PCBs, reductive dehalogenases are usually associated with the cytoplasmic membrane and they are involved in electron transport-coupled phosphorylation processes for energy production (Futagami et al., 2008). Structurally, RDs contain two Fe-S clusters in the catalytic center that are
<table>
<thead>
<tr>
<th>Dechlorination activity</th>
<th>Targeted chlorine</th>
<th>Homolog substrate range</th>
<th>Reactive chlorophenyl groups&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primary chlorophenyl products</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>Flanked <em>para</em></td>
<td>4-6</td>
<td>34, 234, 245, 2345, 23456</td>
<td>(23), 25, 235, 2356</td>
</tr>
<tr>
<td>H</td>
<td>Flanked <em>para</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4-7</td>
<td>34, 234, 245, 2345</td>
<td>3, 24, 25, 235</td>
</tr>
<tr>
<td>H’</td>
<td>Flanked <em>para</em>&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>3-5</td>
<td>23, 34, 234, 245, 2345</td>
<td>2, 3, 24, 25, 235</td>
</tr>
<tr>
<td>N</td>
<td>Flanked <em>meta</em></td>
<td>5-9</td>
<td>234, 236, 245, 2345, 23456</td>
<td>24, 25, 26, 246</td>
</tr>
<tr>
<td>M</td>
<td>Flanked &amp; unflanked <em>meta</em></td>
<td>2-4</td>
<td>3, 23, 25, 34, 234, 236</td>
<td>2, 4, 24, 26</td>
</tr>
<tr>
<td>Q</td>
<td>Flanked &amp; unflanked <em>para</em>&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>2-4</td>
<td>4, 23, 24, 34, 234, 245, 246</td>
<td>2, 3, 25, 26</td>
</tr>
<tr>
<td>LP</td>
<td>Flanked &amp; unflanked <em>para</em>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3-6</td>
<td>24, 245, 246</td>
<td>2, 25, 26</td>
</tr>
<tr>
<td>T</td>
<td>Doubly flanked <em>meta</em>&lt;sup&gt;e&lt;/sup&gt;</td>
<td>7-8</td>
<td>2345</td>
<td>245</td>
</tr>
</tbody>
</table>

<sup>a</sup> The targeted chlorine(s) for each chlorophenyl groups is (are) underlined

<sup>b</sup> The doubly flanked meta chlorine of 234-chlorophenyl groups is also targeted

<sup>c</sup> The meta chlorine of 23-chlorophenyl groups is also targeted

<sup>d</sup> The substrate range of this dechlorination process has not been completely characterized

<sup>e</sup> This dechlorination process has been observed only at 50 to 60 ºC.
Figure 1.9 Phylogeny of known dechlorinating bacteria (from Löffler et al., 2003).
involved in the electron transfer to the chlorinated substrate, and most of these enzymes also contain a corrinoid cofactor, a Co(I) complex that is similar in structure to vitamin B_{12}. A mechanistic model for the reductive dechlorination of organochlorine compounds is shown in Figure 1.10 (Krasotkina et al., 2001). Two pathways are presented in this mechanism. The first one (dashed arrows) involves formation of an organocobalt adduct, in which two electrons are transferred to the compound with removal of chlorine while the second pathway (solid arrows) involves transfer of one electron to the compound with formation of a radical anion, which finally loses a chlorine atom after transfer of another electron.

One of the most important factors governing reductive dechlorination is the redox status of the environment. In oxidized environments, bacteria use O2 as electron acceptor to gain energy from the oxidation of electron donors. Due to the high reduction potential of O2, bacteria using O2 as an electron acceptor gain high amounts of energy, grow quickly, and outcompete other organisms that conduct lower energy-yielding reactions. In water-saturated environments, where O2 is consumed faster than it is supplied by diffusion, bacteria must use alternate electron acceptors for energy generation. The order that electron acceptors are reduced in the environment can be largely predicted from the amount of energy associated with the redox reactions, which can be calculated from the relationships $$
Delta G^0 = \Sigma \Delta G^0_{f(products)} - \Sigma \Delta G^0_{f(substrates)}$$, and $$
Delta G^0 = -nF\Delta E^0$$, where $n$ is the number of electrons transferred during the reaction, $F$ is the Faraday constant (96.48 kJ/V) and $\Delta E^0$ is the difference in potentials of the coupled oxidation-reduction reaction. Using these relationships and tables of thermodynamic values of common electron acceptors (Dolfing, 2003; Madigan et al., 2003a), the amount of free energy generated
Figure 1.10 Mechanistic model for the corrinoid iron-sulfur reductive dehalogenase.

Dashed arrows pathway (upper) involves formation of an organocobalt aduct, while solid arrows pathway (lower) involves electron transfer from Co(I) to the aromatic ring (Krasotkina et al., 2001).
from different environmental redox reactions using H\textsubscript{2} as electron donor are shown in Figure 1.11.

Based on these calculations, the predicted order of electron acceptor reduction is O\textsubscript{2}, Mn\textsuperscript{4+}, NO\textsubscript{3}\textsuperscript{-}, Fe\textsuperscript{3+}, SO\textsubscript{4}\textsuperscript{2-}, and CO\textsubscript{2}. As mentioned earlier, some bacteria can also use PCBs as electron acceptors. The amount of energy generated from the reaction can also be derived from the Gibb’s free energies of formation of the reactants and products, which were given by Holmes et al. (1993). Based on thermodynamic calculations, about 116 kJ would be obtained from the reductive dechlorination of PCB-153 (Fig. 1.12), which is less than the amount of energy derived from denitrification and greater than the amount of energy derived from sulfate reduction. Therefore, it is predicted that reductive dechlorination of PCB-153 would be inhibited by aerobic and denitrifying activities, but not under sulfate-reducing and methanogenic conditions. In fact, Zwiernik et al. (1998) have shown that adding ferrous sulfate to sediment microcosms stimulated extensive meta and para dechlorination of PCBs. They observed an initial dechlorination inhibition upon sulfate addition, but after sulfate was depleted, amended microcosms showed greater dechlorination extention than unamended controls. They proposed that the initial inhibition was due to electron acceptor shift to sulfate, and the greater extention of dechlorinaton was caused by an increase in the population of sulfate reducers. Interestingly, Gibbs free energies of reductive dechlorination reactions with highly chlorinated PCBs tend to be more negative (more favorable) than those with less chlorinated PCBs, which may partially explain bacterial preferences for highly chlorinated congeners as electron acceptors. Other environmental factors that affect
\[
\frac{1}{2}O_2 + H_2 \rightarrow H_2O \quad \Delta G^\circ = -237.3 \text{ kJ/reaction}
\]
\[
MnO_2 + H_2CO_3 + H_2 \rightarrow MnCO_3 + 2H_2O \quad \Delta G^\circ = -194.9 \text{ kJ/reaction}
\]
\[
NO_3^- + H_2 \rightarrow NO_2^- + H_2O \quad \Delta G^\circ = -162.1 \text{ kJ/reaction}
\]
\[
2Fe(OH)_3 + 2H_2CO_3 + H_2 \rightarrow 2FeCO_3 + 6H_2O \quad \Delta G^\circ = -117.7 \text{ kJ/reaction}
\]
\[
SO_3^2^- + H^+ + 4H_2 \rightarrow HS^- + 4H_2O \quad \Delta G^\circ = -36.7 \text{ kJ/reaction}
\]
\[
CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \Delta G^\circ = -32.8 \text{ kJ/reaction}
\]

**Figure 1.11** Standard Gibbs free energy of common redox reactions in the environment with hydrogen as electron donor.
Figure 1.12 Determination of the Gibbs free energy of reaction with PCB 153 as electron acceptor and H$_2$ as electron donor.
reductive dechlorination include temperature, pH, and carbon source availability (Wiegel and Wu, 2000). Therefore, these factors must be considered when evaluating the potential for natural attenuation of PCBs in the environment. Temperature plays an important role in regulating reductive dechlorination by affecting bioavailability and transport of PCBs (Carlson and Hites, 2005; Hermanson et al., 2003), as well as controlling the growth and physiological activity of microbial populations (Madigan et al., 2003b). Reductive dechlorination of PCBs has been observed between 8 and 34 ºC, and 50 to 60 ºC, with optimal dechlorination between 18-30 ºC (Wiegel and Wu, 2000).

Dechlorination pathways may also be affected by temperature, probably by affecting the composition of dechlorinating populations (Wu et al., 1997). For example, the removal of flanked *meta* chlorines, was predominant at temperatures between 8 and 15 ºC, while removal of flanked *meta* and *para* chlorines was observed from 18 to 30 ºC (Wu et al., 1997).

pH may affect the interaction between different dehalogenating and non-dehalogenating microbial population, and also affects adsorption equilibrium of PCBs with organic matter and thus influences their bioavailability (Jota and Hassett, 1991). Maximum dechlorination activity has been observed in the pH range between 5.0 and 8.0 (Wiegel and Wu, 2000), but pH can also affect the dechlorination pathway. For example, flanked *meta* dechlorination has been observed at pH 5.0-8.0, unflanked *para* dechlorination at pH 6.0-8.0, and *ortho* dechlorination at pH 6.0-7.5 (Wiegel and Wu, 2000).

The availability of organic C also plays a major role in affecting reductive dechlorination of PCBs (Nollet et al., 2005). During reductive dechlorination, fermenting
bacteria such as *Clostridium* spp. degrade organic C compounds derived from algae, plants, and animals in the environment to simpler compounds such as acetate and H₂ which are utilized as electron donors by dehalogenating bacteria. If the concentrations of acetate and H₂ are low due to low amounts of organic matter or consumption by methanogens, sulfate reducers, and acetogens (Smidt and de Vos, 2004; Chen et al., 2006), then rates of reductive dechlorination will be decreased (Nies and Vogel, 1990; Wiegel and Wu, 2000).

**Objectives and Hypothesis**

The overall goals of this project are to evaluate *in situ* PCB removal and the effects of several environmental factors on microbial community composition and PCB removal from Ohio River sediments, with particular emphasis on reductive dechlorination. Knowledge of the parameters that control the reductive dechlorination would be useful in designing strategies to improve removal of these pollutants from the Ohio River where PCBs have been found in its entire length.

Specific objectives of the research are:

*Objective 1*. Determine the chemical profiles, bacterial community composition, and PCB loss rates in surface sediments from a specific location in the Ohio River.

*Hypothesis*: Several factors govern biological transformations of PCBs in the environment, such as temperature, nutrient composition, electron donor and acceptor levels, as well as the presence of microorganisms capable of transforming PCBs. The hypothesis tested in this study is that differences in chemical composition with depth will
significantly affect bacterial community composition and PCB removal rates in the sediment profile. For example, it is expected that more reduced conditions in the deeper sections of the sediment will be more conducive to anaerobic bacterial groups and reductive dechlorination, while more oxidized conditions in the upper parts of the sediment profile will favor aerobic transformations of PCBs. Such information is needed to determine the potential for natural attenuation of PCBs, as well as determine the main factors that limit biological PCB removal from this aquatic ecosystem, especially in tide waters where saturated and unsaturated conditions may change often in the upper position of the sediment in the river bank.

Objective 2. Determine effects of temperature and electron donor availability on microbial community and PCB reductive dechlorination rates in Ohio River sediment microcosms.

Hypothesis: Previous studies have shown that PCB dechlorination rates and pathways temperature dependent. Different carbon sources and alternative electron acceptors have also been shown to have an impact on these reactions. The hypothesis tested in this experiment is that these effects are connected with changes in microbial population composition, for example, adding sulfate would increase the population of sulfate reducers in these sediments which in turn may provide hydrogen as electron donor for dechlorinating organisms resulting in an increase in reductive dechlorination rates of PCBs. Therefore, DNA analysis of clone libraries obtained from these microcosms should reveal an enrichment of these microorganisms. This information would be useful for the selection and establishment of environmental conditions favorable to
microorganisms involved in the reductive dechlorination of PCBs, which would lead to the improvement of removal rates of these contaminants from the environment.

**Objective 3.** Determine feasibility of sequential anaerobic-aerobic treatment for the complete removal of PCB-153 from Ohio River sediment microcosms, and the associated changes in microbial community composition during the process.

**Hypothesis:** The reductive dechlorination of PCBs is often limited to congeners with five or more chlorine atoms. Inversely, aerobic degradation is limited to congeners with four chlorine atoms or less. This is due to steric hindrance of the chlorine atoms that impede attack of dioxygenase enzymes at position 2,3 or 3,4 in the biphenyl ring. The hypothesis tested in this experiment is that the reductive dechlorination products of PCB-153 (2,2’,4,4’,5,5’-hexachlorobiphenyl) will undergo aerobic oxidation after oxygen exposure of microcosms that have been previously incubated under anaerobic conditions. PCB-153 is one of the most persistent PCBs in the environment and in human tissues. The chlorine substitution pattern in this congener blocks any possible attack of dioxygenase enzymes, however, either *meta* or *para* dechlorination will lead to free 2,3 or 3,4 positions where aerobic attack can now be possible.

**Dissertation Format**

Individual chapters in this dissertation were prepared as individual manuscripts intended for future publication. In this Chapter, a general introduction of PCB nomenclature, production, toxicology, and fate processes was presented. In Chapter 2, *in situ* PCB removal and bacterial community composition in an Ohio River sediment
profile was investigated. In Chapter 3, the effects of temperature, FeSO₄, and electron donors such as peptone and volatile fatty acids on the PCB reductive dechlorination rate and the bacterial community composition in Ohio River sediments were described. In Chapter 4, the effect of sequential anaerobic and aerobic treatments on removal of PCB-153 in Ohio River sediments was described. Finally, a summary and the conclusions for the present project will be provided in Chapter 5.
CHAPTER 2

NATURAL ATTENUATION OF PCBS AND BACTERIAL COMMUNITY COMPOSITION IN OHIO RIVER SEDIMENTS

Introduction

The Ohio River is one of the most extensively PCB-contaminated rivers in the U.S. According to ORSANCO (2006) and Rostad et al. (1993), sediment samples collected along the 981 mile stretch of the river contained PCB concentrations ranging between 0.002 and 8 ppm. In addition, many fish species in the river contain ≥ 2 ppm PCBs. As a result, the EPA has instituted fish consumption advisories to warn the public about the health hazards associated with consuming PCB-contaminated fish obtained from the river (ORSANCO, 2006).

PCB contamination of the Ohio River impacts the lives of millions of people that utilize the resource for aquatic life preservation, recreation, fish consumption, and as a public water supply. The river starts at the confluence of the Allegheny and Monongahela Rivers in Pittsburgh, Pennsylvania, and ends in Cairo, Illinois, where it flows into the Mississippi River. Six states border the Ohio River including Illinois, Indiana, Kentucky, Ohio, Pennsylvania, and West Virginia, and another five states are part of the river’s 190,000 square-mile basin, including New York, North Carolina, Maryland, Tennessee, and Virginia. The Ohio River is believed to be the main source of PCBs to the Mississippi River (Rostad et al., 1995), which extends for another 950 miles from their confluence until it ends in the Gulf of Mexico.

It has been difficult to identify the main sources of PCBs to the Ohio River. Effluents from wastewater treatment plants have been identified as important point
sources of PCBs (ORSANCO, 2002), but other potential point sources include discharges from steel and aluminum manufacturing plants, power generation plants, and petroleum distribution sites. High levels of PCBs have been detected in the air near areas of high industrial activity, which is believed to contribute significant amounts of PCBs to the river by atmospheric deposition (ORSANCO, 2002). Additional non-point sources of PCBs include runoff and leakage from municipal landfills and hazardous wastes sites.

In many other PCB contaminated water bodies such as Fox River, Hudson River, and Lake Hartwell, SC, it has been shown that PCBs undergo reductive dechlorination in the sediments (Brown et al., 1987; Imamoglu et al., 2004; Magar et al., 2005). In this process, chlorine substituents are replaced by hydrogen atoms, a process that is catalyzed by specialized anaerobic bacteria that inhabit sediments such as o-17, DF-1, and Dehalococcoides ethenogenes (Cutter et al., 2001; Wu et al., 2002; Fennell et al., 2004). Although PCBs are not natural compounds, it is believed that reductive dechlorination is a common process due to the widespread distribution of other chlorinated compounds that are produced by a variety of abiotic and biotic processes in the environment (Gribble 1996; 1998; 2003).

Typically, reductive dechlorination leads to an enrichment of ortho-chlorinated congeners compared to the original PCB congener distribution, which suggests that most bacteria responsible for the process remove chlorines from the meta and para positions of the biphenyl ring (Brown et al., 1987; Lake et al., 1992; Sokol et al., 1994; Bedard and May, 1996). However, other dechlorination patterns have also been observed in some environments, which suggest that other types of bacteria may be involved in the process.
Several factors influence reductive dechlorination in the environment, including temperature, pH, and electron donor/acceptor availability (Wiegel and Wu, 2000). Therefore, these factors must be considered when evaluating the potential for natural attenuation of PCBs in the environment. Temperature plays an important role in regulating reductive dechlorination by affecting bioavailability and transport of PCBs (Hermanson et al., 2003; Carlson and Hites, 2005), as well as controlling the growth and physiological activity of microbial populations (Madigan et al., 2003 pp 151-155). In most studies, reductive dechlorination has been studied at room temperature (25°C), but the reaction has been found to occur at significant rates over a wide temperature range of between 8 and 34 °C, and 50 to 60 °C, with maximum rates observed between 18-30 °C (Wiegel and Wu, 2000). Temperature was also found to control PCB dechlorination pathways, presumably by affecting the composition of dechlorinating populations in Woods Pond sediments (Wu et al., 1997). For example, process N (removal of flanked meta chlorines) was predominant at temperatures between 8 and 15 °C, while process LP (removal of flanked para chlorines) was observed along with process N, from 18 to 30 °C.

The pH of the environment has also been found to be an important factor controlling reductive dechlorination of PCBs. pH may affect the interaction between different dehalogenating and non-dehalogenating microbial populations, and also affects adsorption equilibrium of PCBs with organic matter and thus influences their bioavailability (Jota and Hassett, 1991). Maximum dechlorination activity has been observed in the pH range between 5.0 and 8.0 (Wiegel and Wu, 2000). However, the selectivity of chlorine removal has been shown to be affected by pH. For example,
flanked meta dechlorination has been observed at pH 5.0-8.0, unflanked para
dechlorination at pH 6.0-8.0, and ortho dechlorination at pH 6.0-7.5 (Wiegel and Wu, 2000). Researchers have speculated that changes in dechlorination patterns are due to alterations in the bacterial populations responsible for these reactions.

The availability of organic C also plays a major role in affecting reductive
dechlorination of PCBs (Nies and Vogel, 1990). During reductive dechlorination,
organic C compounds derived from algae, plants, and animals in the environment are
degraded to simpler compounds, such as acetate and H₂ by fermenting bacteria such as
Clostridium spp., which are in turn utilized as electron donors by bacteria that reductively
dechlorinatie PCBs. If the concentrations of acetate and H₂ are low due to low amounts of
organic matter or consumption of these electron donors by methanogens, sulfate reducers,
and acetogens (Smidt and de Vos, 2004; Chen et al., 2006), then rates of reductive
dechlorination will be decreased (Nies and Vogel, 1990; Wiegel and Wu, 2000).

The presence of electron acceptors with reduction potentials greater than 0.5-0.6
V, such as O₂ and NO₃, are expected to inhibit reductive dechlorination of PCBs because
organisms that utilize these electron acceptors outcompete reductive dechlorinators for
electron donors and other substances required for microbial growth (Chang et al., 2004;
Newell and Aziz, 2004). Zwiernik et al. (1998) showed that adding ferrous sulfate to
sediment microcosms promoted reductive dechlorination of PCBs, presumably by two
effects. First, by providing sulfate as electron acceptor that stimulates the growth of
sulfate reducers which in turn might be directly involved in reductive dechlorination.
Second, by providing ferrous iron that removes sulfide as insoluble iron sulfide, reducing
bioavailability of toxic anions. Once sulfate is depleted, the increased population of
sulfate reducers can turn to PCBs as electron acceptors and thus to reductive dechlorination.

In this chapter, the specific objective is to determine the local chemical profiles, the bacterial community composition, and the PCB loss rates at a specific location in the Ohio River. Factors governing biological transformations of PCBs in the environment will be determined, in particular, nutrient concentrations, electron donor and acceptor levels, as well as the presence of microorganisms capable of transforming PCBs. Sediment redox status will be determined based on the porewater chemistry. It is expected that differences in geochemical conditions will significantly affect bacterial community composition and thus, PCB removal rates in the sediment profile. For example, reduced conditions deeper in the sediment will favor anaerobic bacterial groups and reductive dechlorination, while more oxidized conditions in the upper parts of the sediment profile will favor aerobic transformations of PCBs. Such information is needed to determine the potential for natural attenuation of PCBs, as well as determine the main factors that limit biological PCB removal from this aquatic ecosystem.

Materials and Methods

Site description, sample preparation, and collection

Sediments from the littoral zone of river mile 369.5 of the Ohio River (38°37’14.34"N, 83°9’30.84"W) were collected using a petite ponar grab sampler. Approximately 10 L of sediment was collected up to a depth of 30 cm, and was homogenized in a plastic container. 400 mL of sediment were dispensed into three separate 1-L wide-mouth glass containers with Teflon-coated lids. Sediments in the
containers were spiked with 1 mL of a PCB mixture prepared in acetone containing 4000 µg mL$^{-1}$ of each of the following PCBs: 3,3’-dichlorobiphenyl (PCB-11); 4,4’-dichlorobiphenyl (PCB-15); 2,2’,5,5’-tetrachlorobiphenyl (PCB-52); 2,2’,3,3’-tetrachlorobiphenyl (PCB-40); 2,3,4,5-tetrachlorobiphenyl (PCB-61); 2,2’4,4’6,6’-hexachlorobiphenyl (PCB-155); 3,3’,4,4’-tetrachlorobiphenyl (PCB-77); 2,2’,4,4’,5,5’-hexachlorobiphenyl (PCB-153); 2,2’,3,3’,4,4’,5,5’-octachlorobiphenyl (PCB-194); and decachlorobiphenyl (PCB-209, 2000 µg mL$^{-1}$). These congeners were selected to represent a wide range of chlorine substitution patterns that should provide information about the potential dechlorination activities present in these sediments. Except for PCB-209, the final concentration of each PCB in the sediment was 10 mg kg$^{-1}$ on a dry sediment basis. Sediments with PCBs were poured into 35-8 mL cells of a porewater equilibrator that was then covered with a 0.45 µm pore size membrane and a plastic mesh fabric to allow equilibration of porewater constituents in the sediments, but not to allow exchange of sediment with the surrounding environment. Three equilibrators were driven into the river sediment to cover a depth of 35 cm from the surface and were equilibrated for four months. At the end of this period, equilibrators were removed, placed in an ice chest, and stored in the laboratory at -20 ºC until further analysis. While frozen, the contents of each cell in the chamber were transferred to separate glass serum vials and closed with Teflon-coated rubber septa with aluminum crimp. Once the vials were equilibrated to room temperature, the headspace was analyzed by gas chromatography for methane and carbon dioxide. After centrifugation at 2500 rpm for 15 min, the supernatant was removed and saved for analysis of nutrients, metals, and dissolved organic carbon. The sediment was saved for PCB and DNA extraction and analysis.
**PCB extraction**

Approximately 2 g of wet sediment was mixed with 10 g of anhydrous sodium sulfate (to remove excess moisture) and 25 mL of 5:1 hexane:acetone mixture in a wide-mouth 60-mL extraction flask for 6 h on a horizontal shaker. The extracted PCBs were separated from solids by centrifugation at 2500 rpm for 15 min, and 1 mL of the crude extract was cleaned up with HNO₃- and acetone-washed copper powder (1 g) to remove elemental sulfur that was present at elevated levels and interfered with PCB analysis by producing false peaks when using gas chromatography with electron capture detector.

**DNA extraction and PCR amplification, cloning, and sequencing**

Total community DNA was extracted from sediments (0.5 g) with an UltraClean Soil DNA Isolation Kit (MoBio, Solana Beach, CA) using the maximum yield protocol according to the manufacturer’s instructions. This extraction protocol includes a combination of chemical detergent and bead beating steps to lyse cells, and was found to be effective for evaluating bacterial and fungal diversity in soils, sediments, and other environmental samples (Gomes et al., 2003; Hackl et al., 2004; Fierer et al., 2005). The presence of high molecular weight DNA was verified by comparison with molecular weight standards using gel electrophoresis with 0.8% agarose and 1× Tris-acetate-EDTA buffer stained with ethidium bromide dye. The DNA was stored at -20°C until polymerase chain reaction (PCR) amplification.

Separate PCR reactions were conducted for preparation of clone libraries and DGGE profile analysis of 16S rDNA genes in the sediment samples. For clone libraries, a 918-bp fragment of the bacterial 16S rDNA gene was amplified by PCR using 27F
forward primer 5'-AGA GTT TGA TC(A/C) TGG CTC AG-3' and 907R reverse primer 5'-CCG TCA ATT C(A/C)T TT(A/G) GTT T-3' (Lane, 1991; Muyzer et al., 1993). The PCR reaction mixture consisted of 6.5 µL distilled water, 12.5 µL 2× IQ Supermix (Bio-Rad, Hercules, CA), 2.5 µL 2 µM forward and reverse primers, and 1 µL of five times diluted DNA sample. Each PCR reaction had the following final concentrations: 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM each dNTP (dATP, dCTP, dGTP, dTTP), 25 U/ml iTaq DNA polymerase, and 3 mM MgCl₂. DNA extracts were diluted to reduce anomalies such as chimeras, heteroduplex product or mutations that can occur during PCR amplification (Qiu et al., 2001). The PCR was carried out in a MyCycler thermocycler (BioRad, Hercules, CA) with the following temperature program: initial enzyme activation and denaturation of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min of extension at 72°C, and a final extension of 10 min at 72°C. Thirty cycles was the minimum number required to give faint bands of the expected size as determined by comparison with molecular weight standards using gel electrophoresis with 1% agarose and 1× Tris-acetate-EDTA buffer stained with ethidium bromide dye.

For DGGE analysis, a 560-bp fragment of the bacterial 16S rDNA gene was amplified by PCR using 341F-GC forward primer (clamp sequence in bold) 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCG CCC GCC TAC GGG AGG CAG CAG-3' and 907R reverse primer 5'-CCG TCA ATT C(A/C)T TT(A/G) GTT T-3' (Muyzer et al., 1993; Muyzer and Smalla, 1998). The same PCR reaction conditions were used as described above except that a different temperature program was used: initial enzyme activation and denaturation of 3 min at 94°C, followed by 30 cycles
of 30 s at 94°C, 60 s at 50°C, and 90 s of extension at 72°C, with a final extension of 10 min at 72°C.

Based on the sediment pore water chemistry data, four redox zones were identified at 1-9 cm, 10-18 cm, 19-26 cm, and 27-35 cm depth in the sediment profile. PCR products from these zones were combined and purified using Promega Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). The purified products were ligated into Invitrogen pCRII-TOPO cloning vector and transformed into chemically-competent Escherichia coli TOP10F' cells (Invitrogen, Carlsbad, CA). The resulting clones were isolated on Luria Bertani agar plates with 50 µg mL⁻¹ kanamycin at 37°C according to the manufacturer’s instructions. Ninety-four randomly selected white colonies per sample were grown overnight in 2-mL 96-well microplates containing 1.5 mL Luria Broth media and 50 µg mL⁻¹ kanamycin at 37°C on an orbital shaker at 300 rpm.

Plasmids from clones were isolated in 96-well blocks with a Perfectprep Plasmid 96 Vac Direct Bind Kit (Eppendorf, Westbury, NY) and Biomek FX Laboratory Automated Workstation (Beckman Coulter, Fullerton, CA). Nucleotide sequencing reactions were performed with the vector M13F primer and ABI PRISM BigDye v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and resulting sequences were read with an ABI PRISM 3730 DNA analyzer at the University of Kentucky Advanced Genetic Technology Center (Lexington, KY).
Denaturating Gradient Gel Electrophoresis analysis

The DGGE was carried out on the PCR amplicons according to the methods described by Muyzer et al. (1993) and Muyzer (1999). Briefly, the PCR amplicons (25 μl) were separated on 16.5×16.5 cm, 0.7-mm-thick 6% polyacrylamide (37.5:1 [w/v] acrylamide:bisacrylamide) gel with urea/formamide denaturing gradient. The polyacrylamide gel was made with a denaturing gradient ranging from 40 to 60% (where 100% denaturant contained 7M urea and 40% [v/v] formamide), and the electrophoresis was carried out at 60 V for 17.5 h at 60 °C in 1×TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4) in a BioRad-DCODE electrophoresis system. The gels were stained for 30 min in GelStar® nucleic acid stain (Lonza, Rockland, ME) and visualized in a GelDoc 2000 imaging system (BioRad, Hercules, CA).

Analytical methods

Sediment pH was measured with calibrated pH electrode and pH meter model 6071 (Jenco Electronics, LTD, Shanghai). Carbon dioxide in the headspace of vials with sediments was determined using a Shimadzu GC-8A gas chromatograph equipped with thermal conductivity detector (30 °C), with helium as carrier gas and stainless steel Porapak N column (0.3 cm by 2 m) (Supelco, Bellefonte, PA) maintained isothermally at 25 °C. Methane in the headspace of vials with sediments was determined using a Shimadzu GC-14A gas chromatograph equipped with flame ionization detector (FID) at 160 °C, with nitrogen as carrier gas and a stainless steel, 45/60 mesh-packing, Carboxen 1000 column (0.3 cm by 2 m) (Supelco, Bellefonte, PA) maintained isothermally at 110 °C.
Several porewater constituents in sediments were determined after centrifuging sediments and filtering with a 0.45 µm membrane, including metals (Fe, Mn, Al, Ca, Mg) dissolved organic C, sulfate, nitrate+nitrite, ammonium, and phosphate. One mL of supernatant designated for metal cation analysis was acidified with 200 µL of 65% nitric acid, diluted 10 times with deionized and distilled water, and analyzed using a Thermo Jarrell Ash Model 61 Inductively Coupled Argon Plasma (Franklin, MA) at the University of Kentucky Soil Testing Lab. One mL supernatant for dissolved organic C analysis was acidified with 200 µL of 36% hydrochloric acid, diluted five times with deionized and distilled water, and analyzed with a Shimadzu TOC-5000A total organic carbon analyzer (Columbia, MD). Two hundred microliters of supernatant designated for phosphate analyses was mixed with 40 µL of 1.75% (w/v) ammonium heptamolybdate solution in 6.3 N sulfuric acid in a disposable microplate for five min on a microplate mixer and mixed again with 40 µL of 0.035% of malachite green carbinol hydrochloride in 0.35% aqueous polyvinyl alcohol. Color development in the wells was determined by measuring absorbance at 630 nm using a µQuant MQX200 microplate spectrophotometer (Bio-Tek Instruments, Winooski, VE) (D’Angelo et al., 2001). One hundred and twenty microliters of supernatant designated for ammonium analysis was mixed with 50 µL of 0.04% sodium nitroprusside in 2.26% aqueous phenol and 50 µL of 0.084% sodium hypochlorite in 1% sodium hydroxide in a disposable microplate for 30 min on a microplate mixer. Color development in the wells was determined by measuring absorbance at 630 nm using a microplate reader. Twenty microliters of supernatant for nitrate+nitrite analysis was mixed with 200 µL of ammonium chloride buffer (pH 8.5) in the presence of an activated cadmium wire brush in a disposable microplate for 45 min.
on a microplate mixer. After nitrate was reduced to nitrite, samples were mixed with 60 µL of Griess reagent (0.5 % sulfanilamide, and 0.05% N-(1-naphtyl)ethylendiamine dihydrochloride in 1.5 M hydrochloric acid) for 5 min, and the absorbance was determined at 542 nm using a microplate reader. Sulfate in the supernatant was analyzed by ion chromatography with a 1.8 mM carbonate/1.7 mM bicarbonate mobile phase using a Shimadzu HPLC equipped with an IonPac AS4A guard column (4x50 mm) and analytical column (4x250 mm) (Dionex Corp. Sunnyvale, CA), and Model 335 solid phase chemical suppressor module from Alltec (Deerfield, IL).

PCBs in cleaned up extracts were analyzed using a Shimadzu GC-14A gas chromatograph equipped with 63Ni electron capture detector (320°C), autoinjector (250°C), and RTX-1 capillary column (30 m by 0.32 mm with 0.25-µm phase thickness) (Restek Corp., Bellefonte, PA) with the following temperature program: 100°C held for 1 min, ramp to 240°C at 3 ºC min⁻¹, and held at 240°C for 10 min. PCB congeners were identified by comparing retention times with authentic standards obtained from Accustandard (New Haven, CT).

**Phylogenetic analysis of clone library data**

Vector contamination on the sequences obtained from the AGTC was detected with the VecScreen application on the NCBI website (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Vector contamination removal, and sequence editing was performed with MEGA4 software (Tamura et al., 2007). Potential chimeric sequences were detected using the Bellerophon Server (Huber et al., 2004; http://foo.maths.uq.edu.au/~huber/bellerophon.pl), and were removed from
the clone libraries. Operational Taxonomic Units (OTUs) for construction of rarefaction curves were assigned to each sequence using DOTUR (Schloss and Handelsman, 2005; http://www.plantpath.wisc.edu/fac/joh/dotur.html) based on the distance matrix of aligned sequences for each clone library. Taxonomic hierarchy classification, sequence match with nearest neighbors, and clone library comparison for significant differences were performed with the tools available at the Ribosomal Database Project II (http://rdp.cme.msu.edu/).

**Statistical analysis of DGGE and sediment chemistry data**

Microbial community data from DGGE analysis was analyzed by non-parametric multivariate analysis of variance (perMANOVA) using PCORD 5, a software package designed for multivariate analysis of ecological data (Anderson, 2001; McCune and Grace, 2002). This is a distance matrix method that determines a central location for all the observations and central locations for each group of observations to be compared. If different groups have significantly different central locations in multivariate space, then the among-group distances will be relatively large compared to the within group distances, and the resulting test-statistic will be relatively large. The main advantage of perManova is that it does not make assumptions about the distribution of the data that often confound analysis of ecological data by many other multivariate methods (e.g. principal component analysis) (Anderson, 2001).

Four redox zones were identified in the sediment profile, based on porewater chemistry, and one-way analysis of variance for each parameter was obtained using
SAS® 9.1 (SAS Institute Inc. Cary, NC). Means for each zone were compared using the Tukey-Kramer test for multiple comparisons with a 0.05 significance level.

**Results and Discussion**

**Surface water temperature and sediment porewater chemistry**

Surface water temperature in the vicinity of the sampling site during the incubation period ranged from 25 °C in August 1st to 8 °C in November (Fig. 2.1) (USACE, 2006). Wu et al. (1996) determined that dechlorination activity was most rapid in sediments from the Woods Pond in the Housatonic River between 18-34 °C. In the Ohio River, average temperatures in this range only occur between June and September. Therefore, it is likely that reductive dechlorination would be restricted during most times of the year due to low temperature conditions in the Ohio River.

The pH in the sediment profile ranged between 6.8 and 7.1, and did not show a strong gradient with sediment depth (Fig. 2.2A). Wiegel and Wu (2000) indicated that reductive dechlorination of PCBs can occur between pH 5.0 and 8.0, with an optimum at 7.0-7.5. Because pH of Ohio River sediments was in this range, it is unlikely that pH would affect this process in these sediments.

Ammonium concentrations ranged between 3.0 and 92 µM, and generally increased with depth particularly in the 25-30 cm interval (Fig. 2.2B). However, even in the lower depths, ammonium levels in sediments were considerably below the 5.5 mmol L⁻¹ required for optimal growth of bacteria such as *Pseudomonas putida* (Annuar et al., 2006). Therefore, it is possible that nitrogen availability might not be high enough to support PCB transforming populations in the sediments.
Figure 2.1 Average temperatures in the Ohio River at Lloyd Greenup Lock during year 2006 (USACE, 2006)
Figure 2.2 Concentrations of dissolved porewater constituents in the Ohio River sediment profile (Dotted lines indicate separation between redox zones identified in this study).
Dissolved phosphate concentrations were lower than 4.0 µM, and did not show a consistent trend with depth except for elevated levels at the 6 cm, 15 cm, and 24 cm depths (Fig. 2.2C). Dissolved phosphate levels in sediments were comparable with the Michaelis-Menten constants for phosphate, which range between 0.01 and 0.24 µM for many bacteria (Vadstein and Olsen, 1989). However, the P:C ratio was lower than 6 µg P (mg C)\(^{-1}\), thus, it is possible that concentrations of dissolved phosphate limited bacterial growth in the sediments (Vadstein and Olsen, 1989).

Dissolved organic C concentrations ranged between 1500 and 5300 µM, and tended to increase below the 25 cm sediment depth (Fig. 2.2D). High amounts of dissolved organic C and ammonium in the lower sediment depths are likely attributable to anaerobic decomposition of organic matter in these layers. This was supported by an increase of methane from 26.8 to 183 µM in the lower parts of the sediment profile. Dissolved organic C levels were less than the 0.01% that was reported to improve the reductive dechlorination of PCBs (Klasson et al., 1996; Wiegel and Wu, 2000). Therefore, organic carbon availability could be a limiting factor in these sediments. It is likely that organic amendments would improve the reductive dechlorination of PCBs in these sediments because additional carbon sources can stimulate microbial population growth, not only of PCB dechlorinators but also other syntropic populations, e.g. hydrogen producing bacteria.

Nitrate+nitrite concentrations ranged between 6.9 and 105 µM and were highest at the 10 cm and 17 cm depths (Fig. 2.2E). These results suggested that nitrification and denitrification were important reactions at these depths. It is unlikely that reductive dechlorination would be important in these zones of the sediment profile because the
energy yield associated with these electron acceptors is higher than that corresponding to PCBs; therefore nitrate and nitrite will be consumed first.

Dissolved Fe levels ranged between 13.8 and 139 µM, and did not show a consistent trend with depth except for slightly higher levels at the 7 cm, 14 cm, and 34 cm depths (Fig. 2.2F). It is likely that the dissolved Fe measured in this study reflected amounts of Fe$^{2+}$ produced from the reduction of Fe$^{3+}$ oxyhydroxides that commonly occur in water-saturated environments with circumneutral pH. Low levels of Fe$^{2+}$ at other depths may be due to precipitation as iron carbonate and sulfide minerals. Dissolved Mn levels ranged between 33.6 and 86.2 µM (Fig. 2.2G), and followed a similar depth trend as dissolved Fe. It is likely that dissolved Mn reflected amounts of Mn$^{2+}$ produced from the reduction of Mn oxide minerals. The biological reduction of Fe$^{3+}$ and Mn$^{4+}$ would tend to inhibit the reductive dechlorination of PCBs at these depths as they have a similar energy yield compared to PCBs, but the limited solubility of PCBs might prevent their utilization in presence of iron and manganese.

Dissolved sulfate concentrations ranged between 200 and 2300 µM (6.4-73.6 mg L$^{-1}$), and tended to decrease below the 25 cm sediment depth (Fig. 2.2H). From 2003 to 2005, sulfate levels in surface water were 44-90 mg L$^{-1}$ upstream and 33-130 mg L$^{-1}$ downstream of the sampling site (ORSANCO, 2006). Sulfate levels were within these ranges in the upper 25 cm of the profile, but were lower deeper in the profile. These results indicated that sulfate was an important electron acceptor at lower sediment depths. Sulfate reduction in the sediments was consistent with high levels of elemental sulfur in the crude PCB extracts that were detected by gas chromatography (data not shown).
Methane and carbon dioxide also accumulated to high levels in the lower sediment depths (Fig. 2.2I and J). Methane concentrations were between 26.8 and 183 µM, while carbon dioxide was between 1978 and 5042 µM. Interestingly, methane averaged 38.6 µM in the first 22 cm of the profile while the average below this depth was 106 µM, which suggests that methanogenesis was an important microbial process in Ohio River sediments.

Based on the porewater chemistry of the sediment profile, four redox zones were defined as follows: Denitrifying zone, from 1 to 9 cm; Iron-reducing zone, from 10 to 18 cm; Sulfate-reducing zone, from 19 to 26 cm; and Methanogenic zone, from 27 to 35 cm. Table 2.1 shows the means for each porewater constituent in zones defined above. It is predicted that reductive dechlorination would be restricted in the upper 25 cm sediment depths due to the prevalent amounts and reduction of alternate electron acceptors (O₂, nitrate+nitrite, Fe³⁺, Mn⁴⁺, and sulfate) and low amounts of dissolved organic C (Wiegel and Wu, 2000). Below this depth, however, sediments contained higher amounts of ammonium, dissolved organic C, and methane, which indicated that nutrient, electron donor, and redox conditions were more conducive to reductive dechlorination. However, even at the lower sediment depths, reductive dechlorination may be restricted due to low temperature conditions in the sediments.
Table 2.1 Tukey-Kramer Test for Multiple Comparisons of Porewater Chemistry

Parameters in Ohio River Sediments (All values in µM except for pH)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1-9 cm</th>
<th>10-18 cm</th>
<th>19-26 cm</th>
<th>27-35 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.94</td>
<td>6.95</td>
<td>7.02</td>
<td>6.96</td>
</tr>
<tr>
<td>Ammonium</td>
<td>21.5(^{a})</td>
<td>18.6(^{a})</td>
<td>29.8(^{a})</td>
<td>51.8(^{b})</td>
</tr>
<tr>
<td>Nitrite+Nitrate</td>
<td>14.2(^{a})</td>
<td>46.7(^{b})</td>
<td>19.2(^{a,b})</td>
<td>27.3(^{a,b})</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.67</td>
<td>0.39</td>
<td>0.64</td>
<td>0.23</td>
</tr>
<tr>
<td>Sulfate</td>
<td>1453(^{a})</td>
<td>1670(^{a})</td>
<td>1332(^{a})</td>
<td>561(^{b})</td>
</tr>
<tr>
<td>Iron</td>
<td>28.9</td>
<td>42.7</td>
<td>17.1</td>
<td>33.3</td>
</tr>
<tr>
<td>Manganese</td>
<td>50.0(^{a,b})</td>
<td>59.7(^{a})</td>
<td>39.6(^{b})</td>
<td>58.7(^{a})</td>
</tr>
<tr>
<td>Methane</td>
<td>37.8(^{a})</td>
<td>39.3(^{a})</td>
<td>54.1(^{a})</td>
<td>122.3(^{b})</td>
</tr>
<tr>
<td>Carbon Dioxide</td>
<td>2885(^{a})</td>
<td>2847(^{a})</td>
<td>3052(^{a})</td>
<td>4535(^{b})</td>
</tr>
<tr>
<td>Dissolved Organic Carbon</td>
<td>2532(^{a})</td>
<td>2391(^{a})</td>
<td>2004(^{a})</td>
<td>3552(^{b})</td>
</tr>
</tbody>
</table>

Note: Values with letters in common are not significantly different at \(\alpha = 0.05\)
PCB losses from the sediment profile

Small amounts of PCB-61 (2,3,4,5-tetrachlorobiphenyl) (< 10%) were lost at all depths in the sediments during the 4-month incubation period (Fig. 2.3). The appearance of a new peak on gas chromatograms at a retention time of 9.1 min was identified as PCB-23 (2,3,5-trichlorobiphenyl). The production of PCB-23 suggested that PCB-61 was dechlorinated at the para position of the biphenyl ring, as shown in Figure 2.4.

Interestingly, other PCBs with chlorines at the para position were not dechlorinated (e.g. 4,4’-dichlorobiphenyl, 2,2′,4,4’,6,6’-hexachlorobiphenyl, 3,3’,4,4’-tetrachlorobiphenyl, 2,2′,4,4’,5,5’-hexachlorobiphenyl, and 2,2′,3,3’,4,4’,5,5’-octachlorobiphenyl), which suggested that the chlorination of the adjacent aromatic ring inhibited reductive dechlorination of a PCB congener. Since PCB-61 was reductively dechlorinated at all depths, it appears that conditions in the sediments (e.g. temperature, dissolved organic C, electron acceptors, presence of reductive dechlorinating populations, etc) allowed for low rates of reductive dechlorination of this congener. On the other hand, the concentrations of other PCB congeners did not change at any depth during the incubation period, which suggested that in situ conditions were not favorable for reductive dechlorination of these congeners, as discussed in the previous section. Therefore, natural attenuation does not seem to be a viable alternative for removal of most PCBs from Ohio River sediments.
**Figure 2.3** Gas chromatograms of PCB mixtures determined at different depths after four months incubation in the Ohio River sediment profile. Note the appearance of a new peak at 9.1 min retention time, which represents PCB-23.
Figure 2.4 Reductive dechlorination of PCB-61 to PCB-23
DGGE analysis of bacterial communities

The sizes of the genomic DNA extracted from sediments and PCR amplicons using the 341F-GC and 907R primers were in the expected size ranges of 14-15 kb and 620 bp respectively, (Fig. 2.5), which indicated that methods used in the study were effective for extracting and amplifying DNA from the samples.

The number of bands and banding patterns in the different DGGE lanes shown in Fig. 2.6 represent the dominant types of bacterial populations in various redox layers of the sediment profile. PerMANOVA analysis of relative band intensities in the lanes did not reveal any significant differences in microbial community composition in the sediment profile at a p-value of 0.05. One disadvantage of DGGE analysis is that it is often not sensitive enough to show differences in microbial community composition from various habitats, particularly those with extremely high microbial diversity (Burr et al., 2006). Therefore, microbial community composition of the sediments was further explored using the clone library approach.

Clone library analysis of bacterial communities

Four clone libraries were prepared for each redox zone by PCR amplification with primers 27F/907R, with each library containing 53 to 71 chimera-free clones.

Differences in microbial richness in various redox zones were investigated using rarefaction analysis, in which the number of Operational Taxonomic Units (OTUs) in the sample is plotted as a function of the number of sequences evaluated (Fig. 2.7).
Figure 2.5 Agarose gel electrophoresis detection of genomic DNA extraction obtained from sediments collected at different depths in the Ohio River sediment profile (A), and corresponding PCR amplification products using primers 341F-GC/907R for DGGE analysis (B)
Figure 2.6 Denaturating gel gradient electrophoresis analysis of PCR-amplified 16S rDNA obtained from three replicates of four Ohio River sediment depth increments.
Figure 2.7 Rarefaction curves generated from clone libraries obtained from four Ohio River sediment depth increments, as determined by the furthest neighbor assignment algorithm (Schloss and Handelsman, 2005)
Rarefaction curves were prepared at the 3%, 10% and 20% difference levels, which corresponded to comparisons of richness at approximately the species, family/class, and phyla levels, respectively (Schloss and Handelsman, 2005). The highest number of OTUs observed at a given number of sequences were similar for all redox zones at the species and family/class levels (60 and 50, respectively). The number of OTUs at the phyla level was higher for the sulfate-reducing zone with a suggested phyla richness of 31 compared to 25-29 phyla in the other redox zones. Unfortunately, these curves do not approach a plateau, which is observed when there are not enough sequences to represent the population diversity. Therefore, rarefaction provides an underestimation of population richness when a small number of sequences is used for the analysis, and alternative richness indicators might be needed.

Differences in microbial community composition in various redox zones were investigated using the Classification and Library Compare tools available in the online RDP software package (Table 2.2). Bacteria from 20 phyla were represented in clone libraries from all redox zones. However, only 10 or 11 phyla were found in clone libraries from different redox zones, which indicated that there were no significant differences in phyla richness in the different redox zones. These results generally supported the DGGE results. Greater than 90% of clones in the libraries prepared from different redox zones were classified into eight phyla, which generally followed the order Proteobacteria (36-53%), Chloroflexi (11-17%), Bacteroidetes (5-21%), Firmicutes (6-13%), Acidobacteria (3-11%), Actinobacteria (3-6%), Nitrospira (0-4%), and Planctomycetes (0-3%).
Table 2.2 Distribution of bacteria in major taxonomic groups in libraries from Ohio River sediments during *in situ* bioremediation of PCBs for four months, as determined by the Classifier tool available at the Ribosomal Database Project (RDP) (Cole et al., 2007).

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Depth (cm)(^t)</th>
<th>Representative genera in libraries</th>
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\(^t\) Depths are given in centimeters. % indicates the percentage of sequences assigned to each group.
Table 2.2. (continued)

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<th>Representative genera in libraries</th>
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Ferribacterium (1)
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$^\dagger$ Values followed by a different letter are significantly different at p = 0.05; n represents number of sequences in clone library.
Many of the groups that were dominant in Ohio River sediments were also prevalent in other river sediments. Using fluorescent in situ hybridization (FISH), for example, Fazi et al. (2005) discovered that alpha- and beta-Proteobacteria were dominant in three Italian river sediments. Kloep et al. (2006) observed that alpha-, beta-, and gamma-Proteobacteria, Cytophaga-Flavobacteria, and Planctomycetales were dominant in sediments from the Elbe River in Germany, and Peplies et al. (2006) found that beta-Proteobacteria, sulfate-reducers, and methanotrophs bacteria were prevalent in four other German river sediments. Unfortunately, only a small fraction of the bacterial community could be detected by FISH in these studies (<40%), suggesting that more probes would be needed to fully characterize bacterial diversity in these systems (Peplies et al., 2006). This might explain the differences in bacterial composition observed in the various studies. The present study is one of the first to characterize bacterial community composition using the clone library approach.

There were significant differences in the taxonomic makeup of the bacterial communities in the different redox zones (Table 2.2). Bacteria in the class β-Proteobacteria made up the largest fraction of the clone libraries from the denitrifying zone (20%) and also considerable fractions of clone libraries in the iron-reducing zone (19-29%). However, this group made up a smaller fraction of the clone library from the methanogenic zone (<5%). Most of the bacteria in this class were in the order Burkholderiales, which mostly included bacteria with aerobic heterotrophic type metabolism such as Aquabacterium, Hydrogenophaga, Leptothrix, and Rhodoferax spp. High levels of bacteria in this group suggested that surface sediments were relatively oxidized compared to sediments in the lower depths. Because reductive dechlorination
requires strongly reducing conditions, it is unlikely that this process would occur in the 0-26 cm depths. On the other hand, presence of these aerobic bacteria suggests that there is potential for aerobic degradation of PCBs. Although known aerobic PCB degraders were not detected in the clone libraries, phylogenetic analysis suggests presence of related species and will be discussed later.

The second most prevalent class of bacteria in the clone library from the 0-9 depth increment was bacteria in the genus *Anaerolinea* within subdivision I of the phylum Chloroflexi (17% of the clone library) (Table 2.2). There were no differences in the numbers of *Anaerolinea* in clone libraries from other sediment depths. Bacteria in this genus are obligately anaerobic syntrophs that ferment carbohydrates and amino acids primarily to acetate and H$_2$ (Sekiguchi et al., 2003). Interestingly, *Anaerolinea* spp. have been commonly found in many anaerobic dechlorinating environments, and probably support the reaction in these environments by supplying electron donors, such as hydrogen to dechlorinating populations (Yamada et al., 2006). High numbers of both *Anaerolinea* and aerobic bacteria in the class β-Proteobacteria suggested that anaerobic and aerobic microsites were prevalent in the 0-26 cm depth. Because PCBs have been released to the environment as complex mixtures of varying chlorine content, there is not a single process that, by itself, could totally remove PCBs from the environment. Lower chlorinated congener can be readily degraded by aerobic bacteria, but for higher chlorinated congeners it is necessary to reduce the number of chlorines before they can be aerobically degraded, and it is likely that a sequential anaerobic aerobic treatment can completely remove PCBs as shown previously (Master et al., 2002). Therefore, existence
of a mixed aerobic-anaerobic environment in these sediments suggests the possibility of having both processes for the complete removal of PCBs from Ohio River sediments.

The third most important class of bacteria in the clone library from the denitrifying zone was δ-Proteobacteria (11% of the clone library composition) (Table 2.2). The number of bacteria in this class was similar in clone libraries from the iron-reducing and sulfate-reducing zones (6-8%), but it was significantly lower than the clone library from the methanogenic zone (22%). There were differences in the types of δ-proteobacteria in the different depths. In the denitrifying zone, the dominant δ-proteobacteria were in the order Desulfuromonales, and included bacteria in the genera Geobacteria and Geopsychrobacter that have been associated with electricity harvesting from sediments using graphite electrodes as their sole electron acceptors (Holmes et al., 2004), and with oxidation of aromatic hydrocarbons under iron reducing conditions (Rooney-Varga et al., 1999). More recently, Sung et al. (2006) showed that Geobacter lovleyi can couple oxidation of organic substrates not only with metal reduction but also with reduction of tetrachloroethene and triclorethene to dicloroethene. Therefore, presence of these organisms may suggest favorable conditions for reductive dechlorination reactions.

In the methanogenic zone, the dominant δ-proteobacteria were in the orders Desulfurobacterales and Syntrophobacterales. The dominant genera in Desulfurobacterales order included sulfate-reducers such as Desulfo bacterium and Desulfocapsa spp. At this depth, about half of the clones were classified in the order Desulfobacterales, which include sulfate-reducing organisms Desulfobacterium, Desulfonema, and Desulfocapsa spp. The dominant genera in the Syntrophobacterales
order included strictly anaerobic propionate-degrading syntrophe *Smithella* (Liu et al., 1999) and acetate degrading sulfate reducer *Desulfobacca* (Elferink et al., 1999).

Changes in the relative amounts of sulfate reducers in the sediment profile supported the porewater data, which indicated that sulfate reduction was an important process in the lower sediment depths. It is possible that large numbers of sulfate reducers could inhibit reductive dechlorination of PCBs, by competing with reductive dechlorinators for electron donors such as hydrogen, acetate or lactate (Madigan et al., 2003).

The next most important class of bacteria in the clone library from the denitrifying zone was Acidobacteria (10% of the clone library composition) (Table 2.2). The number of bacteria in this class was not significantly different between redox zones. This group of bacteria is of recent discovery and their physiological role is not well understood (Quaiser et al., 2003). However, Eichorst et al. (2007) showed that members of this phylum grow under mildly acidic conditions (pH 6-7), and in the presence of oxygen, but are unable to grow under anaerobic conditions using nitrate or iron as electron acceptors. Therefore, their presence suggests aerobic conditions in the sediment profile with low nutrient levels that may have a negative impact on the reductive dechlorination of PCBs in these environments.

In the phylum Bacteroidetes, the order Sphingobacteriales was abundant in the clone libraries. This group is highly diverse and includes anaerobic species such as the *Cytophaga* species that are also found in marine environments and may be involved in the breakdown of complex organic matter in sediments (Rosello-Mora et al., 1999). The presence of these microorganisms supports the establishment of a mixed aerobic-
anaerobic environment that does not offer favorable conditions for reductive dechlorination.

The order Clostridiales was also higher in the bottom layer and this order includes Desulfitobacterium and Dehalobacter spp. These are obligate anaerobes that have been isolated from environments contaminated with halogenated organic compounds (Villemur et al., 2005). Desulfitobacterium spp. can use a wide range of electron acceptors for anaerobic respiration including nitrate, sulfate, metals, humic acids, and halogenated organic compounds either from natural or anthropogenic origin. Although the substrate specificity varies with the species, they can dehalogenate compounds such as trichloro- and tetrachloroethylene, and haloaromatics such as chlorophenols.

Phylogenetic analysis of the sequences in the clone libraries indicated that several bacteria in sediments were closely related to bacteria with reductive dechlorinating abilities (Fig. 2.8). Eleven clones present at all depths were related to Desulfitobacterium spp. Although bacteria in this genus are not known to dechlorinate PCBs, they are known to dechlorinate closely-related chlorinated aromatic compounds, including chlorophenols, bromophenols, hexachlorobenze, and even hydroxylated PCBs (Villemur et al., 2006).

Several clones were related to several other dehalogenating bacteria such as Desulfuromonas spp, and Trichlorobacter thiogenens, which can dechlorinate solvents like tetrachloro- and trichloroethylene (Smidt and de Vos, 2004) and Desulfomonile tiedjei which can grow with chlorobenzoates as electron acceptor (Mohn and Tiedje, 1992). Additionally, six clones were closely related to Dehalococcoides spp., which are the only known genera with the capacity to reductively dechlorinate PCBs (Field and Sierra-
Figure 2.8 Bootstrapped neighbor-joining phylogenetic tree of 16S rDNA sequences obtained from the in situ reductive dechlorination study and known dehalogenating bacteria. Sequences obtained in the present study are indicated as follows:

InSitu1=Denitrifying zone, InSitu2=Iron-reducing zone, InSitu3=Sulfate-reducing zone, and InSitu4=Methanogenic zone. Numbers at branch points are percentages of 500 resampling bootstrap analysis. The scale bar represents 0.02 changes per nucleotide.
Alvarez, 2008; Yan et al., 2006). The presence of this organism suggests that reductive dechlorination may be possible in Ohio River sediments under suitable biogeochemical conditions.

Several other clones found in the river sediments showed a closer relationship with aerobic PCB degraders such as Burkholderia xenovorans, Comamonas testosteroni, and Pseudomonas spp., with differences less than 5% in their sequences (Fig. 2.9) and the presence of bacterial groups with wide O2 tolerances in the sediments, ranging from obligately aerobic to strictly anaerobic, suggested that the sediment profile contained a strong redox gradient. Such a gradient would tend to support sequential reduction and oxidation of PCBs, although there was no evidence of this during this experiment.

No extensive dechlorination of PCBs was observed in the field study, suggesting that a different set of conditions, besides redox potential, controlled the extent of the reaction. For instance, availability of other electron acceptors such as oxygen, nitrate, iron or manganese may limit the reductive dechlorination of PCBs. In a dynamic system like the river, constant perturbation and mixing of the sediments promotes recycling of redox sensitive elements (Meade and D’Angelo, 2005) that may inhibit the reductive dechlorination. Temperature may have a big impact on the rate, as at temperatures below 20 °C a decrease in dechlorination is observed (Wu et al., 1996; Palekar et al., 2003), and the average temperature in the river during the experiment ranged from 23 °C in August to 7 °C in November when the equilibration chambers were retrieved from the field. Based on this fact it is more likely that the small rate of dechlorination observed for PCB-61, occurred only during the first two months of incubation, when the temperatures were between 19 and 23 °C.
Figure 2.9 Bootstrapped neighbor-joining phylogenetic tree of 16S rDNA sequences obtained from the *in situ* reductive dechlorination study and known aerobic PCB degraders. Sequences obtained in the present study are indicated as follows:

**InSitu** = Denitrifying zone, **InSitu2** = Iron-reducing zone, **InSitu3** = Sulfate-reducing zone, and **InSitu4** = Methanogenic zone. Numbers at branch points are percentages of 500 resampling bootstrap analysis. The scale bar represents 0.02 changes per nucleotide.
In conclusion, porewater chemistry revealed a rather oxidized environment in the surface sediments at the moment of sampling. Below 20 cm, microbial activities were dominated by sulfate reducing processes as well as methanogenesis, which are favorable conditions for reductive dechlorination. Microbial community composition revealed the presence of microorganisms closely related to known dehalogenating bacteria, such as *Desulfitobacterium* spp., and especially *Dehalococcoides* spp. which have been shown to dechlorinate PCBs. However, PCB dechlorination was not extensive as only one single product, PCB-23, was detected at the end of the experiment. Other nutrients such as phosphate and organic carbon were limited, and might have had a negative impact on rates and extent of reductive dechlorination. These limitations along with cold temperatures during part of the experiment might explain the observed low conversion of PCBs into lower chlorinated congeners, and suggest that additional treatments are required to improve natural attenuation of PCBs in this environment. Therefore, it is expected that controls on temperature and nutrient availability might improve the reductive dechlorination of PCBs in Ohio River sediments, which will be discussed in the following chapter.
CHAPTER 3
TEMPERATURE AND ELECTRON DONOR AVAILABILITY EFFECTS ON
PCB REDUCTIVE DECHLORINATION RATES AND MICROBIAL
COMMUNITY COMPOSITION IN OHIO RIVER SEDIMENTS.

Introduction

Polychlorinated biphenyls (PCBs) refer to a family of compounds that were manufactured for many decades as coolants in transformers, capacitors, and other electrical equipment. Millions of tons of PCBs have been dispersed globally in the environment, which have been found to accumulate in aquatic sediments and the food chain due to their hydrophobic and stable chemical characteristics. The main PCB exposure route to humans is consumption of sportfish caught from contaminated environments. Overconsumption of PCB-contaminated fish tissue can cause learning and memory losses, reproductive and immunity deficiencies, and skin and liver damage (Robertson and Hanson, 2001; ATSDR, 2001)

In many aquatic environments, PCBs can be transformed through the concerted activity of anaerobic and aerobic bacteria that inhabit contaminated sediments. In anaerobic zones, bacteria can replace chlorine atoms of highly substituted PCBs with hydrogen atoms through a process known as reductive dechlorination (Brown et al., 1987; Quensen et al., 1988; Bedard and Quensen, 1995; Holliger et al., 1999; Wiegel and Wu, 2000; Smidt and de Vos, 2004). In aerobic zones, less chlorinated PCB congeners can be degraded by many bacteria, such as Alcaligenes, Burkholderia, Pseudomonas, Bacillus, Corynebacterium, and Rhodococcus (Williams et al., 1997; Maltseva et al.,
In aquatic systems with established oxidized and reduced zones, reductive dechlorination and aerobic catabolism can work in tandem to mineralize PCBs to CO₂, H₂O and chloride ions.

Reductive dechlorination of PCBs has been documented in many freshwater, estuarine, and marine environments; little is known, however, about the identity, physiology, or ecology of dechlorinating communities. Ye et al. (1992) concluded that anaerobic spore-forming bacteria dechlorinated PCBs in Hudson River sediments based on results with pasteurized samples. Based on results with specific microbial inhibitors, Williams (1997) concluded that anaerobic Gram-positive bacteria dechlorinated PCBs in Hudson River sediments. Hou and Dutta (2000) concluded that Clostridium species dechlorinated PCBs in Lake Medinah sediments based on phylogenetic analysis of anaerobic enrichment cultures. Using a similar approach, Cutter et al. (2001) and Wu et al. (2002) concluded that Chloroflexi-related strains o-17 (Genbank accession no. AF294958) and Dehalobium chlorocoercia DF-1 (Genbank accession no. AF393781) dechlorinated PCBs in Baltimore Harbor sediments. To date, Dehalococcoides ethenogenens strain 195 is the only strain available in pure culture with PCB-dechlorinating activity (Fennel et al., 2004; Seshadri et al., 2005).

Reductive dechlorination in most anaerobic environments is a syntrophic process between dechlorinating bacteria and many non-dechlorinating bacterial populations. Dechlorinating bacteria typically use simple electron donors such as H₂ and acetate that are provided during oxidation of more complex substrates by fermenting and syntrophic bacteria (Drzyzgal and Gottschal, 2002; Becker et al., 2005). Conversely, it has been known for many years that hydrogenotrophic bacteria (e.g. methanogens and sulfate-
reducers) lower H$_2$ to levels that allow fermentation of organic substrates to be thermodynamically favorable (Fukuzaki et al., 1990). In addition, transcription and activity of dehalogenase enzymes are believed to be strongly regulated by the presence of electron acceptors including O$_2$ and sulfur oxyanion species (Townsend and Suflita, 1997; Pop et al. 2004), the concentrations of which are largely controlled by aerobic and anaerobic bacterial groups. Thus, it is important to understand the environmental factors that regulate many bacterial groups involved in these interactions in order to optimize conditions for reductive dechlorination in natural habitats.

Several physical and chemical conditions can have major effects on bacterial groups in anaerobic environments. In methanogenic paddy soils, for example, thermal regime profoundly influenced fermentative and syntrophic production of H$_2$ and low molecular weight fatty acids, which in turn affected methanogenesis rates in the system (Chin and Conrad, 1995). It is expected that temperature would also play an important role in controlling reductive dechlorinators. In addition, Wu et al. (1996; 1997) hypothesized that temperature influences on PCB dechlorinating populations caused changes in dechlorination rates and patterns in Woods Pond sediments. This could be important in the Ohio River as temperatures ranged widely between 5 and 35 ºC during the year (USACE, 2006).

The types of electron acceptors available in the system can have positive or negative effects on PCB dechlorination. Ferrous sulfate amendments to Hudson River sediments, for example, stimulated PCB dechlorination presumably by enriching for sulfate-reducing, PCB-dechlorinating bacteria and by removing toxic sulfide species as iron sulfide precipitates (Zwiernik et al., 1998). In another study, nitrate and sulfate
inhibited Aroclor 1242 dechlorination in Hudson River sediments, presumably by acting as preferred electron acceptors for some members of the dechlorinating community (Rhee et al., 1993). Results from the previous chapter suggest that several electron acceptors, such as nitrate, Fe$^{+3}$, Mn$^{+4}$ and sulfate, could be important controllers of reductive dechlorination in Ohio River sediments. While environmental conditions are likely to play a large role in regulating bacterial community structure and processes, few studies have investigated these effects in anaerobic sediments.

The objectives of this study were to: (i) characterize the bacterial composition of river sediments using the clone library approach, (ii) determine the effects of anaerobic treatment conditions (e.g. temperature, electron donors, and FeSO$_4$) on bacterial community composition in PCB-contaminated sediments, and (iii) evaluate treatment effects on removal of 2,4,5-2’,4’,5’-hexachlorobiphenyl (PCB-153), which is one of the dominant PCB congeners in many sediments including those from the Ohio River. It was hypothesized that treatments would elicit major changes in key bacterial populations that are either directly or indirectly involved in PCB dechlorination. Association between bacterial taxa and treatment conditions identified in this study could be useful for assessing in situ geochemical conditions that regulate anaerobic PCB removal dechlorination in these types of environments.
Materials and Methods

Site description and sample collection

The Ohio River, a 1600-km river located in the east-central US, is extensively contaminated with PCBs. Sediments from the river contain between 0.01 and 8.5 ppm PCBs and many fish species contain >2 ppm PCBs. As a result, the river is under several fish consumption advisories due to PCB contamination (ORSANCO, 2002; USEPA, 2004). PCBs in the river mainly consist of penta- and hexa-chlorinated biphenyls, particularly PCB-153 and 2,3,5,2’,4’,5’, hexachlorobiphenyl (Gundersen et al., 1998; USEPA, 2006).

Sediments from the littoral zone of the Ohio River (river mile 369.5) were collected using a petite ponar grab in May, 2004. Sediments were transported to the laboratory in an ice chest, and subsamples for phylogenetic analysis and PCB degradation experiments were stored at -80°C and 4°C, respectively. Sediments up to 20 cm had in situ redox potential of 40±37 mV, temperature 15°C, pH 7.9, total carbon 3.2%, dissolved organic carbon (11 mg kg⁻¹), sulfate (90 mg kg⁻¹), total nitrogen 0.11%, and Mehlich III extractable nutrients (mg kg⁻¹) P (22), K (79), Ca (2310), Mg (246), Zn (18), Cd (0.3), Cr (0.5), Ni (4), Pb(0.7), Cu (1.9), Mo (0). At this location, sediments did not have detectable amounts of PCBs, as determined by gas chromatography with an electron capture detector (detection limit<0.05 ppm).

Microcosm treatments and sampling

Thirty-six microcosms were prepared to evaluate bioremediation treatment effects on changes in [¹⁴C]-PCB-153 levels and bacterial community composition in sediments
over a period of 177 d. Congener PCB-153 was selected because it is one of the most important congeners in the river (USEPA 2006). Sediments (10-mL containing 7 g dry sediment) were added to 60-mL glass serum bottles, sealed with teflon-coated rubber stoppers, made anaerobic by purging with N₂, and amended with deoxygenated inorganic nutrient and vitamin solution (5-mL) (Owens et al., 1979).

After one week, when methane was detected in the headspace by GC-FID, one set of 18 microcosms was spiked with [¹⁴C]-PCB-153 solution (0.05 mL of 30,940 µg mL⁻¹ acetone and 4.2 × 10⁶ disintegrations min⁻¹ (dpm) mL⁻¹ (Sigma, St. Louis, MO). Polyurethane foam plugs (PUF) (0.5 cm × 0.5 cm) and vials (2 mL) with 1 M NaOH (1 mL) were suspended inside the bottles with stainless steel wire to trap [¹⁴C]-volatile organic (VOC) and ¹⁴CO₂, respectively, and the microcosms were made anaerobic again by purging with nitrogen gas. The second set of 18 microcosms designated for community DNA analysis was prepared the same way as the first set except that the microcosms were spiked with non-labelled PCB-153 was used.

Six treatments prepared in triplicate were imposed on the two sets of microcosms, which included incubations at 10°C, 25°C, 40°C, amendment with 5 mM FeSO₄ at 25°C, amendment with 22.5 mM peptone on a carbon basis at 25°C, and amendment with 2.5 mM each of acetate, propionate, and butyrate at 25°C. On days 0, 35, 104, and 177, microcosms with [¹⁴C]-PCB-153 were sampled for headspace ¹⁴CO₂, ¹⁴CH₄, and [¹⁴C]-VOC analysis. Headspace ¹⁴CO₂ was determined by analyzing radioactivity in the 1 M NaOH trap. [¹⁴C]-VOCs were determined by analyzing radioactivity trapped in the PUF plugs. Headspace ¹⁴CH₄ was purge with air into a 900 °C furnace, and the ¹⁴CO₂ from combustion was in a 1 M NaOH. After each sampling, new PUF plugs and 1 M NaOH
traps were added and the headspace was purged with N2 to re-establish anaerobic conditions. Microcosms with non-labelled PCB-153 were also sampled for PCBs and genomic DNA (day 177 only).

**PCB extraction**

PCBs were extracted from sediments (1 mL) in 16 mL serum bottles with 5:1 hexane:acetone (10 mL) on a horizontal shaker for 6 h. After centrifugation at 2500 × g for 5 min, crude extracts were cleaned up with HNO₃- and acetone-washed copper powder (1 g) and subsequently analyzed for PCB congeners by gas chromatography with an electron capture detector. Preliminary spike recovery experiments showed that this procedure yielded >95% recovery of PCBs from sediments.

**DNA extraction, PCR, cloning, and sequencing**

Total community DNA was extracted from sediments (0.5 g) with an UltraClean Soil DNA Isolation Kit (MoBio, Solana Beach, CA) using the maximum yield protocol according to the manufacturer’s instructions. This extraction protocol includes a combination of chemical detergent and bead beating steps to lyse cells, and was found to be effective for evaluating bacterial and fungal diversity in soils, sediments, and other environmental samples (Gomes et al., 2003; Hackl et al., 2004; Fierer et al., 2005). The presence of high molecular weight DNA was verified by comparison with molecular weight standards and gel electrophoresis with 0.8% agarose and 1× Tris-acetate-EDTA buffer stained with ethidium bromide dye. The DNA was stored at -20°C until polymerase chain reaction (PCR) amplification.
A 1.36 kb fragment of the bacterial 16S rDNA gene was amplified by PCR using 27F forward primer 5'-AGAGTTTGATC(A/C)TGGCTCAG-3' and 1392R reverse primer 5'-ACGGGCGGTGTGT(A/G)C-3' (Lane, 1991). The PCR reaction mixture consisted of 19 µL distilled water, 10 µL 5× TaqMaster (Eppendorf, Westbury, NY), 5 µL 10× PCR buffer, 5 µL 2 mM dNTPs, 5 µL 2 µM forward and reverse primers, 0.25 µL 5 U µL⁻¹ Taq DNA polymerase, and 1 µL of five times diluted DNA sample. DNA extracts were diluted in order to dilute DNA and contaminants that can cause PCR bias (Qiu et al., 2001). PCR amplification was carried out in a MyCycler thermocycler (BioRad, Hercules, CA) with the following conditions: initial enzyme activation and denaturation of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min of extension at 72°C, and a final extension of 10 min at 72°C. Thirty cycles was the minimum number required to give faint bands of the expected size as determined by comparison with molecular weight standards and gel electrophoresis with 1% agarose and 1× Tris-acetate-EDTA buffer stained with ethidium bromide dye.

PCR products were purified using Promega Wizard SV Gel and PCR Clean-Up System (Promega, Madison WI) and pooled from the three replicates in order to reduce stochastic PCR bias (Acinas et al., 2005). The pooled, purified products were ligated into Invitrogen pCRII-TOPO cloning vector and transformed into chemically-competent Escherichia coli TOP10F' cells (Invitrogen, Carlsbad, CA). The resulting clones were isolated on Luria Bertani agar plates with 50 µg mL⁻¹ kanamycin at 37°C according to the manufacturer’s instructions. Ninety-four randomly selected white colonies per sample were grown overnight in 2-mL 96-well microplates containing 1.5 mL Luria Broth media.
and 50 µg mL⁻¹ kanamycin at 37°C on an orbital shaker at 300 rpm, and two wells were reserved for UK-AGTC to performed quality controls.

Plasmids from clones were isolated in 96-well blocks with a Perfectprep Plasmid 96 Vac Direct Bind Kit (Eppendorf, Westbury, NY) and Biomek FX Laboratory Automated Workstation (Beckman Coulter, Fullerton, CA). Nucleotide sequencing reactions were performed with the vector M13F primer and ABI PRISM BigDye v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and resulting sequences were read with an ABI PRISM 3730 DNA analyzer at the University of Kentucky Advanced Genetic Technology Center (Lexington, KY).

**Phylogenetic analysis**

Sequences were aligned against a phylogenetically-diverse set of 16S rRNA sequences in the GreenGenes database using the Nearest Alignment Space Termination (NAST) algorithm (http://greengenes.lbl.gov/cgi-bin/nph-NAST_align.cgi) and Clustal W algorithm available in MEGA3 (http://www.megasoftware.net/; Kumar et al., 2004). Aligned sequences were evaluated for potential anomalies (e.g. chimeras) using MALLARD with *E. coli* as the reference sequence (http://www.cardiff.ac.uk/biosi/research/biosoft/). Anomalous sequences were excluded from further analysis.

Phylogenetic affiliations of 16S rDNA sequences were determined by the Basic Local Alignment Search Tool (BLAST) 2.2.14 available at the National Center for Biotechnology Information (NCBI) webpage (http://www.ncbi.nih.gov/BLAST/Blast.cgi) and the Classifier and Sequence Match tools available at Ribosomal Database Project II
Differences in community composition between libraries were determined using the Library Compare tool available at RDP. Bacterial nomenclature followed *Bergey's Manual of Systematic Bacteriology* Release 6.0.

Sequences generated in this study were deposited in the GenBank database under the accession numbers EF392901-EF393578.

**Analytical methods**

Total carbon and nitrogen of oven-dried (60°C) sediment samples were determined with a LECO CN-2000 analyzer (St. Joseph, MI). Soil pH was determined in a 1:1 soil/water ratio. Concentrations of bioavailable elements (P, K, Ca, Mg, Zn, Cd, Cr, Ni, Pb, Cu, Mo) were estimated by equilibrating sediment (2.0 g) for 5 min with Mehlich III solution (20-mL) and filtering the extract with a Whatman #2 filter paper (Mehlich, 1984). Elements in the filtered extracts were analyzed with a Thermo Jarrell Ash Model 61 Inductively Coupled Argon Plasma (Franklin, MA). Sulfate was determined by ion chromatography with a Shimadzu LC-10AD VP liquid chromatograph (Columbia, MD). Dissolved organic carbon of sediment pore water was determined using a Shimadzu TOC-5000A Analyzer.

Radioactivity trapped in 1 M NaOH traps was determined by mixing an aliquot with scintillation cocktail and measuring activity using a Packard 2200 CA Tri-Carb liquid scintillation counter (Downers Grove, IL). Radioactivity in PUF was determined by extracting the PUF plug with acetone and mixing with scintillation cocktail as described above.
PCB transformation products in hexane:acetone extracts were analyzed using a Shimadzu Model 14A gas chromatograph equipped with $^{63}$Ni electron capture detector (320°C), autoinjector (250°C), and RTX-1 capillary column (30 m by 0.32 mm with 0.25-µm phase thickness) (Restek Corp., Bellefonte, PA) with the following temperature program: 100°C held for 1 min, ramp to 240°C at 3 min$^{-1}$, and held at 240°C for 10 min. PCB congeners were identified by comparing retention times with authentic standards obtained from Accustandard (New Haven, CT).

**Results and Discussion**

**Treatment Effects on PCB removal**

Preliminary experiments were conducted to evaluate natural PCB attenuation potential in surficial sediments by monitoring *in situ* changes in Aroclor 1254 congener concentrations between August and November, when temperatures were in the optimal range for PCB degradation (USACE, 1995; Wu et al., 1996; 1997). No PCB congeners were lost from sediments in these investigations (data not shown), which signified that natural attenuation of PCBs was not an important process at this location or time frame in the river.

Because PCBs were not degraded under *in situ* conditions, additional laboratory studies were performed to evaluate whether several anaerobic treatments that were shown to be effective at promoting reductive dechlorination in other studies would be effective in Ohio River sediments. In the lab investigations, up to 67% of PCB-153 was removed in 177 d, which signified that there was considerable potential for PCBs to be removed from sediments under certain environmental conditions (Fig. 3.1).
Figure 3.1 Anaerobic losses of $^{14}$C-2,4,5-2',4',5'-hexachlorobiphenyl (PCB-153) from Ohio River sediments under different treatment conditions: (a) 10°C, 25°C, and 40°C, (b) peptone, volatile fatty acid (VFAs) mixture. PCB-153 losses in the FeSO$_4$ treatment were similar to the 25°C treatment. Each bar represents the standard deviation of the average of three replicates.
As expected, temperature played an important role in controlling PCB-153 removal rates in the lab-incubated samples (Fig. 3.1). PCB-153 removal was greater at 25°C than at 40°C or 10°C, which suggested that losses were microbiologically-mediated rather than lost by volatilization or other abiotic processes. Similar temperature effects on removal rates were observed in Hudson River and Woods Pond sediments (Mohn and Tiedje, 1992; Wu et al., 1996; 1997). The fact that PCBs were lost in the lab incubation under anaerobic conditions at 25°C but not in the in situ experiment was likely attributed to differences in redox and temperature conditions in the two experiments that significantly affected key bacterial populations, as discussed below. Results from the lab incubation suggested that considerable amounts of PCBs could be removed if sediments were maintained at 25°C under anaerobic conditions for extended periods.

In contrast to temperature, FeSO₄ amendments did not significantly affect PCB removal in lab-incubated sediments (data not shown). This was in contrast to results in Hudson River sediments, in which FeSO₄ amendments improved PCB removal, presumably by enriching for sulfate-reducing, PCB-transforming bacteria (Zwiernik et al., 1998). One likely explanation for the lack of FeSO₄ response in the current study was that sediments contained high sulfate levels of 1 mM. Under these conditions, additional sulfate inputs would not be expected to have a major effect on bacterial groups or PCB dechlorination rates.

Both the peptone and the fatty acid mixture amendments significantly increased PCB removal from sediments (Fig. 3.1). Similar organic carbon amendment effects on anaerobic PCB removal were observed in other studies (Nies and Vogel, 1990). Organic carbon likely stimulates PCB removal by serving as a carbon and energy source for PCB
transforming bacteria and other beneficial organisms in the dechlorinating community. Indeed, many bacterial groups associated with reductive dechlorinating activity were enriched in these treatments, as discussed below. These results, as well as dissolved organic carbon levels in sediments of only 11 ppm, indicated that PCB transformers in native sediments were electron donor-limited and that such amendments would likely improve anaerobic PCB removal rates in this system.

In all treatments in which PCB-153 was decreased, there were concomitant increases in 2,4,5-2',4'-pentachlorobiphenyl (PCB-99) and 2,4-2',4'-tetrachlorobiphenyl (PCB-47) (Fig. 3.2). $^{14}$CO$_2$, $^{14}$CH$_4$ and [$^{14}$C]-VOC were not detected in any treatments, which indicated that PCB-153 mineralization and volatilization were not major processes in the anaerobic sediments. These results demonstrated that PCB-153 and PCB-99 were lost primarily by reductive dechlorination, and that chlorines were preferentially removed from the meta positions of the biphenyl ring. Chlorine removal from the meta position is consistent with Pathway N, although additional pathways may have become apparent if additional congeners were evaluated (Bedard and Quensen, 1995; Williams, 1997). PCB dechlorination at the meta position is common in other aquatic sediments, including those from the Hudson River (NY), Woods Pond (MA), and Silver Lake (MA) (reviewed by Bedard and Quensen, 1995). In the in situ study, the lower chlorinated congeners in Aroclor 1254 were stable and so if produced would also pose health risks.

**River Sediment Bacterial Community Composition**

One of the main goals of this study was to evaluate the composition of the dominant bacterial inhabitants in sediments because of their possible relationship to
Figure 3.2 Polychlorinated biphenyl concentrations during reductive dechlorination of PCB-153 to PCB-99 and PCB-47 using peptone as organic carbon amendment. Each bar represents the standard deviation of the average of three replicates.
important physical and chemical conditions and PCB removal in this system. Duplicate libraries were prepared from the freshly collected sediments (ORSFC1 and ORSFC2), which were found to contain similar distributions of bacteria (Table 3.1). Diverse groups of bacteria were detected, including difficult-to-lyse Gram-positive bacteria. These results suggested that the DNA and PCR methods were reproducible and that many of the artifacts commonly associated with these methods were minimal in this study.

Most sequences in the duplicate libraries (83-88%) were distributed into ten phyla, including Proteobacteria (primarily in classes beta-, alpha-, and delta-Proteobacteria) > Bacteroidetes (mainly in order Sphingobacterales) = Chloroflexi (Anaerolineales) > Nitrospira = Actinobacteria = Gemmatimonadetes = Acidobacteria > Planctomycetes = Spirochaetes = Firmicutes (Table 3.1). The remaining 12-17% of sequences could not be classified with confidence into any known phyla.

None of the sequences in the clone libraries from freshly-collected sediments were related to PCB dechlorinators (e.g. Dehalococcoides, o-17, DF-1 and Clostridium) or aerobic PCB degraders, which could at least partially explain why Aroclor 1254 was so stable in sediments in the in situ study. PCB dechlorinators were obviously present in sediments, however, as evidenced by PCB-153 conversion to PCB-99 and PCB-47 in the lab studies. It is possible that PCB dechlorinators would have been detected if more clones or specific primers were evaluated. Nevertheless, these groups did not appear to be numerically dominant or active in the natural environment, which was probably attributed to unfavorable physical and chemical conditions as suggested by the types of other bacteria that were dominant in the sediments.
Table 3.1 Distribution of bacteria in major taxonomic groups in duplicate libraries (ORS FC1 and ORS FC2) from Ohio River sediments before anaerobic treatments, as determined by the Classifier tool available at the Ribsomal Database Project (RDP) (Cole et al., 2007). Values in parentheses are number of sequences in the libraries. There were no significant differences between libraries at p-value=0.05, as determined by the Library Compare tool available in RDP.

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The physiological characteristics of bacterial groups that made up a sizeable fraction of clone libraries (>3% of sequences in libraries) provided several clues as to the chemical conditions and reasons for PCB recalcitrance in sediments. The most abundant sequences were classified in the genus *Anaerolinea* within subdivision I of the phylum Chloroflexi (9% of sequences in both of the duplicate libraries). *Anaerolinea* spp. are obligately anaerobic syntrophs that ferment carbohydrates and amino acids primarily to acetate and H$_2$ (Sekiguchi et al., 2003). Interestingly, *Anaerolinea* spp. are commonly found in anaerobic dechlorinating environments, and probably support the reaction by supplying electron donors to dechlorinating populations. The large presence of this group suggests that at least part of the sediment profile was anaerobic and would be able to support reductive dechlorination in sediments under certain conditions.

Also prevalent in the clone libraries were sequences classified in the genus *Gemmatimonas* within subdivision I of phylum Gemmatimonadetes (3-8% of sequences) (Table 3.1). *Gemmatimonas* are aerobic and mesophilic bacteria that utilize many types of simple sugars, acetate, formate, and benzoate as carbon and energy sources (Zhang et al., 2003). The large presence of this group suggested that part of the sediment profile was aerobic. Reductive dechlorination would not be expected in these zones. However, if PCBs were transformed by aerobic bacteria, then *Gemmatimonas* could play a role in PCB removal by degrading benzoate, which is a major and toxic intermediate of aerobic PCB degradation (Bevinakatti and Ninnekar, 1992; Boyle et al., 1992; Vrana et al., 1996).

Sequences classified in the genus *Chitinophaga* within the order Sphingobacteriales and phylum Bacteroidetes were also abundant in clone libraries from
freshly-collected sediments (2-7% of sequences) (Table 3.1). *Chitinophaga* are aerobic/microaerophilic bacteria that hydrolyze a wide range of biopolymers (e.g. chitin, proteins, and polysaccharides) and degrade many simple carbohydrates to organic acids (Pankratov et al., 2006). The presence of *Chitinophaga* spp. also suggested that part of the sediment profile was oxidized.

Sequences affiliated with the genus *Geobacter* within the order Desulfuromonales under the class delta-Proteobacteria were also prevalent in clone libraries from freshly-collected sediments (3-4% of sequences) (Table 3.1). *Geobacter* are not known to transform PCBs. However, they may play a role in PCB removal by degrading toxic PCB degradation intermediates or other pollutants under different terminal electron acceptor reducing conditions that are likely to occur in contaminated sediments (Lovley et al 1993; Wischgoll et al., 2005; Sung et al., 2006).

The presence of bacterial groups with wide O₂ tolerances in the sediments, ranging from obligately aerobic to strictly anaerobic, suggested that the sediment profile contained a strong redox gradient. Such a gradient would tend to support sequential reduction and oxidation of PCBs, although there was no evidence of this in the *in situ* experiment. This suggested that other factor(s), such as electron donor availability, limited these activities in the sediments, as shown in the lab study.

Many of the groups that were dominant in Ohio River sediments were also prevalent in river sediments from other systems, which were determined by fluorescent *in situ* hybridization (FISH) using various probes for different organisms (Fazi et al., 2005; Kloep et al., 2006; Peplies et al., 2006). For example, Fazi et al. (2005) discovered that alpha- and beta-Proteobacteria were dominant in three Italian river sediments, Kloep et
al. (2006) observed that alpha-, beta-, and gamma-Proteobacteria, Cytophaga-Flavobacteria, and Planctomycetales were dominant in sediments from the Elbe River in Germany, and Peplies et al. (2006) found that beta-Proteobacteria, sulfate-reducers, and methanotrophic bacteria were prevalent in four other German river sediments. In these studies, only a small fraction of the bacterial community was detected (<40%), suggesting that many more probes would be needed to fully characterize bacterial diversity in these systems (Peplies et al., 2006). Results from this study suggest that additional probes to evaluate bacterial diversity in river sediments might include those for *Anaerolinea, Gemmatimonas, Chitinophaga, Geobacter*, and other groups listed in Tables 3.1 and 3.2.

**Temperature Effects on Anaerobic Bacterial Community Composition**

It was expected that temperature would significantly affect bacterial community composition, based on its influence on PCB removal in this and other studies. Indeed, proteobacterial sequences were significantly higher in the 10°C library compared to the 25°C and 40°C libraries, especially with regard to sequences in the class alpha-Proteobacteria and order Rhizobiales (Table 3.2). The opposite trend was observed with Bacteroidetes-related sequences. Most of the Bacteroidetes sequences that were enriched in the 25°C library were classified as hydrolytic bacteria *Chitinophaga* spp., which was surprising considering the O₂ requirements of this genus. These results suggest that this group might play a critical role in organic carbon cycling in both anaerobic and aerobic sediments. Most of the Bacteroidetes-related sequences enriched in the 40°C library were affiliated with the strictly anaerobic fermentative bacteria *Anaerophaga* (Denger et
Table 3.2 Distribution of bacteria in major taxonomic groups in libraries from Ohio River sediments after exposure to anaerobic treatments for 177 d, as determined by the Classifier tool available at the Ribosomal Database Project (RDP) (Cole et al., 2007). Values in parentheses are number of sequences in the libraries.

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% of clones

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Acidobacterium (1)
Magnetobacterium (2), Nitrospira (2)
Prosthecobacter (1)
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WS3 (3)
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† Values followed by a different letter are significantly different at p=0.05.

‡ Values followed by * are significantly different than values in the 25°C library at p=0.05.
Interestingly, at least one *Anaerophaga* strain produces biosurfactant that increases the solubility of hydrophobic compounds. Syntrophaceae sequences within the class delta-Proteobacteria were also significantly enriched in the 40°C library compared to the 10°C and 25°C libraries. Most of these sequences were classified as *Smithella*, an anaerobic propionate-degrading syntroph (Liu et al., 1999). The fact that fermentative and syntrophic bacteria were significantly reduced at 10°C was consistent with previous results that showed this group to be particularly sensitive to low temperature in methanogenic paddy soils (Chin and Conrad, 1995).

Reduced levels of fermentative and syntrophic populations in the 10°C library could at least partially explain why PCB dechlorination rates were negligible in this treatment, since these groups are essential for providing electron donors to PCB dechlorinators. The fact that PCB dechlorination was low in the 40°C treatment, despite having high levels of these groups, suggested that PCB dechlorinators were strongly inhibited at this temperature.

**FeSO₄ Amendment Effects on Anaerobic Bacterial Community Composition**

It was expected that FeSO₄ amendments to anaerobic sediments would increase levels of sulfate reducing bacteria, particularly strains with PCB dechlorination abilities as proposed by Zwiernik et al. (1998). However, FeSO₄ amendments did not have major effects on any bacterial group compared to unamended sediments (Table 3.2). As mentioned previously, a likely explanation is that sediment sulfate concentrations of 1 mM did not limit the growth of sulfate reducing bacteria, which included *Desulfo bacterium, Desulfuromonas, Desulfonema, Desulfobacca* and *Desulfitobacterium*. 
Under high sulfate conditions, it is likely that some other factor limited sulfate reducing bacteria growth, such as electron donor availability.

**Organic Carbon Effects on Anaerobic Bacterial Community Composition**

It was expected that amendment of sediments with peptone and a mixture of fatty acids would significantly affect bacterial communities, based on their effects on PCB removal rates as discussed earlier. As expected, both treatments significantly increased levels of Clostridiales sequences compared to unamended sediments (Table 3.2). A majority of these sequences, which made up 11-13% of clone libraries, were closely related (94% similar) to two uncultured clones from other anaerobic reductive dechlorinating environments, namely clone SJA-118, a member of an anaerobic trichlorobenzene-transforming microbial consortium (von Wintzingerode et al., 1999) and clone Peptococcaceae KB-1 1, a member of an anaerobic chloroethene-dechlorinating microcosm (GenBank accession number AY780555) (Fig. 3.3). These sequences were also closely related (90-91%) to several cultured reductive dechlorinating *Desulfitobacterium* strains, including tetrachloroethene-dechlorinators *Desulfitobacterium* sp. Viet-1 (GenBank accession number AF357919), *Desulfitobacterium hafniense* Y51 (Villemur et al., 2002), and chlorophenol-dechlorinator *Desulfitobacterium hafniense* (previously *D. frappieri*) PCP-1 (Thibodeau et al., 2004; Villemur et al., 2005).

Although *Desulfitobacterium* spp. are not known to dechlorinate PCBs, at least two lines of evidence suggest that this population may have conducted the reaction in Ohio River sediments. First, the *Desulfitobacterium* population(s) was closely related to
Figure 3.3 Bootstrapped neighbor-joining phylogenetic tree of sequences in the phyla Firmicutes obtained from the present study and closest relatives in the Ribosomal Database Project release 9.48. Sequences obtained in the present study are indicated in bold with the following abbreviations: ORSFC1 and ORSFC2=freshly-collected sediment (before anaerobic treatment), ORS10C=10°C anaerobic treatment, ORS25C=25°C anaerobic treatment, ORS40C=40°C anaerobic treatment, ORSFAM=volatile fatty acid mixture anaerobic treatment, ORSFES=FeSO₄ anaerobic treatment, ORSEP=peptone anaerobic treatment. Numbers at branch points are percentages of 500 resampling bootstrap analyses. The scale bar represents 0.02 changes per nucleotide.
strains with well-known dechlorination abilities, some of which are able to dehalogenate compounds with structures very similar to PCBs, such as hydroxylated PCBs (Wiegel et al., 1999). Second, the *Desulfitobacterium* population was largest in samples with the greatest PCB dechlorination, which would be expected if the reaction was coupled to energy generation by dehalorespiration, a capability possessed by some members of this genus (Finneran et al., 2002; Villemur et al., 2006). Results from several other studies suggest that PCB dechlorination was conducted by bacteria with *Desulfitobacterium*-like characteristics, including anaerobic, spore-forming bacteria (Ye et al., 1992), anaerobic Gram-positive bacteria (Williams, 1997), and sulfate-reducing bacteria (Zwiernik et al., 1998). As pointed out by others (Nonaka et al., 2006; Villemur et al., 2006), additional work is needed to isolate desulfitobacteria populations and characterize their dechlorination potential, particularly because of their metabolic versatility and ability to survive in a wide range of environments.

In conclusion, results from this study showed that Ohio River sediment populations held large potential for PCB reductive dechlorination, although this potential may not often be realized because of unfavorable temperature, redox, and substrate availability conditions. Changes in these conditions were associated with several bacterial taxa, suggesting that they could be used as indicators to guide and monitor anaerobic bioremediation activities *in situ*. Further work is needed to substantiate the accuracy and sensitivity of these potential indicator taxa for predicting geochemical conditions and processes in other anaerobic systems. Additional work is also needed to determine potential mineralization of PCB reductive dechlorination products in a sequential anaerobic/aerobic treatment, conditions that can be established in the field due to
fluctuations in river level. Finally, much debate is still present regarding the microorganisms responsible for PCB reductive dechlorination. PCR amplification of gene targeting reductive dehalogenases may provide additional information on the presence of potential PCB dechlorinators that cannot be identified or detected using the 16S rDNA-based clone library approach. In chapter 4, these objectives will be addressed using PCB-153 as model compound and PCR primers targeting reductive dehalogenase genes that have been identified.
CHAPTER 4

SEQUENTIAL ANAEROBIC-AEROBIC TREATMENT OF PCB-153 IN OHIO RIVER SEDIMENTS.

Introduction

In the previous chapter it was shown that reductive dechlorination of PCBs in Ohio River sediments has great potential, and that controlled conditions of nutrient availability, temperature and redox status, have a major impact on dechlorination rates. For higher chlorinated PCBs this is a requirement to improve their aerobic degradation that otherwise would be minimal. Lower chlorinated congeners are readily oxidized by several aerobic bacteria such as *Pseudomonas, Alcaligenes, Achromobacter*, *Burkholderia, Comamonas, Sphingomonas, Ralstonia, Acinetobacter, Rhodococcus, Corynebacterium*, and *Bacillus* (Abraham et al., 2002, Field and Sierra-Alvarez, 2008).

This degradation occurs via dioxygenation of the aromatic ring that can occur at positions 2,3 or 3,4. The enzymes responsible for this reaction are known as biphenyl dioxygenases (Borja et al., 2005; Furukawa, 2000; Pieper, 2005). However, the aerobic degradation rate usually decreases with increased chlorine content, and PCBs with *ortho* chlorines are particularly resistant, therefore, aerobic degradation of highly chlorinated PCBs is severely restricted, and reduction of chlorine content is required for this reaction to occur.

Reductive dechlorination reaction is an alternative for degradation of PCBs. In this reaction, PCBs are used as electron acceptors for energy production, resulting in the substitution of chlorine atoms with hydrogen, and thus producing lower chlorinated congeners. Early evidence of the importance of reductive dechlorination of PCBs in the environment were alterations in congener profiles in PCB contaminated aquatic
sediments compared to those of the original commercial mixtures (Brown et al., 1987; Abramowicz, 1995; Wu et al., 1998). Typically, less chlorinated, ortho-chlorinated congeners tend to accumulate and highly chlorinated meta- and para-chlorinated congeners tend to be depleted in these environments (Quensen et al., 1988). Interestingly, toxicity is also reduced during reductive dechlorination because PCB toxicity depends largely on substitutions at those positions because of structural similarities with polychlorinated dioxins (PCCDs).

As both aerobic degradation and reductive dechlorination have been well studied (Borja et al., 2005; Field and Sierra-Alvarez, 2008), it is expected that exposure to anaerobic conditions followed by aerobic conditions could result in the complete removal of PCBs from the environment. Because reductive dechlorination does not completely eliminate the PCB problem under anaerobic conditions, highly chlorinated congeners will be transformed into lower chlorinated congeners that otherwise could not be transformed under aerobic conditions into open ring intermediates and finally into carbon dioxide and water. Previous studies have demonstrated the feasibility of sequential anaerobic-aerobic treatment of PCBs. For example, Master et al. (2002) showed that weathered Aroclor 1260 can be treated in a sequence of four months of anaerobic incubation followed by 28 days of aerobic incubation. The results were an average substitution of about one chlorine atom per molecule during the anaerobic phase, with no changes in molar concentration, and about 60% total removal of PCBs by the end of the aerobic phase. Evans et al. (1996) also showed that the sequential anaerobic-aerobic treatment of weathered Aroclor 1248 improved the removal of PCBs from 67% to 70% compared to aerobic treatment alone. Although this might represent a small improvement in PCB removal, the
proportion of penta and hexachlorinated congeners in the aerobic treatment was higher, proving that the overall treatment reduces both the concentration and the chlorine content in the mixture. In both studies the aerobic phase was inoculated with enriched cultures of *Burkholderia* sp. LB400, a known PCB degrader (Rein et al., 2007; Rodrigues et al., 2006) to improve the degradation rates, but the existence of a native microbial community capable of aerobic degradation was not verified.

Therefore, one of the objectives in the research reported in this chapter is to determine the feasibility of sequential anaerobic-aerobic degradation of PCBs in Ohio River sediments, particularly PCB-153. Additionally, previous results from the *in situ* bioremediation in the Ohio River suggests that potential aerobic degraders are present, and therefore enriched cultures will not be used. Finally, the ultimate goal in this project is to identify the species involved in the reductive dechlorination of PCBs in this environment, and it is expected that using primers designed to detect particular reductive dehalogenase genes will help answer this question.

**Materials and Methods**

**Site description, sample preparation, and collection**

Sediments from the littoral zone at river mile 369.5 of the Ohio River (38°37'14.34"N, 83°9'30.84"W) were collected using a petite ponar grab sampler. Sediments were transported to the laboratory in an ice chest, and subsamples for DNA analysis and PCB degradation experiments were stored at -80°C and 4°C, respectively. At this location, sediments did not have detectable amounts of PCBs, as determined by gas chromatography with an electron capture detector (detection limit<0.05 ppm).
Approximately 10 L of sediment was collected and homogenized in a plastic container, and dispensed into 2.5 L glass bottles equipped with teflon-lined screw caps. Sediments were stored at 4 °C until further use.

To evaluate the potential for a sequential anaerobic-aerobic bioremediation of PCBs, a total of twenty microcosms were prepared. Briefly, sediments (10-mL containing 7 g dry sediment) were added to 120-mL glass serum bottles, sealed with teflon-coated rubber stoppers, and amended with deoxygenated inorganic nutrient and vitamin solution (5-mL) (Owens et al., 1979). These microcosms were designated for PCB and DNA analysis and were spiked with $[^{14}\text{C}]$-PCB-153 (0.05 mL of 31 mg mL$^{-1}$ acetone and $4.2 \times 10^6$ disintegrations min$^{-1}$ (dpm) mL$^{-1}$ (Sigma, St. Louis, MO) to a final concentration of 100 mg kg$^{-1}$ on a dry sediment basis. Fifteen of these microcosms were purged with nitrogen gas, the rest with air, and then incubated in the dark for 150 days. Air-purged microcosms were repeatedly purged with air every 3 days to ensure aerobic conditions were established, and the purged headspace was collected through polyurethane foam (PUF) plugs to trap volatile organic compounds, and then through 1 M aqueous NaOH to trap carbon dioxide that may have been produced during the process. At the end of the 150 day period, three serum bottles incubated anaerobically and three incubated aerobically were removed for PCB and DNA analysis. The rest of the bottles were incubated for another 100 days under the same conditions with the exception of six anaerobic bottles that were purged with air and treated aerobically for the rest of the incubation period. These bottles represent the anaerobic-aerobic sequential treatment. After 50 and 100 days (200 and 250 days total incubation time), three bottles from each
treatment, anaerobic, aerobic, and anaerobic-aerobic, were removed for PCB and DNA analysis.

**PCB extraction**

Approximately 2 g of wet sediment was mixed with 10 g of anhydrous sodium sulfate (to remove excess moisture) and 25 mL of 5:1 hexane:acetone mixture in a wide-mouth 60-mL extraction flask for 6 h on a horizontal shaker. The extracted PCBs were separated from solids by centrifugation at 2500 rpm for 15 min, and 1 mL of the crude extract was cleaned up with HNO₃ and acetone-washed copper powder (1 g) to remove elemental sulfur that was present at elevated levels and interfered with PCB analysis by gas chromatography with electron capture detector.

**DNA extraction and PCR amplification, cloning, and sequencing**

Total community DNA was extracted from sediments (0.5 g) with an UltraClean Soil DNA Isolation Kit (MoBio, Solana Beach, CA) using the maximum yield protocol according to the manufacturer’s instructions. This extraction protocol includes a combination of chemical detergent and bead beating steps to lyse cells, and was found effective for evaluating bacterial and fungal diversity in soils, sediments, and other environmental samples (Gomes et al., 2003; Hackl et al., 2004; Fierer et al., 2005). The presence of high molecular weight DNA was verified by comparison with molecular weight standards using gel electrophoresis with 0.8% agarose and 1× Tris-acetate-EDTA buffer stained with ethidium bromide dye. The DNA was stored at -20°C until polymerase chain reaction (PCR) amplification.
Separate PCR reactions were conducted for preparation of clone libraries and DGGE profile analysis of 16S rDNA genes in the sediment samples. For clone libraries, a 918-bp fragment of the bacterial 16S rDNA gene was amplified by PCR using 27F forward primer 5'-AGA GTT TGA TC(A/C) TGG CTC AG-3' and 907R reverse primer 5'-CCG TCA ATT C(A/C)T TT(A/G) GTT T-3' (Lane, 1991; Muyzer et al., 1993). The PCR reaction mixture consisted of 6.5 µL distilled water, 12.5 µL 2× IQ Supermix (Bio-Rad, Hercules, CA), 2.5 µL 2 µM forward and reverse primers, and 1 µL of five times diluted DNA sample. Each PCR reaction had the following final concentrations: 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM each dNTP (dATP, dCTP, dGTP, dTTP), 25 U/ml iTaq DNA polymerase, and 3 mM MgCl2. DNA extracts were diluted in order to dilute DNA and contaminants that can cause PCR bias (Qiu et al., 2001). The PCR was carried out in a MyCycler thermocycler (BioRad, Hercules, CA) with the following temperature program: initial enzyme activation and denaturation of 5 min at 94ºC, followed by 30 cycles of 1 min at 94ºC, 1 min at 60ºC, and 2 min of extension at 72ºC, and a final extension of 10 min at 72ºC. Thirty cycles was the minimum number required to give faint bands of the expected size as determined by comparison with molecular weight standards using gel electrophoresis with 1% agarose and 1× Tris-acetate-EDTA buffer stained with ethidium bromide dye.

Amplified products from three replicates of each treatment were pooled in order to reduce stochastic PCR bias (Acinas et al., 2005) and purified using Promega Wizard SV Gel and PCR Clean-Up System (Promega, Madison WI). Purified products were ligated into Invitrogen pCRII-TOPO cloning vector and transformed into chemically-competent *Escherichia coli* TOP10F' cells (Invitrogen, Carlsbad, CA). The resulting
clones were isolated on Luria Bertani agar plates with 50 µg mL\(^{-1}\) kanamycin at 37°C according to the manufacturer’s instructions. Ninety-four randomly selected white colonies per sample were grown overnight in 2-mL 96-well microplates containing 1.5 mL Luria Broth media and 50 µg mL\(^{-1}\) kanamycin at 37°C on an orbital shaker at 300 rpm.

Plasmids from clones were isolated in 96-well blocks with a Perfectprep Plasmid 96 Vac Direct Bind Kit (Eppendorf, Westbury, NY) and Biomek FX Laboratory Automated Workstation (Beckman Coulter, Fullerton, CA). Nucleotide sequencing reactions were performed with the vector M13F primer and ABI PRISM BigDye v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and resulting sequences were read with an ABI PRISM 3730 DNA analyzer at the University of Kentucky Advanced Genetic Technology Center (Lexington, KY).

**Reductive dehalogenase gene analysis**

For the detection of reductive dehalogenase genes in this study several primer sets were used. Similar PCR conditions as described before were used, except for the PCR temperature program which is described after each primer set:

- DHAR1000F [G(A/T)A GCA GGT (C/T)TR GGA (G/C)AA] and DHU1350R [CC(A/G) TAG CC(A/C/G) AA(G/T) AT(A/T) TCA TC(A/C) AT] developed based on three known reductive dehalogenase (RDH) genes (pceA from *Dehalospirillum multivorans*, cprA from *Desulfitobacterium dehalogenans*, tceA from *Dehalococcoides ethenogenes* (Rhee et al., 2003) The following PCR thermocycling program was used: 2 min of denaturation at 94°C, followed by 35 cycles of 30-s at
94°C, 1-min at 46°C, and 30-s at 67°C with a final elongation step of 7 min at 65°C, and finally cooling at 4°C.

- SpDr11F (TTG GAT GAG GCC TTG AAC GC) and SpDR9R (GCG CTG CAT AAT AGC CAA GC), developed based on pceA gene from *Dehalobacter restrictus* and *Desulfitobacterium* spp. TCE1, PCE-S, and Y51 (Regeard et al., 2004). The following PCR thermocycling program was used: 3 min of denaturation at 94°C, followed by 26 cycles of 30-s at 94°C, 30-s at 55°C, and 1 min at 72°C with a final elongation step of 10 min, and finally cooling at 4°C.

- SpDr1F (CGT TGG ACC TAT TCC ACC TG) and SpDr1R (CAA GAA CGA AGG CAA TCA CA) also based on pceA gene from *Dehalobacter restrictus* and *Desulfitobacterium* spp. Y51 (AY013365) (Regeard et al., 2004). The following PCR thermocycling program was used: 3 min of denaturation at 94°C, followed by 26 cycles of 30-s at 94°C, 30-s at 53°C, and 1 min at 72°C with a final elongation step of 10 min, and finally cooling at 4°C.

- SpSm1F (TCG TTG CAG GTA TCG CTA TG) and SpSm1R (TTC AAC AGC AAA GGC AAC TG) based on pceA gene from *Sulfospirillum multivorans* (AF022812) (Regeard et al., 2004). The following PCR thermocycling program was used: 3 min of denaturation at 94°C, followed by 26 cycles of 30-s at 94°C, 30-s at 52°C, and 1 min at 72°C with a final elongation step of 10 min, and finally cooling at 4°C.

- SpDe1F (GCT TTG GCG GTG ATG ATA AG) and SpDe1R (GTT ATA GCC AAG GCC TGC AA) based on tceA gene from *Dehalococcoides ethenogenens* (AF228507) (Regeard et al., 2004). The following PCR thermocycling program was used: 3 min of denaturation at 94°C, followed by 26 cycles of 30-s at 94°C, 30-s at
50°C, and 1 min at 72°C with a final elongation step of 10 min, and finally cooling at 4°C.

- DHC974F (GGG AGT ATC GAC CCT CTC) and DHC1212R (GGA TTA GCT CCA GTT CAC ACT G) based on 16S rDNA sequences of *Dehalococcoides* spp. (Hendrickson et al., 2002). The following PCR thermocycling program was used: 2 min of denaturation at 95°C, followed by either 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C and finally cooling at 4°C.

- cprA1023F (AAA CGA CGC GAT TAC CTT TG) and cprA1202R (CCT GCT TTA TGG AAC CAG GA) based on the cprA gene from *Desulfitobacterium dehalogenans* (AF115542) (This study). This set of primers was designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), and the PCR thermocycling program used was as follows: 3 min of denaturation at 94°C, followed by 26 cycles of 30-s at 94°C, 30-s at 54°C, and 1 min at 72°C with a final elongation step of 10 min, and finally cooling at 4°C.

**Phylogenetic analysis**

Sequences were aligned against a phylogenetically-diverse set of 16S rRNA sequences in the GreenGenes database using the Nearest Alignment Space Termination (NAST) algorithm (http://greengenes.lbl.gov/cgi-bin/nph-NAST_align.cgi) and Clustal W algorithm available in MEGA4 (http://www.megasoftware.net/; Kumar et al., 2004). Aligned sequences were evaluated for potential anomalies (e.g. chimeras) using the Bellerophon Server (Huber et al., 2004, and were removed from the clone libraries. Phylogenetic affiliations of 16S rDNA sequences were determined by the Basic Local
Alignment Search Tool (BLAST) 2.2.14 available at the National Center for Biotechnology Information (NCBI) webpage (http://www.ncbi.nih.gov/BLAST/Blast.cgi) and the Classifier and Sequence Match tools available at Ribosomal Database Project II (RDP II) Release 9.48 webpage (http://rdp.cme.msu.edu/index.jsp) (Cole et al., 2007). Differences in community composition between libraries were determined using the Library Compare tool available at RDP. Bacterial nomenclature followed *Bergey's Manual of Systematic Bacteriology* Release 6.0.

**Analytical methods**

PCBs in cleaned up extracts were analyzed using a Shimadzu GC-14A gas chromatograph equipped with $^{63}$Ni electron capture detector (320°C), autoinjector (250°C), and RTX-1 capillary column (30 m by 0.32 mm with 0.25-µm phase thickness) (Restek Corp., Bellefonte, PA) with the following temperature program: 100°C held for 1 min, ramp to 240°C at 3 min$^{-1}$, and held at 240°C for 10 min. PCB congeners were identified by comparing retention times with authentic standards obtained from Accustandard (New Haven, CT). Preliminary spike recovery experiments showed that this procedure yielded >95% recovery of PCBs from sediments.

Headspace methane was measured using a Shimadzu GC-14A gas chromatograph equipped with flame ionization detector (FID) at 160 °C, with nitrogen as carrier gas and a stainless steel, 45/60 mesh-packing, Carboxen 1000 column (0.3 cm by 2 m) (Supelco, Bellefonte, PA) maintained isothermally at 110 °C. Headspace carbon dioxide was measured using a Shimadzu GC-8A gas chromatograph equipped with thermal conductivity detector (30 °C), with helium as carrier gas and stainless steel Porapak N
column (0.3 cm by 2 m) (Supelco, Bellefonte, PA) maintained isothermally at 25 °C. Sulfate was analyzed by ion chromatography using a Shimadzu (Columbia, MD) SLC-10A system controller with the following modules: LC-10AD solvent delivery module, FCV-10AL gradient flow controller, CDD-6A conductivity detector, all from Shimadzu, and a Model 335 solid phase chemical suppressor module from Alltec (Deerfield, IL). Separation was achieved under isocratic conditions with carbonate buffer (1.8 mM carbonate/1.7 mM bicarbonate) in an IonPac AS4A guard column (4x50 mm)/analytical column (4x250 mm) system (Dionex Corp. Sunnyvale, CA).

Radioactivity trapped in 1 M NaOH traps was determined by mixing a 500 µL aliquot with scintillation cocktail and measuring activity using a Packard 2200 CA Tri-Carb liquid scintillation counter (Downers Grove, IL). Radioactivity in PUF plugs was determined by extracting the PUF plug with acetone and mixing with scintillation cocktail as described above.

Results and discussion

Sequential anaerobic aerobic treatment of PCB-153

Anaerobic treatment of PCB-153 occurred with conversions between 60 and 68% at the end of the 150 and 250 days, respectively (Fig. 4.1). PCB-99 and PCB-47 were the main detected dechlorination products, with a greater accumulation of PCB-47. There is no evidence that reductive dechlorination reactions removed two chlorine atoms per molecule in a single step. However, when PCB-99 is formed, the molecule might still be in close proximity to the catalytic active site of the reductive dehalogenase
Figure 4.1 PCB-153 conversion under anaerobic, aerobic, and sequential anaerobic/aerobic conditions. Initial PCB-153 concentration was 270 nmol/g on a dry basis. Each value represents the mean of three replications ± one standard deviation.
(RDH), thus decreasing diffusion problems and energetic barriers associated with transport of these molecules to the active site. In other words, transport to the RDH active site is a limiting step for PCB-153, but not for PCB-99, and that might be the reason for a greater accumulation of PCB-47. Under aerobic conditions, there were no significant changes on PCB-153 concentration. This congener does not have free positions 2 and 3, or 3 and 4, for a potential biphenyl dioxygenase attack, and therefore it is particularly resistant to aerobic degradation. However, meta dechlorination provides free 2,3 positions in one ring for PCB-99, and in both rings for PCB-47, making them amenable for aerobic degradation in these sediments. At the end of the 250 days period on the sequential anaerobic-aerobic treatment, PCB-47 concentration appears to be smaller than at 200 days, but not significantly different. However, from PCB-153 concentration it can be concluded that its concentration is smaller in this set of microcosms. A mass balance on total PCB molar concentration indicates that initially there were 250-270 nmol PCBs per gram of dry sediment. But after 250 days of treatment, the anaerobic-aerobic treatment contained only 200 nmol per gram. Assuming that PCB-153 was not lost under aerobic conditions, this suggests that lower chlorinated products were transformed under aerobic conditions. Analysis of the PUF and aqueous NaOH traps, however, did not reveal any detectable radiation, which suggested that any aerobic transformation products remained in the system and might correspond to hydroxylated PCBs or chlorobenzoic acids that are not detected by regular PCB extraction and analysis procedures.
Detection of reductive dehalogenase genes

PCR amplification reactions using the degenerate primer set DHAR1000F-DHU1350R did not show the expected 450 bp fragment in the anaerobic treatment. Therefore this set was tested with DNA isolated from pure cultures of known dehalogenators such as *Desulfitobacterium* spp., *Dehalobacter restrictus*, *Desulfuromonas michinagensis*, and *Geobacter lovleyi* (Fig. 4.2). Only *Desulfitobacterium hafniense* and *Dehalobacter restrictus* yield strong bands for the expected PCR product while *Desulfitobacterium dehalogenans* yields a faint band. Therefore it is possible that these primers might not capture all the RDH genes present in the anaerobic microcosms. In addition, PCR products were not detected using more specific primers targeting individual RDH genes such as SpDr11F/SpDr9R, SpDr1F/SpDr1R, and SpSm1F/SpSm1R, and SpDe1F/SpDe1R, which suggested organisms such as *D. restrictus*, *S. multivorans*, and *Dehalococcoides* spp. were not present in these sediments. These results were surprising considering that *Dehalococcoides* spp. were detected using 16S rDNA based primers DHC974F/DHC1212R that were designed to detect these species (Fig. 4.3). Interestingly, this primer set also provided positive results for microcosms exposed to aerobic conditions suggesting that *Dehalococcoides* can survive under aerobic conditions for prolonged periods.

PCR amplification using primer pair cprA1023F/cprA1202R was successful at detecting RDH genes corresponding to cprA, which is consistent with the detection of *Desulfitobacterium* spp. in the sediment clone libraries (Fig. 4.4). Although cprA has not been shown to be involved in the reductive dechlorination of PCBs, it has been shown to
Figure 4.2 PCR amplification of reductive dehalogenase DNA from pure cultures

(Primers DHAR1000F and DHAR1350R)
**Figure 4.3** PCR amplification products of 16S rDNA of *Dehalococcoides* spp. with primers DHC974F/DHC1212R
Figure 4.4 PCR amplification products of cprA gene of *Desulfotobacterium dehalogenans* with primers cprA1023F/cprA1202R
Changes in microbial community structure under anaerobic and aerobic conditions

There were significant differences in microbial community structure in sediments under different redox conditions. The greatest differences were between the anaerobic treatment and treatments that contained an aerobic phase. For example, the clone library from the 250 d anaerobic treatment (Table 4.1) was enriched in bacteria in the phyla Bacteroidetes (20% vs 3-5% in the aerobic treatments), Chloroflexi (18% vs 6-9%), and Firmicutes (26% vs 9%) but it was relatively depleted in bacteria from the phylum Proteobacteria (22% vs 52-56%). The enrichment/depletion pattern observed in the anaerobic clone library is similar to clone libraries from anaerobic treatments using organic amendments shown in Chapter 3. Additionally, the observed pattern for the aerobic clone libraries is similar to the freshly collected sediment shown in Chapter 3, suggesting that river sediments were mostly under aerobic conditions at the time of collection.

Several clones closely related to reductive dechlorinators were detected in sediments incubated anaerobically for 250 days (Fig. 4.5). These results further validate the use of the clone library approach and bacterial groups to indicate redox conditions in aquatic sediments. About 19 clones closely related to species such as *Desulfitobacterium chlororespirans* and *Desulfitobacterium hafniense* Y51, and several clones related to
Table 4.1 Distribution of bacteria in major taxonomic groups in libraries from Ohio River sediments during sequential anaerobic-aerobic treatment of PCB-153 for 250 days, as determined by the Classifier tool available at the Ribosomal Database Project (RDP) (Cole et al., 2007).

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<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Aerobic (n=206)</th>
<th>Anaerobic/Aerobic (n=196)</th>
<th>Anaerobic (n=247)</th>
<th>Representative genera</th>
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Table 4.1. (continued)

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<td>Aerobic (206)</td>
<td>Anaerobic/Aerobic (196) %</td>
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Table 4.1. (continued)

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<th>Anaerobic/Aerobic (196)</th>
<th>Anaerobic (247)</th>
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Values followed by a different letter are significantly different at p= 0.05, Number in parentheses indicate number of sequences in clone library.
**Figure 4.5** Bootstrapped minimum-evolution phylogenetic tree of 16S rDNA sequences isolated from Ohio River sediments incubated anaerobically for 250 days, as well as known aerobic PCB degraders, and dehalogenating bacteria. Numbers at branch points are percentages of 500 resampling bootstrap analyses. The scale bar represents 0.05 changes per nucleotide.
other dehalogenators such as *Desulfomonile tiedjei* and *Anaeromyxmbacter dehalogenans*, and *Dehalococcoides* spp. were detected. As mentioned previously *Dehalococcoides* spp. are the only recognized PCB dechlorinators, and because the number of clones is small, no significant differences were detected between the treatments. Evidence indicates that both *Desulfitobacterium* and *Dehalococcoides* spp. are present and most probably they are forming part of a consortia that reductively dechlorinates PCBs in Ohio River sediments. This tree also indicates that there are clones related to aerobic PCB degraders such as *Pseudomonas*, *Burkholderia*, and *Comamonas* spp. Because of redox conditions, aerobic degradation of PCB-153 was not an important process in this treatment, however the presence of these organisms suggests that they can tolerate and survive under these conditions and develop into an aerobic PCB degrading consortium, once appropriate conditions are established.

Phylogenetic trees were also constructed for the aerobic and sequential anaerobic-aerobic treatment of PCB-153 (Figs. 4.6 and 4.7 respectively). In the aerobic treatment, there were more clones related to aerobic PCB degraders than related to reductive dechlorinators. There were approximately 28 clones associated with aerobic PCB degraders mentioned previously, while the presence of closely related dechlorinators was scarce. In this case, presence of bacteria related to dechlorinators suggests tolerance to aerobic conditions, presumably by inhabiting anaerobic microsites that persist during the aerobic treatment. A similar result was observed for the sequential anaerobic-aerobic treatment, in which a considerably higher number of clones related to aerobic PCB degraders were present (11 clones), when compared to the anaerobic treatment (5 clones).
Figure 4.6 Bootstrapped minimum-evolution phylogenetic tree of 16S rDNA sequences isolated from Ohio River sediments incubated aerobically for 250 days, as well as known aerobic PCB degraders, and dehalogenating bacteria. Numbers at branch points are percentages of 500 resampling bootstrap analyses. The scale bar represents 0.05 changes per nucleotide.
Figure 4.7 Bootstrapped minimum-evolution phylogenetic tree of 16S rDNA sequences isolated from Ohio River sediments incubated anaerobically for 150 days and then aerobically for 100 days, as well as known aerobic PCB degraders, and dehalogenating bacteria. Sediments Numbers at branch points are percentages of 500 resampling bootstrap analysis. The scale bar represents 0.02 changes per nucleotide.
In conclusion, the anaerobic treatment of PCB-153 produced PCB-99 and PCB-47 as dechlorination products, while its aerobic treatment did not cause any changes, despite the presence of bacteria that were closely related to aerobic PCB degraders. The dechlorination is also consistent with the presence of microorganism related to known dehalogenating bacteria such as Desulfitobacterium and Dehalococcoides spp. which was confirmed not only by phylogenetic analysis of 16S rDNA sequences but also by specific PCR primer design targeting both ribosomal and reductive dehalogenase genes for these species. The sequential anaerobic-aerobic treatment of PCB-153 suggests that aerobic degradation of PCB dechlorination products is possible after changing conditions from anaerobic to aerobic, but it was not conclusive. Therefore additional analysis is required with particular attention to PCB metabolites such as hydroxylated PCBs to determine the true potential of sequential anaerobic-aerobic treatment of PCBs in Ohio River sediments.
CHAPTER 5

SUMMARY AND CONCLUSIONS

In situ studies were conducted to evaluate factors affecting the natural attenuation of PCBs in Ohio River sediments and local microbial community composition (Chapter 2). Laboratory studies were conducted to determine temperature and nutrient availability effects on PCB reductive dechlorination rates (Chapter 3) and redox conditions effects on PCB bioremediation (Chapter 4). During the course of this project it was shown that PCB reductive dechlorination is a feasible process in Ohio River sediment microcosms, under controlled conditions of nutrient availability, temperature and redox status. Unfortunately, local conditions in the river are not favorable, mainly because of limiting factors such as organic carbon, phosphorus or nitrogen, and also because temperatures in the river are below optimal values during most of the year.

Are local conditions conducive to natural attenuation of PCBs in Ohio River sediments? PCB losses during the in situ study were negligible. The chemical profile determined at this location indicated a redox gradient from denitrifying to methanogenic conditions, but nutrients such as ammonium, phosphate and organic carbon were present in limited supplies. 16S rRNA clone libraries obtained from these sediments indicated the prevalence of aerobic bacteria such as Sphingomonas, Pseudomonas and Burkholderia spp. supporting the presence of a rather oxidized environment, but also indicated the presence of potential aerobic PCB degraders that might contribute to natural attenuation of PCBs. Known dehalogenating bacteria such as Dehalococcoides, Desulfitobacterium, Clostridium and Bacillus spp. were also detected in clone libraries from sediments,
indicating that reductive dechlorination can occur. However, cold temperatures during most of the year, limited availability of nutrients in the river, and rather oxidized sediments have created a set of unfavorable conditions that restrict natural attenuation and therefore it would not be an important process for PCBs in the Ohio River.

**Did temperature and carbon amendments influence PCB reductive dechlorination rates and microbial community structure in Ohio River sediments?**

Cold (10 °C) and high (40 °C) temperatures cause a negative impact on dechlorination rates. Cold temperatures completely inhibited PCB dechlorination while dechlorination at high temperatures was reduced to only 10% compared to the 25 °C treatment. Microbial community structure also changed under the influence of temperature. Low temperatures caused enrichment in the Alphaprotobacteria class and particularly in the Rhizobiales order but a decrease in the phylum Bacteroidetes. On the other hand, high temperatures caused Bacteroidetes to increase and Alphaproteobacteria to decrease, so Bacteroidetes is proposed as an indicator species for temperature conditions in the river. Carbon amendments caused increased bacteria in the phyla Firmicutes and Chloroflexi but decreased bacteria in the phylum Bacteroidetes, thus, it is proposed that bacteria in these groups can serve as indicators for organic carbon availability in the river. PCB reductive dechlorination rates were also greater in anaerobic treatments with carbon amendments, and, consequently, it is proposed that the Firmicutes may play an important role in these reactions. However, the role as active PCB dechlorinators is still unclear as *Dehalococcoides* spp. were also detected in these treatments and they are known to reductively dechlorinate PCBs.
Is it possible to achieve complete removal of PCBs through a sequential anaerobic-aerobic treatment?

Control over redox status in Ohio River sediment microcosms improved PCB reductive dechlorination rates. However, this process alone cannot completely remove PCB from the environment. Laboratory studies showed that PCB-153 reductive dechlorination rates can be improved by adding organic amendments with an average of two chlorine atoms removed per molecule. However, aerobic degradation of its dechlorination products was not detected to a great extent, and additional work may be required to focus on potential metabolites to determine whether the biphenyl ring is undergoing oxygenation and possibly ring-opening reactions. DNA analysis showed that sediments with high PCB dechlorination are associated with enrichment of bacteria from phyla Bacteroidetes, Chloroflexi and Firmicutes, but with a decrease in the population of Proteobacteria. Particularly Desulfitobacterium spp. were detected in high number in the clone libraries, but also Dehalococcoides spp, were detected, suggesting that these species are actively involved in the reductive dechlorination of PCBs. Dehalococcoides spp. have been shown to dechlorinate PCBs, but the role of Desulfitobacterium spp. might still be in debate because these microorganisms have an aromatic reductive dehalogenase that might also be directly involved in PCB dechlorination. This study also showed the presence of microorganism related to aerobic PCB degraders in these sediments that, although aerobic degradation products were not detected, may accomplish the ultimate goal of complete mineralization of PCBs in the environment.
Future Directions

Up to this point, primer design for the detection of RDH genes has been limited for the small number of sequences available in the current databases to describe these genes. However, more studies dedicated to characterization of these enzymes will also increase the number of sequences available, and thus will allow more efficient primer design that might more successful in capturing the diversity of dechlorinating bacteria in aquatic environments, such as the Ohio River. Using such primers at different locations in the river, especially those located near “hot spots” or power generation plants, might reveal not only the diversity of dechlorinating bacteria in this ecosystem but also the potential differences in the spatial distribution of these organisms along the river.
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**Abstracts**


