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Metabolic and Phenotypic Characterization of Aerobic PCB Degrading Bacteria Isolated from Kalamazoo River Sediments

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METABOLIC AND PHENOTYPIC CHARACTERIZATION OF AEROBIC PCB DEGRADING BACTERIA ISOLATED FROM KALAMAZOO RIVER SEDIMENTS

by

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The Kalamazoo River is contaminated with polychlorinated biphenyls (PCBs) which are neurotoxins and suspected carcinogens. The goal of this study was to isolate, identify, and metabolically characterize PCB degrading strains of aerobic bacteria. Biphenyl enrichments resulted in the isolation of putative PCB degrading strains from different locations along the river. Based on 16S rRNA gene analysis the isolated strains were identified as belonging to the genera of *Achromobacter*, *Acidovorax*, *Cellulosimicrobium*, *Paenibacillus*, *Pseudomonas*, and *Rhodococcus*. These strains were screened for their PCB degrading ability and congener specificity. All of the isolated strains degraded PCBs via the 2,3-dioxygenase pathway. The range of PCBs degraded was limited to mono- to tri-substituted congeners. In addition, the isolated strains oxidized 2,4'-CB but accumulated problematic intermediate metabolites. These results indicate that most of the aerobic-PCB-degrading bacteria isolated in this study from the Kalamazoo River have a moderate potential to degrade the lesser chlorinated PCBs, while one strain (D1) was found to have above average PCB degrading capabilities.

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CHAPTER I

INTRODUCTION

Structure of Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) are a group of nonpolar, chlorinated aromatic hydrocarbons with the chemical formula $C_{12}H_{10-n}Cl_n$ (Fig. 1A). The chemical structure consists of a biphenyl backbone with one to ten chlorine substituents. There are 209 theoretical congeners ranging from monochlorobiphenyl to decachlorobiphenyl. Congeners with an equal number of substituents are referred to as homologs, and homologs with varying substitution patterns are referred to as isomers. The most abundant congeners found in commercial mixtures of PCBs are *ortho*-substituted. As a result, the preferred conformation of the molecule is with the two phenyl-moieties at right angles to each other. Congeners with one or no *ortho*-substitutions can more readily assume a flat or coplanar configuration (Erickson, 1997).

PCBs have four equivalent *ortho*-sites (2,6,2' and 6'), four equivalent *meta*-sites (3,5,3'and 5') but only two equivalent *para*-sites (4 and 4') (Fig. 1B). PCBs can have several names for the same structure (Erickson, 1997). For example, the International Union of Applied Chemistry's (IUPAC) name of the structure in Figure

1C is 2,2',5,5'-tetrachlorobiphenyl. However, for the remainder of the text it will be referred to as 2,5,2',5'-chlorobiphenyl or 2,5,2', 5'-CB.

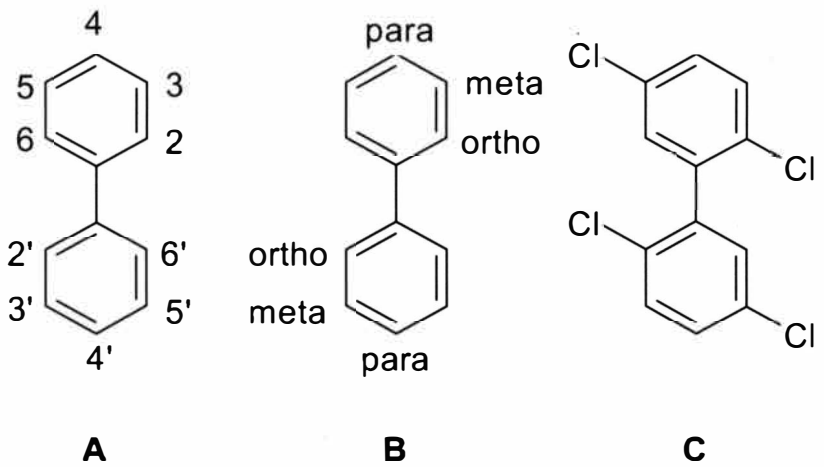

Figure 1. Structure and Nomenclature of PCB Molecules. Structure A demonstrates the numbering system of a PCB molecule. Structure B demonstrates the site designations (*ortho, meta*, and *para*). Structure C is 2,5,2',5'-chlorobiphenyl.

The Swann Chemical Company (Anniston, AL) manufactured PCBs in the United States (US) from 1929 to 1935. Monsanto (St. Louis, MO) purchased the company in 1935 and continued manufacturing PCBs until 1977 under the trade name Aroclor. Commercial PCB formulations are complex mixtures of isomers and were synthesized in a batch process by the direct chlorination of biphenyl with chlorine gas in the presence of a $FeCl_3$ catalyst. The degree of chlorination was regulated by the reaction time. Of the 1.4 billion pounds manufactured worldwide by 1976, 93% were

produced in the US. Commercial Aroclor mixtures have a four-digit designation (e.g. Aroclor 1242). The first two digits (e.g. 12) indicate the number of carbons in the molecule, however, there are exceptions (e.g. Aroclor 1016). The last two digits (e.g. 42) indicate the average percentage of chlorine per weight in the mixture. Aroclor 1242 has an average of 3.1 chlorine substituents per molecule, Aroclors 1248, 1254, and 1260 average approximately four, five, and six, respectively. PCBs are chemically and physically stable and as a result nonflammable, making them ideal for use as the main component in heat transfer fluids. PCBs were also used in a wide variety of products including dielectric fluids, hydraulic fluids, lubricating oils, pesticides, paints, adhesives, sealants, plastics, and of major significance to this study, carbonless copy paper (Erickson, 1997). The same properties that made PCBs ideal for industrial purposes have also resulted in their persistence in the environment.

PCB Toxicology

PCB toxicology has been studied extensively in regards to lethality, carcinogenicity, reproductive toxicity, hepatotoxicity, immunotoxicity, neurotoxicity, dermal toxicity, enzyme induction, endocrine effects, thymic atrophy, inhibition of growth, and porphyria (Safe, 1994). The majority of these studies were performed with commercial mixtures of Aroclors which contain approximately 70 to 80 congeners and can vary from batch to batch making it difficult to pinpoint the exact etiologic agent. Commercial mixtures also contain contaminants like polychlorinated terphenyls (PCTs), polychloroquarterphenyls (PCQs), and polychlorinated

dibenzofurans (PCDFs) which can also confound toxicity test results (Erickson, 1997).

The first reported incidence of PCB toxicity occurred in 1933 when employees at the Swann Chemical plant developed chloracne following exposure to high concentrations of the offending compound (Erickson, 1997). Occupational exposures to PCBs are generally 10-100 times greater than the concentrations found in fish. The consumption of fish is the principle route of PCB exposure to the general population (Brown and Lawton, 1993), and this is also the case for the Kalamazoo River system (Blasland *et al.*, 2000).

The toxicological effects of PCBs are directly related to the structure of the molecule. Different classes of PCBs have different toxicological profiles. Non-*ortho*-substituted and mono-*ortho*-substituted congeners assume a flat or coplanar orientation due to the lack of steric hindrance which occurs between multiple *ortho*-chlorine substituents. The flat congeners have configurations similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and bind to the aryl hydrocarbon (Ah) receptors of vertebrates in the same manner as TCDD (Erickson, 1997). The binding of coplanar congeners to Ah receptors results in the induction of arylhydrocarbon hydroxylase (AHH) and ethoxyresorfurin-*O*-deethylase (EROD) (biochemical markers of exposure), and may result in hepatocarcinomas by inhibiting intercellular communication or by stimulating cell proliferation (Silberhorn *et al.*, 1990). Other structural classes of PCBs have been implicated in neurotoxicity, estrogenicity, endogenous protein binding, and inhibition of oxidative phosphorylation (Safe, 1994).

Results of a two year gavage study performed on female rats by the National Toxicology Program (NTP) indicate that the congener 2,4,5,2',4',5'-CB (PCB 153) may be carcinogenic as evidenced by cholangioma of the liver and increased incidences of neoplastic lesions of the liver, thyroid gland, ovary, oviduct and uterus (National Toxicology Program, 2006).

In 1999 the Agency for Toxic Substance and Disease Registry (ATSDR) reported that there was not enough evidence to support claims that PCBs were human carcinogens. One year later, however, the ATSDR recanted that statement and was now claiming that recent human studies had provided evidence that PCBs were indeed carcinogenic. During the intervening period only one study pertaining to the subject was published, and it supported the ATSDR's previous claim that there is not enough evidence to link PCBs to cancers in humans (Golden *et al.*, 2003.)

The possible neurotoxic effects of PCBs became evident following the Yusho (Japan, 1968) and Yucheng (Taiwan, 1979) incidents when rice oil was accidentally contaminated with PCBs. The children of mothers who consumed the tainted oil had lower body weights and heights, were hyperactive, experienced behavioral problems, and scored lower on IQ tests. PCDFs, contaminants found in all commercial mixtures of PCBs, could not be ruled out as being the etiologic agent (Schantz, 1996).

Studies of the neurobehavioral effects induced by *in utero* low level exposure to environmental PCBs were met with conflicting results. One group reported that prenatal exposure to PCBs resulted in shorter gestation periods, lower birth weights, decreased head circumference, and below average scores on the Brazelton Neonatal

Assessment Battery. A second group did not find any evidence of reduced birth weights and head circumferences, or deficits in memory function. Both groups were, however, in agreement on the Brazelton test results. The second group also noted small delays in psychomotor development but which did not persist beyond two years (Schantz, 1996).

Studies on the effects of PCBs on the nervous system determined that neonates and young children of parents who consumed large quantities of Great Lakes fish experienced delayed motor skills, hyporeflexia and low psychomotor scores between the ages of six months and two years. These effects could have also been attributed to dioxins. Monkeys exposed postnatally to PCB mixtures of similar composition and concentration to those found in human breast milk exhibited learning deficits for significant periods of time following exposure. The *ortho*-substituted congeners appear to be more involved than coplanar congeners in the impairment of cognitive ability. Both dioxin-like (coplanar) and nondioxin-like (*ortho*-substituted) congeners have been shown to induce neurobehavioral changes in monkeys, rats, and mice (Faroon *et al.*, 2001).

PCBs in the Environment

Environmental PCBs were first detected in Swedish fish and at that time were claimed to be as toxic as dichlorodiphenyltrichloroethane (DDT) (Jensen, 1966). PCBs are ubiquitous pollutants of the environment which can be found in marine plants and animals, mammals, birds, bird eggs, and humans. They have low solubility in water and low vapor pressures resulting in high octanol/water partition coefficients

(K_{OW}). K_{OW} represents the ratio of PCBs found in octanol versus water in an equilibrated octanol/water system. PCBs are highly lipophilic, and as a result, accumulate in fatty tissues with the highest concentrations being found at the top of the food chain, a phenomenon referred to as biomagnification. Ninety-nine percent of environmental PCBs are found in soils and sediments. The coplanar and highly-chlorinated congeners adsorb to these matrices stronger than nonplanar and lightly-chlorinated congeners. PCBs have strong affinities for the organic components of soils and sediments, and as a result, are relatively immobile in both matrices (Erickson, 1997).

PCBs in the Kalamazoo River

Ironically, PCBs were released into the Kalamazoo River as a result of the paper recycling process from paper manufacturing plants in Kalamazoo and Plainwell (Blasland *et al.*, 2000). Carbonless copy paper was developed by the National Cash Register Company and is often referred to as NCR paper. Aroclor 1242 was incorporated into copy paper as an ink carrier or solvent. NCR paper was manufactured by the Appleton Coated Paper Company (Appleton, WI) from 1954 to 1971 (Appleton Papers, 1987), and utilized approximately 44 million pounds of Aroclor 1242 in the process. PCBs from the deinked and repulped copy paper were either integrated into new paper products, including cereal boxes, or entered the company's waste stream (Carr *et al.*, 1977). The raw paper wastes were pumped to clarifiers that separated the wastewater (effluent) from the solid matter (primary sludge). The wastewater was either treated at the municipal wastewater treatment

plant or discharged directly into the river. The primary sludge, consisting of water, clay, and fibrous materials to which PCBs strongly adsorbed, was placed in dewatering lagoons (Blasland *et al.*, 2000). The paper companies were not the sole source of the pollutant; industries including Auto Ion located in Kalamazoo, Bentler Industries of Comstock, and the Eaton Corp. located in Battle Creek, Marshall, and Kalamazoo were among fourteen industries with documented PCB discharges into the river. This would account for the Aroclor 1254 found in Morrow Lake sediments and the Aroclor 1260 detected in fish throughout the river. PCBs are still entering the river through the eroding banks of the Michigan Department of Natural Resources (MDNR) former impoundments located in Plainwell, Otsego, and Trowbridge (Blasland *et al.*, 2000). It has been estimated that approximately 230,000 (Blasland *et al.*, 2000) to 350,000 lbs (USEPA, 2003) of PCBs currently reside in Kalamazoo River sediments and in the floodplain of the three MDNR former impoundments. A three mile section of the Portage Creek and an approximate 80 mile stretch of the Kalamazoo River from Morrow Dam to Lake Michigan (Fig. 2) are part of the USEPA's superfund remediation project (Allied Paper, Inc./Portage Creek/Kalamazoo River/Superfund Site).

The environmental consulting firm of Blasland, Bouck, and Lee (Syracuse, NY) was contracted by the Kalamazoo River Study Group (KRSG) to perform a remedial investigation and feasibility study starting in 1993. Their objective was to characterize the site in regards to the chemical nature of the wastes, sources of contamination, the extent of contamination, to identify contaminant migrant

pathways, to prepare a risk assessment and feasibility study, and finally, propose a remediation strategy. Their long-term goal was to reduce the PCB concentrations in indigenous species of fish.

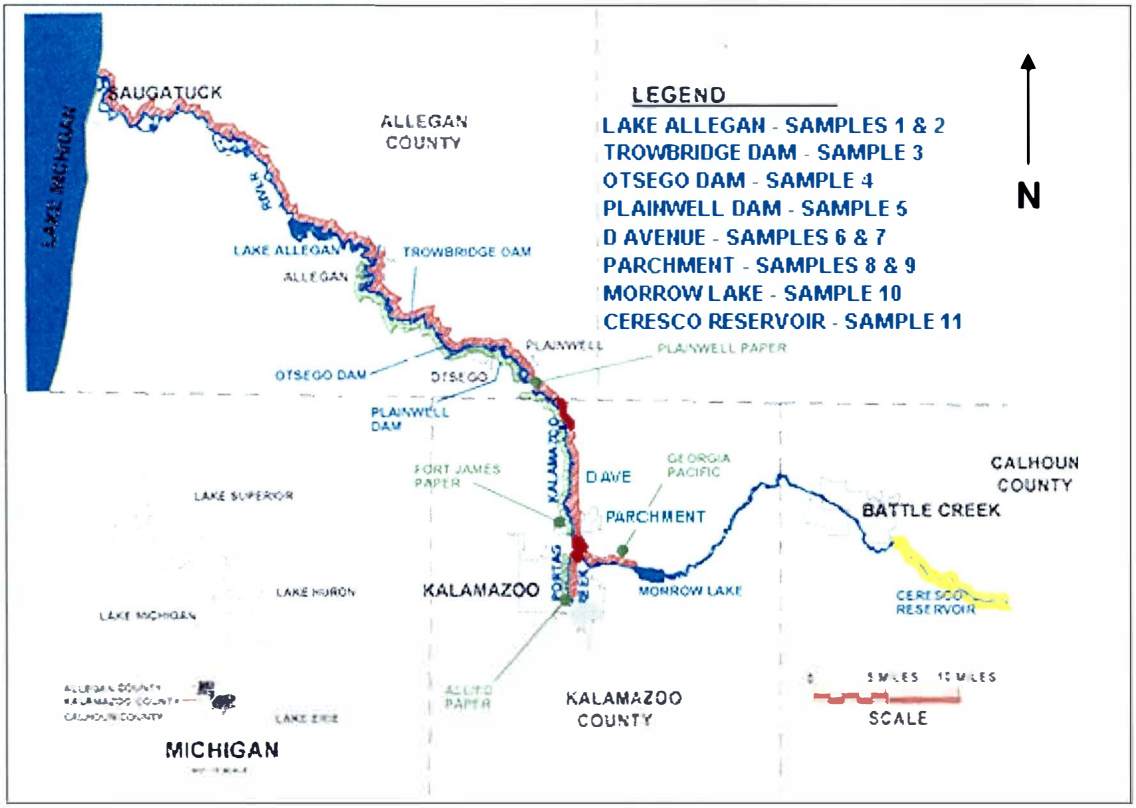

Figure 2. Map of the Kalamazoo River and Superfund Site. Stretches include the USEPA's Superfund site (red), source of PCBs (green), sediment sampling sites (blue), and reference site (yellow). Map taken from the Department of Environmental Quality (DEQ) website and modified.

CHAPTER II

LITERATURE REVIEW

PCB Remediation

There are several options available for the remediation of PCBs including; incineration, vitrification, chemical dechlorination, solvent extraction, stabilization, and base catalyzed decomposition (Erickson, 1997). The majority of the processes require the removal of sediment from the site, and by doing so makes PCBs more available to the environment. The biological treatment or bioremediation of contaminated sediments is a natural process that takes advantage of indigenous microorganisms. For PCBs, a two-step procedure that requires both anaerobic and aerobic microorganisms is envisioned, and is referred to as sequential anaerobic-aerobic remediation. The first step occurs under anaerobic conditions and involves the removal of chlorine from the more highly substituted congeners. The second step occurs under aerobic conditions and involves the degradation of lesser-chlorinated congeners to chlorobenzoic acid (CBA) by the dioxygenase pathway. The history of anaerobic dechlorination, aerobic degradation, and sequential remediation will be discussed with an emphasis on the biphenyl (*bph)* operon of aerobic PCB degrading bacteria.

Anaerobic Dechlorination of PCBs

An organism that can utilize a substance as a source of energy which others in the same community cannot has a competitive advantage. A limiting factor for growth in an anaerobic environment is the presence of final electron acceptors necessary for respiration. An anaerobic microorganism capable of utilizing PCBs as an electron acceptor would have a competitive advantage over neighboring anaerobes in contaminated sediments and therefore be selected for (Brown *et al.*, 1987).

When analyzed by gas chromatography (GC), Aroclor 1242 manifests 55 unique peaks corresponding to the congeners making up the mix. The commercial mixture actually contains 75 individual congeners but exhibits only 55 peaks due to the tendency of some congeners to coelute during GC analysis (Quensen and Tiedje, 1997). Brown *et al.* (1987) observed what they believed to be the dehalogenation of PCBs from river sediments upon examination of several hundred chromatograms from six PCB spill sites. Weathered samples of Aroclor 1242 in Hudson River sediments showed marked reductions in tri-, tetra-, penta- and hexachlorinated congeners with a concurrent increase in mono- and dichlorinated congeners as evidenced by a shift in the chromatographic peaks. The chromatograms revealed six distinct dechlorination patterns attributed to the presence of several distinct localized populations of anaerobic microorganisms. Dechlorination by anaerobic microorganisms does not decrease the molar concentration of PCBs; it does however, decrease the concentration of highly chlorinated congeners.

Quensen *et al.* (1988) were the first to successfully demonstrate the reductive dechlorination of Aroclor 1242 by unidentified anaerobic microorganisms under laboratory conditions. They have shown that anaerobic microorganisms extracted from Hudson River sediments reductively dechlorinated the vast majority of the PCB congeners found in the commercial mixture. The most rapid dechlorination was noted at higher concentrations (700 ppm) with 53% of the total chlorine being eliminated in a 16-week period with a concurrent increase in mono- and dichlorobiphenyls (from 9 to 88%). Dechlorination occurred primarily from the *meta*- and *para*-positions. The dechlorination patterns were designated M (*meta*) and Q (*para*) respectively, whereas pattern C is a combination of patterns M and Q. Removal of chlorine from the *meta*- and *para*-positions resulted in an accumulation of *ortho*-substituted congeners, most notably 2,6-CB. All of the highly chlorinated congeners undergoing dechlorination are those that are exceedingly toxic or are known to bioaccumulate in higher animals. The lesser-chlorinated congeners formed by dechlorination are those that may undergo aerobic degradation. Removal of chlorine from the *meta*- and *para*-positions reduces the dioxin-like toxicity and makes the molecule more amenable to aerobic degradation by creating two adjacent unsubstituted sites necessary for enzymatic attack (e.g. by 2,3-dioxygenase) (Quensen *et al.*, 1988).

In a follow up study, Quensen *et al.* (1990) utilized sediments from two separate PCB contaminated sites to determine the rate, extent, and pattern of degradation of Aroclors 1242, 1248, 1254, and 1260 *in vitro*. All four mixes of Aroclor were dechlorinated from 15 to 85% at the *meta*- and *para*-positions.

Anaerobic bacteria from the Hudson River, NY, contaminated with Aroclor 1242, dechlorinated Aroclor 1242 more effectively than strains from Silver Lake, MA, which is contaminated with Aroclor 1260. The Silver Lake strains, however, dechlorinated Aroclor 1260 more rapidly than the Hudson River strains, and showed a preference for chlorine substituents located at the *meta*-position. The rate of dechlorination for both the Hudson River and Silver Lake strains decreased as the degree of chlorination increased. The dechlorination patterns suggested that the Hudson River strains were more effective than the Silver Lake strains at removing the last *para*-chlorine. Together, the results suggested that distinct strains of PCB dechlorinating microorganisms existed at the two separate sites, each with their own mode of PCB dehalogenation (Quensen *et al.,* 1990).

The first *in vitro* demonstration of *ortho*-dechlorination was performed by utilizing unidentified anaerobic microorganisms residing in Woods Pond sediment contaminated with Aroclor 1260 (Van Dort and Bedard, 1991). Woods Pond is a shallow impoundment on the Housatonic River located in Lennox, MA. A methanogenic slurry was prepared in the laboratory from Woods Pond sediments and amended with the congener 2,3,5,6-CB. After a 37-week incubation period the single congener (2,3,5,6-CB) was dechlorinated to yield the following products; 2,5-CB (21%), 2,6-CB (63%), and 2,3,6-CB (16%). Since then, there have been several reports of *ortho*-dechlorinating activity by anaerobic microorganisms (Berkaw *et al.*, 1996; Cutter *et al.*, 1998; Wu *et al.*, 1998, 2000).

The first PCB dechlorinating microorganism to be identified was bacterium *o*-17. Its 16S rRNA gene shared a high sequence homology to that of green non-sulfur bacteria and with *Dehalococcoides etheneogenes* (Cutter *et al.*, 2001). Cultures of bacterium *o*-17 required amendment with acetate in order for the *ortho*-dechlorination of 2,3,5,6-CB to 2,3,5-CB and 3,5-CB to occur. The only other anaerobic dechlorinating microorganism identified to date is bacterium DF-1 which reductively dechlorinated doubly flanked congeners when supplemented with formate. The 16S rRNA gene sequence of DF-1 shared the highest sequence homology with a species of *Dehalococcoides* and was 89% homologous to that of bacterium *o*-17 (Wu *et al.*, 2002).

Aerobic Biodegradation of PCBs

The degradation of the lesser chlorinated PCB congeners, mono- to hexachlorinated, found in commercial mixtures of Aroclor by aerobic strains of bacteria has been well documented (Ahmed and Focht, 1973; Kaiser and Wong, 1974; Tucker *et al.*, 1975; Furukawa and Matsumura, 1976; Sayler *et al.*, 1977; Clark *et al.*, 1979; Bopp, 1986; Bedard *et al.*, 1986a). Lunt and Evans reported in 1970 that pure cultures of gram-negative bacteria hydroxylated biphenyl at the 2,3-position followed by *meta*-cleavage between C-3 and C-4 (extra-diol distal cleavage) of the same ring. Alternatively, it was determined that ring fission actually occurs between C-1 and C-2 (extra-diol vicinal cleavage) (Catelani *et al.*, 1973). Bacterial strains capable of utilizing biphenyl as the sole source of carbon and energy did so via the enzymes of the upper- and lower-pathways encoded by the genes of the of the *bph* operon (Fig. 3a

and 3b). PCBs are degraded fortuitously to CBAs in the presence of biphenyl; a phenomenon referred to as cometabolism. However, a few strains are capable of utilizing certain mono- and di-chlorinated biphenyls as the sole source of carbon and energy (Ahmed and Focht, 1973; Barton and Crawford, 1988; Kim and Picardal, 2001). CBAs are further degraded by indigenous bacteria to carbon dioxide, chloride, water, and biomass by way of the β-keto adipate pathway and tricarboxylic acid cycle (Reiner, 1971; Abramowicz, 1990). The *bph* operon of most gram-negative strains is chromosomally located (Furukawa *et al.*, 1989), while several gram-positive strains of *Rhodococcus* were found to have biphenyl catabolic genes distributed between the chromosome (Asturias and Timmis, 1993), plasmids (Masai *et al.*, 1997), and transposons (Merlin *et al.*, 1997). The enzymes of the upper-pathway include a multicomponent biphenyl dioxygenase (*bphA1A2A3A4*) (Furukawa *et al.*, 1986; Erickson and Mondello, 1992), a dihydrodiol dehydrogenase (*bphB*) (Furukawa and Miyazaki, 1986), a dihydroxybiphenyl dioxygenase (*bphC*) (Furukawa and Miyazaki, 1986; Kimbara *et al.*, 1989), and a hydrolase (*bphD*) (Furukawa and Miyazaki, 1986; Kimbara *et al.*, 1989; Hofer *et al.*, 1993). Although there are variations, the genes encoding the enzymes of the upper-pathway of the typical biphenyl utilizing strain are ordered accordingly, *bphA1A2A3A4BCD* (Furukawa *et al.*, 1979, 1987, 1989; Furukawa and Miyazaki, 1986; Kimbara *et al.*, 1989; Hayase *et al.*, 1990; Erickson and Mondello, 1992; Hofer *et al.*, 1993). The first enzyme of the pathway is closely related to the multicomponent toluene dioxygenase (*todC1C2BA*) of *Pseudomonas putida* (Taira *et al.*, 1992). A common feature of aromatic ring-hydroxylating

dioxygenases is the presence of a Rieske-type Fe_2S_2 cluster and a mononuclear iron center, which are located within the large subunit of the iron-sulfur protein (ISP) (Erickson and Mondello, 1992; Haddock *et al.*, 1993). The BphA1A2 proteins associate as a heterohexamer ($A1_3A2_3$) making up the large (α) and small (β) subunits of the ISP, respectively (Broadus and Haddock, 1998; Maeda *et al.*, 2001) and are responsible for substrate specificity (Kimura *et al.*, 1998; Kumamaru *et al.*, 1998; Hurtubise, 1998). The BphA3 protein is a ferredoxin and the BphA4 protein a ferredoxin reductase (Erickson and Mondello, 1992). The *bphA* gene products form a short electron transport chain passing two electrons from NADH + H^+ to ferredoxin reductase, to ferredoxin, and finally to a mononuclear Fe^{2+} within the ISP, activating molecular oxygen for insertion into the biphenyl ring (Mason and Cammack, 1992; Haddock *et al.*, 1993; Broadus and Haddock, 1998). Most strains insert oxygen at the 2,3-position of the lesser-chlorinated biphenyl ring (Fig. 3a structure II), oxidizing the substrate to 2,3-dihydrodiol (Gibson *et al.*, 1973; Haddock *et al.*, 1995). Others claim that it is the position of the chlorine substituents and not the number of substituents that determines the site of attack (Arnett *et al.*, 2000). The initial attack by the BphA enzyme (2,3-dioxygenase) requires two adjacent unsubstituted carbons; however, some strains (*Burkholderia* LB400 and *Alcaligenes* H850) have the ability to dehalogenate from the *ortho*-position and therefore require only one nonchlorinated carbon at the adjacent *meta*-position for 2,3-dioxygenase attack. *Burkholderia* LB400 and *Alcaligenes* H850 were shown to be capable of initiating a dioxygenase attack at the 3,4-position; this also required two adjacent open sites (Bopp, 1986; Bedard *et al.*,

1986a, 1987; Bedard and Haberl 1990; Seeger *et al.*, 1995, 1999). The BphB protein is a tetramer and a member of the short-chain alcohol dehydrogenase/reductase family (Sylvestre *et al.*, 1996; Hulsmeyer *et al.*, 1998) which converts 2,3-dihydrodiol (Fig. 3a structure II) to 2,3-dihydroxybiphenyl (structure III) with the concurrent reduction of NAD^+ to NADH (Gibson *et al.*, 1973). Most BphC proteins are octamers of identical subunits that utilize a nonheme Fe^{2+} center to cleave 2,3-dihyroxybiphenyl at C-1 and C-2 (extra-diol vicinal cleavage) generating 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA; Fig. 3a structure IV) (Catelani *et al.*, 1973; Furukawa *et al.*, 1987; Khan *et al.*, 1996). Some strains of *Rhodococcus* are known to have multiple copies of *bphC* (Asturias and Timmis 1993; Asturias *et al.*, 1994; Taguchi *et al.*, 2004). The *bphD* gene product is a serine hydrolase that belongs to a family of proteins having a α/β fold, and hydrolyze HOPDA (Fig. 3a structure IV) to benzoic acid (structure V) and 2-hydroxypenta-2,4-dienoic acid (structure VI) (Seah *et al.*, 1998, 2000).

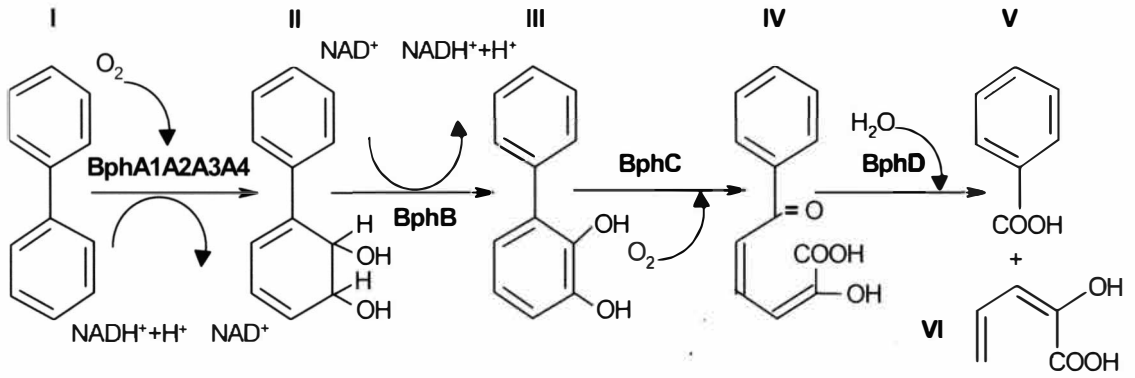

Figure 3a. The Catabolic Upper-Pathway for Biphenyl and the Organization of the *bph* Operon. Substrates for the upper-pathway include; biphenyl (I), 2,3-dihydrodiol (II), 2,3-dihydoxybiphenyl (III), 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA IV). Biphenyl is catabolized to benzoic acid (V) and pentadienoic acid (VI).

The *bphEFG* genes encode the enzymes of the lower-pathway and include a hydratase, an aldolase and a dehydrogenase, respectively. The enzymes of the lower-pathway convert 2-hydroxypenta-2,4-dienoic acid to acetylaldehyde with the concurrent production of pyruvate and acetyl CoA, which enters the tricarboxylic acid cycle (Hofer and Timmis 1994; Kikuchi *et al.*, 1994) (Fig. 3b).

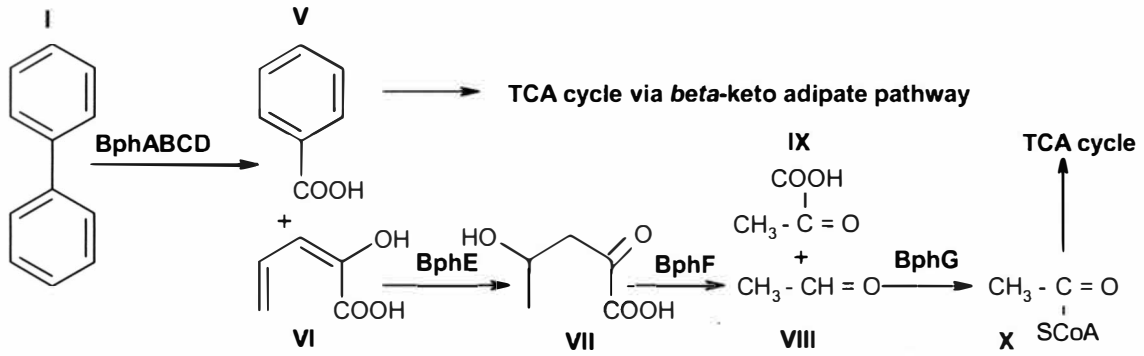

Figure 3b. The Catabolic Lower-Pathway for Biphenyl and the Organization of the *bph* Operon. Substrates for the lower-pathway include; pentadienoic acid (VI) is catabolized to oxovaleric acid (VII), acetaldehyde (VIII) and pyruvic acid (IX). Acetylaldehyde is converted to acetyl CoA (X), which enters the TCA cycle.

The *bphH* gene encodes the enzyme glutathione S-transferase, the function of which is unknown and is not required for the utilization of biphenyl, but may play a role in dehalogenation or detoxification (Bartels *et al.*, 1999). Regulation of the operon varies among different strains; however, the *bph* operon of *Pseudomonas* KF707 is well characterized and under the control of two transcriptional activators, BphR1 and BphR2. The BphR1 protein belongs to the GntR-type transcriptional regulatory family. It initiates the transcription of the genes encoding the enzymes of the lower-pathway, and is enhanced by the presence of HOPDA (Watanabe *et al.*, 2000). The BphR2 protein belongs to the LysR-type transcriptional regulatory family and initiates transcription of the genes encoding enzymes of the upper-pathway (Watanabe *et al.*, 2003). The cistronic organization of the typical gram-negative *bph* operon is *bphR1A1A2(orfX)bphA3A4BCEFGHDR2*. The biphenyl dioxygenase gene

sequences of *Burkholderia* LB400 and *Pseudomonas* KF707 are nearly identical; however, they exhibit significantly different substrate specificities (Erickson and Mondello, 1993). The function of *orfX* is still unresolved; it could possibly encode a transcriptional repressor (Watanabe *et al.*, 2003).

PCB degrading strains of aerobic bacteria are categorized into two catabolic groups based on their ability to degrade an assortment of PCB congeners. The first group comprises strains with *Burkholderia* LB400-like specificities, which degrade a broad range of congeners with up to 6 chlorine-substitutions, including di-*ortho*-substituted, but are relatively ineffective at degrading di-*para*-substituted congeners. Strains within this group may also have dehalogenase and/or 3,4-dioxgenase activity (Gibson *et al.*, 1993; Mondello *et al.*, 1997). LB400-like strains attack the *ortho*-substituted ring of 2,4'-CB resulting in the formation of CBAs as depicted in Figure 4 (Maltseva *et al.*, 1999).

The second group includes strains with *Pseudomonas* KF707-like specificity, which degrade a much narrower range of congeners with up to 4 chlorine-substitutions, but are relatively effective at degrading di-*para*-substituted congeners. Strains within this group do not have dehalogenase and/or 3,4-dioxygenase activity (Gibson *et al.*, 1993; Mondello *et al.*, 1997). KF707-like strains attack the *para*-substituted ring of 2,4'-CB which results in the formation of problematic *meta*-cleavage products rather than CBAs as depicted in Figure 4 (Maltseva *et al.*, 1999).

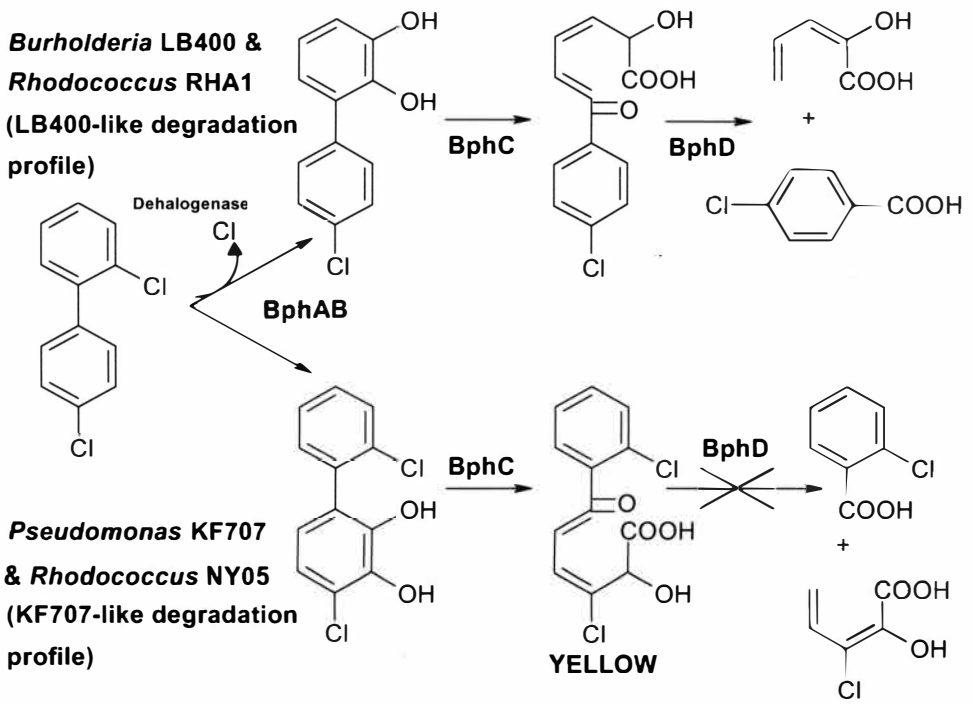

Figure 4. Alternate Pathways for the Degradation of 2,4'-CB by Aerobic Bacteria with Contrasting Degradation Profiles. Strains with dehalogenase activity (*Burkholderia* LB400) oxidize the *ortho*-substituted ring of 2,4'-CB resulting in the formation of CBAs. Other strains (*Rhodococcus* NY05) oxidize the *para*-substituted ring of 2,4'-CB resulting in the formation of the dead-end metabolite 3,8-dichloro-HOPDA which is yellow in color.

Sequential Anaerobic-Aerobic Remediation of PCBs

The first *in vitro* testing of the sequential technique was performed by amending anaerobically dechlorinated river sediments with hydrogen peroxide to initiate aerobic degradation. Positive results were noted by increased bacterial counts and an approximate 85% reduction in PCB concentrations (Anid *et al.*, 1993). The

fact that both anaerobic-dechlorinating, and aerobic-PCB-degrading microorganisms, were found together in the same sediment support the potential for *in situ* bioremediation.

Microcosms created from Hudson River sediments were utilized to determine the degradative fate of Aroclor 1242 by indigenous microorganisms (Fish and Principe, 1994). Discernable aerobic and anaerobic layers were evident by two to four weeks, with significant PCB degradation noted by 140 days. PCBs located in superficial sediments underwent both aerobic degradation and anaerobic dechlorination. This resulted in an approximate 72% decrease in total PCB concentrations. In anaerobic subsurface sediments, PCBs underwent dechlorination from the *meta*- and *para*-positions (patterns M and Q). PCB concentrations remained constant, however, the average number of chlorines decreased by approximately 41%. These results suggested that the naturally occurring microorganisms from Hudson River sediments had the potential to mineralize PCBs that had previously been released into the river system (Fish and Principe, 1994).

Unknown anaerobic-PCB-dechlorinating microorganisms extracted from Hudson River sediments were utilized to dechlorinate weathered Aroclor 1248 from contaminated soil deficient in organic carbon (Evans *et al.*, 1996). Sequential anaerobic-aerobic treatment was compared to single treatments with anaerobic or aerobic microorganisms. The anaerobic treatment alone resulted in the loss of chlorine substituents from the *meta*-positions of highly chlorinated congeners. This was followed by aerobic treatment which consisted of exposure to oxygen, an

inoculation with *Burkholderia* LB400, and the addition of biphenyl. The total concentration of PCBs was decreased by 70% following the sequential anaerobic-aerobic treatment compared to a 67% reduction with just the aerobic treatment. Compared to anaerobic or aerobic treatments performed separately, the sequential treatment did result in the greatest reduction in total PCB concentration albeit by only 3% (Evans *et al.*, 1996).

Soil contaminated with weathered Aroclor 1260 underwent *in vitro* sequential anaerobic-aerobic treatment (Master *et al.*, 2002). The anaerobic treatment lasted for four months and resulted in complete or partial transformation to lesser chlorinated congeners, with the major products being 2,4,2',4'-CB and 2,4,2',6'-CB. The average number of chlorine substituents per PCB molecule was reduced by approximately 19% and as expected the molar concentration of PCBs remained the same. After 28 days of aerobic treatment, *Burkholderia* LB400 degraded the major products of the anaerobic treatment. The total concentration of PCBs was reduced by approximately 66%. Strain LB400, however, was unable to degrade weathered Aroclor in soil that had not been treated anaerobically (Master *et al.*, 2002).

Maltseva *et al.* (1999) utilized four strains of aerobic-PCB-degrading bacteria to test the susceptibility of congeners representing the primary products from the anaerobic dechlorination of Aroclor 1242 (patterns M and C) to aerobic degradation. Of the four bacterial strains tested, the two with LB400-like degradation profiles (*Burkholderia* LB400 and *Rhodococcus* RHA1) degraded 2,4'-CB and 2,4,4'-CB to 4-CBA, which can be further degraded by other microorganisms. Conversely, the

other two strains (*Rhodococcus* NY05 and *Comamonas* VP44) with KF707-like degradation profiles transformed the congeners into the problematic *meta*-cleavage product 3,8-dichloro-HOPDA (Fig. 4). The *ortho*-directed strains readily degraded seven of the eight congeners when tested individually, with the most recalcitrant being 2,6-CB, which has been found to inhibit dihydroxybiphenyl dioxygenase (Dai *et al.* 2002). The degradation rates of congeners 2,2-CB and 2,4,4'-CB were reduced when they were supplied in a mixture. Overall, the strains mounting an attack on the *ortho*-substituted ring were more efficient at degrading individual congeners and their mixes, with a greater production of CBAs than the strains directing an attack on the *para*-substituted ring (Maltseva *et al.*, 1999).

Recalcitrant Congeners and Problematic Metabolic Intermediates

In summary, based on the literature cited above, the sequential anaerobic-aerobic treatment appears to be a viable option for the remediation of PCB contaminated river sediments. There are, however, several major drawbacks regarding the generation of toxic intermediates in the aerobic degradation scheme. Commercial mixtures of Aroclor contain 70 to 80 unique congeners or compounds (Erickson, 1997); some are more resistant to degradation than others (Furukawa *et al.* 1979, Bedard and Haberl, 1990). Certain metabolic steps can be rate limiting during the degradation of PCBs by aerobic bacteria, with bottlenecks occurring at the level of biphenyl dioxygenase (BphA), dihydroxybiphenyl dioxygenase (BphC), and HOPDA hydrolase (BphD). These are the first, third, and fourth enzymes of the *bph* pathway, respectively. The oxidation of 2-CB results in the production of dihydrodiols and

dihydroxybiphenyls which were found to be highly cytotoxic, even for short periods, as evidenced by cell lysis (Camara *et al.*, 2004). Accumulation of dihydroxy compounds occurred when the following congeners were oxidized: 2,6-CB, 2,3,6-CB, 2,4,2',5'-CB, 2,5,2',5'-CB, and 2,4,5,2',5'-CB (Furukawa *et al.*, 1979; Bedard and Haberl, 1990). The oxidation of 2,2'-CB resulted in the formation of hydrogen peroxide, which is known to be cytotoxic and to inhibit the degradation of other congeners (Imbeault *et al.*, 2000). The aromatic ring cleavage of PCBs is catalyzed by 2,3-dihydroxybiphenyl-1,2-dioxygenase (DHBD). It has been demonstrated that the *ortho*-substituted congener 2,6-CB strongly inhibited DHBD by the generation of a superoxide anion. The k_{cat} for 2,6-CB was 7,000 times smaller than the k_{cat} of nonchlorinated dihydroxybiphenyl (DHB), and 2,6-CB bound with a high affinity resulting in the competitive inhibition of DHB (Bartels *et al.*, 1984; Adams *et al.*, 1992; Vaillancourt *et al.*, 1998, 2002; Dai *et al.*, 2002). To make matters worse, 2,2'-CB and 2,6-CB accumulated as a result of preferential dehalogenation from the *meta*- and *para*-positions by anaerobic microorganisms (Quensen *et al.*, 1988).

Accumulation of problematic *meta*-cleavage products, chlorinated HOPDAs, occurred with the degradation of 2,4'-CB, 2,4,4'-CB, and 2,5,4'-CB, all of which had the 2,4'-Cl substitution in common (Furukawa *et al.*, 1979; Bedard and Haberl, 1990). Individual PCB degrading strains preferentially attacked either the 2-Cl ring or the 4'-Cl ring of 2,4'-CB (Maltseva *et al.*, 1999). Oxidation of the 4'-Cl ring resulted in the formation of 3,8-dichloro-HOPDA, which is known to inhibit the *bphD* gene product HOPDA hydrolase (Fig. 4), which in turn inhibited the degradation of all congeners

(Seah *et al.*, 2000). HOPDAs can also be converted to chloroacetophenones by ultra violet (UV) radiation, which are highly toxic (Baxter and Sutherland, 1984).

Remediation Strategy for the Kalamazoo River

Following a lengthy investigation by Blasland *et al.* (2000), five remediation strategies were proposed ranging from no further action to submerged sediment removal. During the course of their investigation Blasland *et al.* (2000) found no "hot spots" in river sediments, determined that 70% of PCB containing sediments had settled in Lake Allegan, and large quantities of PCB laden sediments were exposed in the banks of the three MDNR former impoundments and were currently the leading source of PCB loading to the river system. Blasland *et al.* (2000) were also able to determine that natural attenuation, which includes physical, chemical, and biological processes to degrade or isolate PCBs, was active in the Kalamazoo River as evidenced by decreasing PCB concentrations in surface water, sediments, and fish over a period of two decades. As a result, Blasland *et al.* (2000) proposed placing of physical barriers along the banks of the MDNR former impoundments to prevent erosion, letting natural attenuation processes run their course, and closely monitoring the natural processes.

Blasland's *et al.* (2000) assessment of the natural attenuation process included laboratory bench scale models of an aerobic bioslurry treatment, and an anaerobic *in situ* treatment of river sediments. Aerobic treatments were assessed by comparing degradation rates under varying conditions: 1) the addition of nutrients, 2) the addition of nutrients and biphenyl, and 3) the addition of nutrients, biphenyl and

proprietary PCB-degrading bacteria provided by a company named Envirogen of Lawrenceville, NJ. From the aerobic treatments, treatment 3 resulted in the largest reduction in PCB concentrations from 18.1 ppm to 5.5 ppm. Treatment 2 reduced concentrations from 18.1 ppm to 7.5 ppm, and treatment 1 reduced concentrations from 18.1 ppm to 11.8 ppm, a reduction of 70, 58, and 35%, respectively. The anaerobic treatment involved the addition of a carbon source, or amendment with active PCB dehalogenating microorganisms. Neither of these treatments enhanced dechlorination activity. Based on the results stated above, Blasland *et al.* (2000) rejected sequential anaerobic-aerobic remediation as a practical *in situ* remediation option. Sequential bacterial remediation is a major part of the natural attenuation scheme and without it the process relies almost entirely on the burial of contaminated sediments by cleaner sediments deposited by way of erosion.

Overall Goals For this Study

The goal of this study was to isolate aerobic-PCB-degrading bacterial strains from river sediments in order to taxonomically identify, and metabolically characterize them in regards to their PCB degrading abilities. Kalamazoo River sediments were also tested for the presence of environmental PCBs and for signs of reductive dechlorination activity by anaerobic microorganisms. This was completed in an effort to isolate PCB degrading bacteria with novel substrate specificities, and to supplement the assessment of the natural attenuation processes claimed to be active in the Kalamazoo River system.

Isolation of putative PCB degraders was accomplished through focused sampling of river sediments. Focused sampling entails sampling from sites of known PCB discharge, or in stretches with heavy silt deposits, to which PCB's are known to adsorb. Putative PCB degrading strains were selected for via biphenyl enrichment culturing. The rationale behind this is to supplement the culture with biphenyl while exhausting existing sources of carbon. Serial enrichments resulted in the exclusive growth of biphenyl utilizing strains which may be potential PCB degraders. Putative PCB degraders were screened and identified by performing two variations of the polymerase chain reaction (PCR). Isolated strains were tested as resting cells for their ability to degrade a select group of PCB congeners. Substrates for resting cell assays included a mix of congeners representative of those found in commercial mixes of Aroclor 1242, and the recalcitrant congeners 2,4'-CB and 2,6-CB. The problematic congeners 2,4'-CB and 2,6-CB were tested in an attempt to discover strains capable of resolving some of the "bottlenecks" encountered in the aerobic degradation scheme. Bacterial strains with novel substrate specificities could possibly enable the degradation of the most resistant *ortho*-chlorinated PCB congener, 2,6-CB, which accumulates during the course of anaerobic dechlorination, and the development of toxic metabolites that accumulate during the aerobic degradation of 2,4'-CB. Doing so could possibly contribute to making the sequential anaerobic-aerobic treatment of PCB contaminated sediments a feasible option for future remediation projects.

CHAPTER III

MATERIAL AND METHODS

Study Site

The Kalamazoo River is located in southwestern Michigan and runs through the counties of Jackson, Calhoun, Kalamazoo, and Allegan for approximately 162 miles (Fig. 2). The river flows west from the city of Jackson to Kalamazoo, then northwest from Kalamazoo to Saugatuck, where it empties into Lake Michigan. An approximate 80 mile stretch between Morrow Lake and Lake Michigan is impounded by seven dams and fed by twelve tributaries. The river bottom consists primarily of sand and gravel in free flowing areas, with large quantities of silt deposited in the slower reaches and impoundments. The Trowbridge, Otsego, and Plainwell impoundments were drained in the early 1970's releasing 1.1 million cubic yards of sediment containing over 32,000 lbs of PCBs, the majority of which now reside in the Allegan City impoundment and Lake Allegan (Blasland *et al.*, 2000). This study site encompasses an approximate 65 mile stretch of the Kalamazoo River from the Ceresco Reservoir (background control) to Lake Allegan.

Sediment Sampling

Focused sampling was utilized rather than random sampling. Sediment cores were taken manually with a Geoprobe Hand Corer™ with a 3.0 cm bore and a 60 cm barrel. Rinsing with 95% ethanol (ETOH) prior to use sanitized the probe tip, acetate

liners, and end caps. Sediment core samples were obtained by pounding the probe into the river bottom until the barrel was full (~ 60 cm). Sediment cores were contained within the acetate liners. End caps were placed on the liners and labeled with the location, date, and orientation. Sediment cores were immediately placed in a cooler with ice (~4°C) out of direct sunlight for transport to Western Michigan University (WMU). Samples were stored at 4°C for approximately 72 hr prior to use in determining PCB concentrations and for microbial analysis. Additional samples, for microbial analysis only, were obtained from Kevin Kahmark (Dept. of Chemistry, WMU) consisting of dry primary sludge stored at 4°C for several weeks. The primary sludge was taken from three locations: the south shore of the former Otsego impoundment, the banks of the former Trowbridge impoundment, and from the Trowbridge Dam boat launch. The PCB concentrations were not determined for the primary sludge samples.

Preparation of Sediments

Sediments for PCB extraction and use in enrichment cultures were prepared in a laminar flow hood (The Baker Co., Sanford, MA). The procedure was initiated by disinfecting the outside of the acetate liners with 95% ETOH. Sediments were exposed by cutting the liners lengthwise with a sterile metal scalpel, exposing the contents. The top 20 cm of sediments were dispensed into sterile beakers and homogenized with a sterile metal spatula. For quantitative determination of PCB concentrations, several grams of each sample were allowed to desiccate in a fume-hood for 24 hr, followed by a light grinding with a mortar and pestle. Sediments for

cultural enrichments were prepared in the same manner, except they were not allowed to desiccate and were not ground.

PCB Extraction from Sediments

PCBs from sediments were Soxhlet extracted using a procedure described by Quensen and Tiedje (1997) with the following modifications. A surrogate (2,4,6,2',4',6'-CB) was not used and octachloronaphthalene (OCN) was added immediately after clean-up. No correction was made for extraction efficiency and therefore no estimate of extraction efficiency could be made and results are reported as uncorrected (J.F. Quensen III, MSU, personal communication).

Gas Chromatography Analysis

PCB analysis was performed with a gas chromatograph with an electron capture detector (GC-ECD model 5890; Hewlett-Packard, Palo Alto, CA) equipped with a Hewlett-Packard Ultrabond 2 capillary column (type SE-54 equivalent; 50 m by 0.2 mm). The carrier gas was He (20 cm/s) and N_2 was the makeup gas. Two µl of sample was added by the split injection technique (10:1), the inlet was held at 220°C and the detector at 325°C. An initial oven temp of 140°C was held for 1.0 min, then ramped at 2°C/min to 300°C, and held for 4 additional min.

A three level internal standard calibration table based on peak area was used for quantitation of Aroclors. The level 1 standard consisted of a mixture of Aroclor 1242 (10 µg/ml), Aroclor 1260 (10 µg/ml), 2-CB (10 µg/ml), 3-CB (10 µg/ml), 4-CB (10 µg/ml), 2,2'-CB (7.5 µg/ml), 2,6-CB (2.5 µg/ml), 2,2',4,4',6,6'-CB (1 µg/ml), and

OCN (1.6 µg/ml). The level 2 standard contained twice as much of each component except for OCN which remained the same (1.6 µg/ml). The level 3 standard contained four times as much as in the level 1 standard, except OCN remained the same. The calibration curve for each peak consisted of a power function forced through the origin (John F. Quensen III, MSU, personal communication).

A four level internal standard calibration table based on peak area was used for quantitation of PCB congener mixes 1B and 2B. The level 1 standard contained 0.5 µg/ml of each component in mix 1B and 2B and 2.0 µg/ml of 2,2',4,4',6,6'-CB. Standard levels 2, 3, and 4 contained two, four, and eight times as much as in the level 1 standard, except 2,2',4,4',6,6'-CB was always 2.0 µg/ml. The calibration curve for each peak consisted of a power function forced through the origin (John F. Quensen III, MSU, personal communication).

Bacterial Strains and Cultural Conditions

The bacterial strains used in this study were isolated from Kalamazoo River sediments by successive biphenyl enrichments. *Rhodococcus erythreus* NY05, a confirmed PCB degrader, was previously isolated from soil collected in the state of New York (Pellizari *et al.*, 1996) and served as a reference strain throughout the study. Bacterial enrichment cultures were grown aerobically in liquid K1 mineral medium (Zaitzev *et al.*, 1985) supplemented with ground biphenyl (1.0 g/l unless stated otherwise) as the sole source of carbon and energy. For solid medium 1.8 g/l of Noble agar (Difco, Detroit, MI) was added. A few crystals of biphenyl were placed on

the lid of each petri dish, sealed with parafilm™, and vapors from the crystals served as the sole carbon source.

Cultural Biphenyl Enrichment

All cultural enrichments were performed in a laminar flow hood (The Baker Co., Sanford, MA). A total of 2.5 g of homogenized sediment was dispensed into sterile Erlenmeyer flasks containing 250 ml of K1 mineral medium supplemented with biphenyl (1.0 g/l) and capped with sterile aluminum foil. *R. erythreus* NY05 served as the reference strain (positive control) to test the medium. All samples were incubated at 28°C in a rotary shaker at 200 rpm (New Brunswick Scientific, Edison, NJ). Subcultures were prepared every 14 days by inoculating 100 ml of fresh K1 with 1.0 ml of the previous culture. Bacterial colonies were isolated from enrichment cultures via the spread plate technique by inoculating plates with 100 µl of serially diluted (10^{-1} - 10^{-6}) bacterial culture. A few crystals of biphenyl were placed on the lid of each dish and sealed with parafilm™. These plates were placed lid-down in a cell culture incubator (GMI, Ramsey, MN) at 28°C. Isolated colonies were picked from spread plates and purified three times by streak-plating twice on solid K1 medium, and once on 0.1 trypticase soy agar (TSA). All plates were incubated at 28°C.

Phenotypic Analysis

Gram stains were performed with the following reagents; crystal violet, Gram's iodine, 95% ETOH, and 0.25% safranin. Spore stains were performed via the Schaeffer-Fulton method with the following reagents; malachite green and 0.5%

safranin. A cytochrome oxidase test was performed on all gram-negative strains with oxidase reagent (250 mg N-N-N-tetramethyl:*p*-phenyline 0.1%, 25 mg ascorbic acid 1.0%, 25 ml H_2O). Oxidase-positive strains were tentatively identified with a BBL® Oxi/Ferm™ Tube II (Becton Dickinson, Cockeysville, MD).

Total Genomic DNA Extraction from Cells

Total genomic DNA was extracted from cells as described by Ausubel *et al.* (1995). The genomic DNA was precipitated with ice-cold (-20°C) 100% ETOH and collected by spooling onto a sterile glass rod. The precipitate was dissolved in 100 µl of 1X Tris-EDTA (TE) and stored at -20°C for use in rep-PCR and 16S rRNA gene analysis.

DNA Quantitation

Extracted genomic DNA from the river isolates was quantitated and tested for purity spectrophotometrically (Model DU 6000, Beckman, Fullerton, CA) as described by Ausubel *et al.* (1995).

Rep-PCR

Reagents for one PCR-mixture include in a total volume of 25.0 µl: 5.0 µl of 5X Gitschier Buffer, 10.8 µl of mΩ H_2O, 0.2 µl of bovine serum albumin (BSA 20 mg/ml), 2.5 µl of dimethylsulfoxide (DMSO), 3.0 µl of 40 mM dNTP-mixture, 1.0 µl of primer 1 REP-1R: 5'-IIIICgICgICATCIggC-3' (0.3 µg/µl), 1.0 µl of primer 2 REP-2I: 5'-ICgICTTATCIggCCTAC-3' (0.3 µg/µl) (Rademaker *et al.*, 1997), 0.5 µl of Taq

polymerase (5U/µl *Taq* DNA Polymerase, SIGMA, St. Louis, MO), and 1.0 µl of template genomic DNA (10-100X diluted to ~ 50 ng/µl). One reaction minus template DNA was prepared to serve as a control. PCR was performed in an Eppendorf Mastercycler (Westbury, NY). Thermal cycling conditions were as follows: 95°C for 7 min, 35 cycles of 94°C for 1 min, 40°C for 1 min, and 65°C for 8 min with a final extension of 65°C for 8 min. Once complete, 10 µl of PCR product was loaded onto a 1.5% agarose gel and run in an electrophoresis cell (Embi Tec, San Diego, CA) at 50 V for 25 minutes in 0.5% Tris-acetate-EDTA (TAE) buffer.

16S rRNA Gene PCR

Reagents for one PCR mixture include in a total volume of 50.0 µl: 5.0 µl of 10X PCR reaction buffer, 40 µl of mΩ H_2O, 0.5 µl of BSA (20 mg/ml), 1.0 µl of 40 mM dNTP-mixture, 1.0 µl of primer 1, 10 µM stock (Primer #1 - 8-27F sequence 5' - AGA GTT TGA TCM TGG CTC AG), and 1.0 µl of primer 2, 10 µM stock (Primer #2 - 1392-1407R sequence 5' - ACG GGC GGT GTG TRC) (Weisburg *et al.*, 1991; Reysenbach *et al.*, 1992), 0.5 µl of Taq polymerase (1U/µl RED*Taq* DNA Polymerase, SIGMA, St. Louis, MO), and 1.0 µl of template genomic DNA (10-100X diluted to ~ 50 ng/µl). One reaction minus template DNA was prepared to serve as a control. PCR was performed in an Eppendorf Mastercycler (Westbury, NY). Thermal cycling conditions were as follows: 95°C for 5 min, 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 2 min with a final extension of 72°C for 10 min. Once

complete, 3.0 µl of PCR product plus 2.0 µl of loading dye was loaded onto a 1% agarose gel and run in an electrophoresis cell (Embi Tec, San Diego, CA) at 25 V for 30 minutes in 0.5% TAE buffer.

Gel Visualization

Following electrophoresis, gels were stained with a ethidium bromide solution (0.5 µl/ml from liquid stock) containing 0.5% TAE buffer for a minimum of 10 min. DNA was visualized by placing the gel on a Fotodyne Foto/Prep Ultraviolet transluminator (Hartland, WI) using the analytical UV setting. Photographs were taken with the Fotodyne-FCR-10 camera with an ethidium bromide filter using Polaroid 667 black and white film (Exposure time 1.0 sec, F-stop 4.5).

DNA Purification

Products from the 16S rRNA gene PCR were purified using the 15-minute batch purification method by Wizard PCR Preps DNA Purification Systems (Promega, Madison, WI) with a vacuum manifold as per protocol. The purified 16S rRNA genes were quantitated and tested for purity spectrophotometrically (Beckman DU-6000, Fullerton, CA) as described by Ausubel *et al.* (1995) and stored at -20°C for future use in DNA sequencing.

DNA Sequencing

DNA sequencing was performed on the 16S rRNA gene PCR products at Michigan State University's Genomics Technology Support Facility (GTSF). Fifty nanograms of template DNA and 30 pmoles of primer 8-27F were submitted in 1.5 ml

microcentrifuge tubes according to GTSF's specifications: 5 µl DNA (10 ng/µl) + 3 µl primer (10 µM) + 4 µl mΩ H_2O = 12 µl total (www.genomics.msu/protocols). Sequences were obtained from GTSF's Finch server. The Basic Local Alignment Search Tool (BLAST) was performed via the National Center for Biotechnology Information's (NCBI) BLAST program (Altschul *et al.*, 1990) and GTSF's Ribosomal Data Base II (RDB II) Naive Bayesian Classifier Version 1.0 and Seqmatch Version 3 release 9.38 (http:/www.rdp.cme.msu.edu). The sequences were compared to those deposited in GenBank and RBD II. The BLAST program compares nucleotide sequences to sequence databases and calculates the statistical significance of matches.

Rapid Screening for Bacteria which Oxidize the *ortho*-substituted Ring of 2,4'-CB

Liquid cell cultures were prepared as described above (cultural conditions) with fresh isolated colonies until an OD_{600} of approximately 1.0 was reached (Model DU 6000, Beckman, Fullerton, CA). One and one-half ml of cell culture was dispensed into 2.0 ml sterile clear-glass vials. Ten µl of 2,4'-CB in acetone (100 mM initial concentration) was added to each liquid culture and incubated at room temperature until a yellow precipitate was noted (approximately 20 min.). Isolated strains were also screened for their ability to degrade persistent *ortho*-substituted congeners (2,2'-CB, 2,6-CB, 2,4'-CB 2,2',4'-CB, and 2,4,2',4'-CB) in resting cell assays, as described below.

Growth Curves of Isolates

Fifty ml of liquid K1 mineral medium supplemented with 0.5 g/l biphenyl was dispensed into sterile Klett flasks with metal caps. Flasks were prepared in duplicate for each isolate and *Rhodococcus* NY05, inoculated with 20 µl of preculture (OD_{600} ~1.0), and incubated at 28°C while shaking at 200 rpm (New Brunswick Scientific, Edison, NJ). Turbidity measurements were taken with a Klett photoelectric colorimeter (Scienceware, Pequannock, NJ) every 4 hrs for the first 72 hrs and every 12 hrs for the remaining 64 hrs. Late log phase for each isolated strain was determined by plotting the data in a spreadsheet and graphing.

Resting Cell Assays

Modified versions of the resting cell assay previously described by Bedard *et al.* (1986b) were performed. Liquid cell cultures for the resting cell assays were prepared as described above (growth curves) and incubated until late log phase was attained. The cells were harvested, washed three times in fresh liquid K1 medium by centrifuging for 10 min at 5000*g* (Sorval RC5B, Asheville, NC), and resuspended in fresh K1 medium to an OD_{600} of 2.0 (Beckman DU 6000, Fullerton, CA). Nine-hundred-ninety µl aliquots of each cell suspension were dispensed into 8 ml sterile glass vials and sealed with sterile Teflon™ coated screw-caps. Cell suspensions were allowed to incubate at room temp for 1 hr to metabolize residual biphenyl.

Resting cell assay version #1 was designed to test a strain's ability to initiate a dioxygenase attack on a select mix of congeners representative of those found in Aroclor 1242. Samples were screened in triplicate as both live and dead cell

suspensions. Cultures for use as dead cell controls were inactivated by placing the vials into a 90°C water-bath for 20 min. The congener mixes (1B and 2B) were gifts from John F. Quensen III (MSU) and each congener had a concentration of 200 µg/ml. Mix 1B contained the following congeners; 2,4'-, 4,4'-, 2,4,4'-, 2,5,2',5'-, 2,3,2',5'-, 2,3,2',3'-, 2,4,3',4'-, 2,4,5,2',3'-, 3,4,3',4'-, 2,4,5,2',4',5'-CB. Mix 2B contained the following congeners; 2,2'-, 2,3-, 2,5,2'-, 2,5,4'-, 2,4,2',4'-, 2,5,3',4'-, 2,4,5,2',5'-, 2,3,4,2',5'-, 2,4,5,2',4',5'-CB. One-half of the samples received 10.0 µl of mix 1B, while the other one-half received 10.0 µl of mix 2B by injection with a 10 µl Hamilton syringe. All vials were placed in a rotary shaker (New Brunswick Scientific, Edison, NJ), in the dark, and allowed to incubate for 24 hr at 28°C and 200 rpm. Following incubation, live cell suspensions were inactivated by placing the vials into a 90°C water-bath for 20 min.

Resting cell assay version #2 was designed to test a strain's ability to initiate a dioxygenase attack on the doubly *ortho*-substituted congener 2,6-CB. The assay was performed as described above except 10 µl of 2,6-CB (10 mM initial concentration) was dispensed into each sample instead of either mix 1B or 2B. Also, the surrogate (2,4,6,2',4',6'-CB), and the internal standard (OCN) were not used. As a result, reports were compiled utilizing the external standard method. Strains to be tested were screened in duplicate as live-cell suspensions and singularly as dead-cell controls. PCB's from resting cell assays #1 and #2 were solvent extracted as described below.

PCB Extraction from Cell Suspensions

To quantitate PCB degradation, 40 µl of the surrogate 2,4,6,2',4',6'-CB (50 mM initial concentration) was injected into each vial of cell culture (to serve as a control for measuring extraction efficiency) with a fine-point 50 µl Hamilton syringe prior to extraction. PCBs were extracted from resting cell suspensions by dispensing 2.0 ml of hexane/acetone (1:1) to each vial, and shaking (by inversion) for 20 min. The solution was allowed to settle and the top phase was collected from each cell suspension. This was repeated three times. The extracts were dispensed into glass conical tubes and the volume was reduced by evaporation in a fume-hood to approximately 1.0 ml (Bedard *et al.*, 1986b). The remaining extracts were dispensed into 2.0 ml GC vials, topped with Teflon™ coated caps, crimped shut, and stored at 4°C awaiting GC analysis as described above.

Determining the Site of Initial Dioxygenase Attack on 2,4'-CB

Cell suspensions were prepared as described above (growth curves). Ten µl of 2,4'-CB (10 mM initial concentration) was injected into each cell suspension with a 10 µl Hamilton syringe. All vials were placed in a rotary shaker (New Brunswick Scientific, Edison, NJ), in the dark, and allowed to incubate for 96 hr at 28°C and 200 rpm. Intermediate metabolites (HOPDA's) were extracted by withdrawing 1.0 ml of cell suspension from each sample, dispensing into 1.5 ml microcentrifuge tubes, and centrifuging at 15,000*g* for 5 min (Model 5415D, Eppendorf, Westbury, NY). Following centrifugation, the top 0.5 ml of supernatant was transferred to a 2.0 ml quartz cuvette. The volume was brought up to 2.0 ml by the addition of 1.5 ml fresh

K1 medium (dilution factor 4:1). The OD of each sample was measured at 396 nm at 0, 6, 12, 24, 48, and 96 hrs and plotted on a graph (Model DU 7000, Beckman, Fullerton, CA). Absorption was also measured at wavelengths of 360, 370, 380, 396, 400, 410, 420, and 434 nm after a 12 hr incubation period to determine the wavelength of maximum absorption (λ max). Liquid K1 mineral medium served as the blank (Maltseva *et al.*, 1999).

CHAPTER IV

RESULTS

PCB Analysis of River Sediments

Sediment core samples were taken in order to detect and quantitate environmental PCBs, to determine if, and to what extent reductive dechlorination had occurred, and to isolate biphenyl/PCB degrading bacteria for catabolic and taxonomic analysis. The samples were taken from eleven locations along the Kalamazoo River from the Ceresco Reservoir to Lake Allegan (Fig. 2). Eight of the eleven core samples had approximately the same composition consisting of silt (0 – 10 cm), sand (10 – 30 cm), and gravel (30 – 60 cm). The Trowbridge Dam, D Ave, and Ceresco Reservoir samples consisted entirely of silt (0 – 60 cm).

GC analysis of environmental PCB extracts taken from Trowbridge Dam and D Avenue sediments revealed concentrations of 20.5 and 12.5 ppm, respectively (Table 1). Zero to trace amounts of PCBs was detected in Lake Allegan, Otsego Dam, Plainwell Dam, Parchment, Morrow Lake, and Ceresco Reservoir sediments. Nevertheless, Blasland *et al.* (2000) have detected significant concentrations of PCBs at all sites except the Ceresco Reservoir (Table 1).

Table 1

PCB Concentrations in Sediments and Locations from which Strains were Isolated.

Location	Sample depth (cm) this study	PCB concentration (ppm) this study	Sample depth (cm) BBL	PCB concentration (ppm) BBL (max)	# of biphenyl degraders isolated and designation in this study
Lake Allegan 1	0 – 20	0.0	30 – 60	61*	0
Lake Allegan 2	0 – 20	0.0	0 - 5	73*	0
Trowbridge Dam	0 – 20	20.5	60 – 90	120*	0
Otsego Dam	0 – 20	0.0	15 – 30	18*	0
Plainwell Dam	0 – 20	0.2	5 – 30	140*	0
D Avenue	0 – 20	12.5	5 – 15	8.2*	4 – D1, D2, D3, D4
Parchment 1 (Mosel Ave)	0 - 20	0.1	30 - 50	67*	1 – P1
Parchment 2 (G Ave)	0 - 20	**	15 – 35	14*	2 – P2A, P2B
Parchment 3 (Springbrook)	0 –20	**	5 - 30	2.7*	2 – P3A, P3B
Morrow Lake	0 - 20	0.0	30 - 60	4,5*	0
Ceresco Reservoir	0 - 20	0.1	0 - 5	0.0*	0
Primary sludge 1 (Otsego)	0 – 5	**	0 - 5	160*	2 – O1A, O1B
Primary sludge 2 (Trowbridge)	0 – 5	**	0 - 5	91*	1 – T1
Primary sludge 3 (Trowbridge)	0 - 5	**	0 - 5	91*	2 – T2A, T2B

Blasland, Bouck, and Lee (BBL) (2000). Remedial Investigation and Feasibility Study (RI/FS). *Maximum PCB concentrations detected from sites. **Not tested.

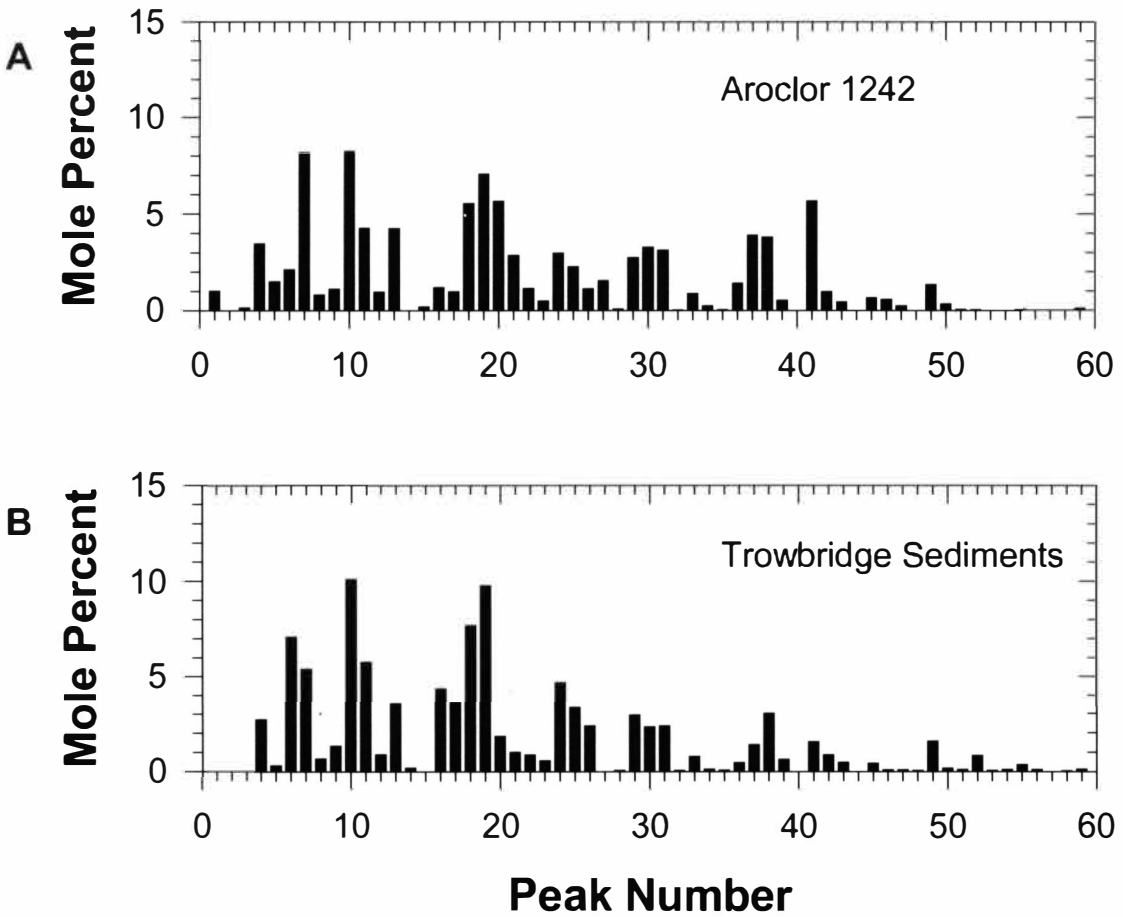

Figure 5. Graphical Representation of Chromatographic Profiles. Histograms of Aroclor 1242 (A) and PCB extracts taken from Trowbridge Dam (B). Each bar represents one, and in some cases, two PCB congeners.

Chromatographic profiles based on peaks present and their approximate amounts were generated for both the Trowbridge Dam and D Ave extracts. Environmental PCBs extracted from Trowbridge Dam sediments (Fig. 5B) were found to be most similar to Aroclor 1242 (Fig. 5A), while the PCBs extracted from D Ave sediments (Fig. 6B) were found to be most similar to Aroclor 1248 (Fig. 6A) (John F. Quensen III, MSU, personal communication).

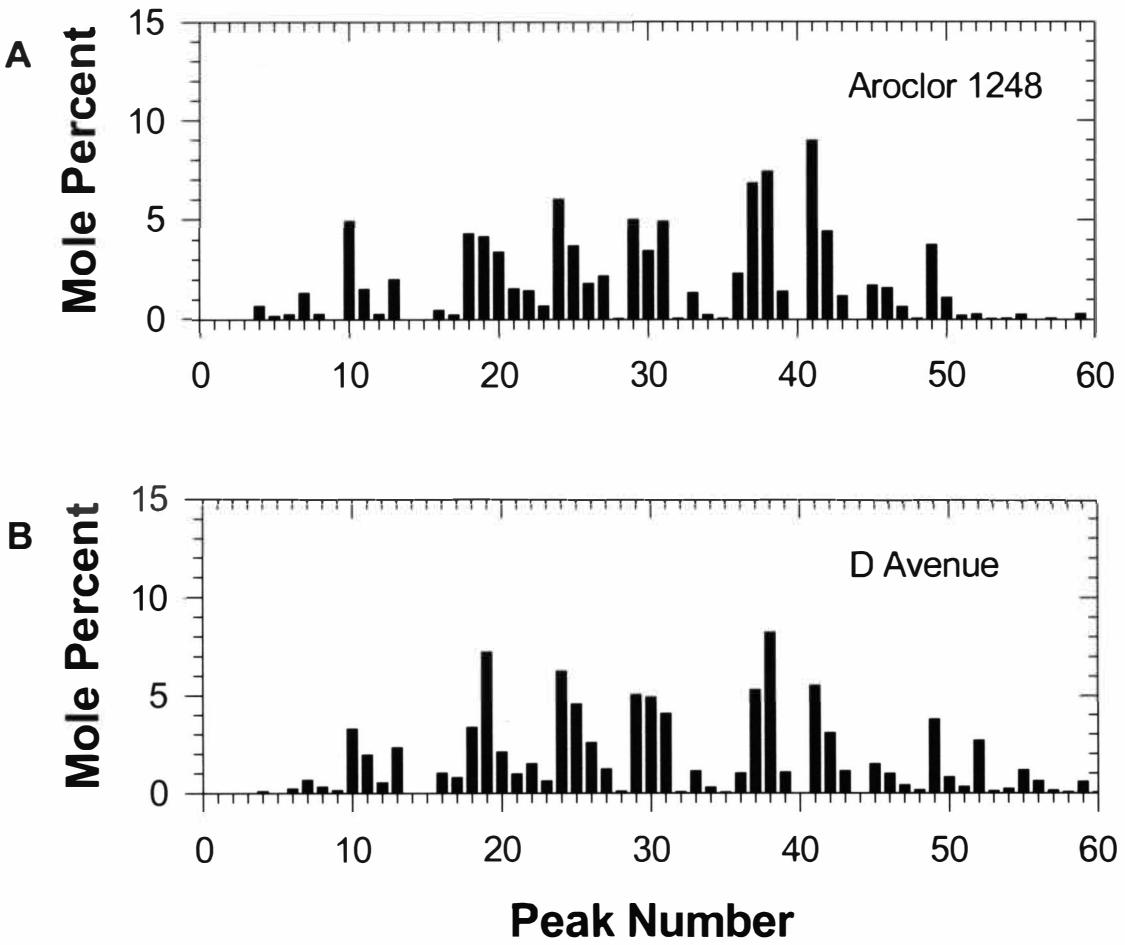

Figure 6. Graphical Representation of Chromatographic Profiles. Histograms of Aroclor 1248 (A) and of PCBs extracted from the D Avenue site (B).

When calculated, the average number of chlorines for each sample did not show evidence of dechlorination when compared to values for Aroclors 1242 and 1248. There were some differences observed between the Trowbridge Dam and D Ave samples and their respective Aroclors, however, no distinct dechlorination patterns (M and Q) were observed (John F. Quensen III, MSU, personal communication).

Isolation of Biphenyl/PCB Degrading Bacterial Strains

PCBs are degraded fortuitously by the enzymes of the *bph* operon and do not support the growth of the bacterial strains of interest; therefore, PCBs were not utilized as substrates in the enrichment cultures. Instead, biphenyl was used as the sole source of carbon in order to isolate biphenyl/PCB degrading strains from river sediments. Successive biphenyl enrichments resulted in the isolation of fourteen putative PCB degrading strains from seven separate locations along the Kalamazoo River (Table 1). Four strains were isolated from D Ave (D1, D2, D3, D4), two strains from the Otsego impoundment (O1A, O1B), five strains from three locations in Parchment (P1, P2A, P2B, P3A, P3B), and three strains were isolated from two locations at the Trowbridge impoundment (T1, T2A, T2B). It is noteworthy to mention that strains D4 and T2B manifest a dark precipitate when grown in both liquid and solid media supplemented with biphenyl as the sole carbon source, where as all others appeared white/cream.

Genetic Analysis

Repetitive Extragenic Palindromic (rep)-PCR was performed on all fourteen isolated strains: D1, D2, D3, D4, O1A, O1B, P1, P2A, P2B, P3A, P3B, T1, T2A, T2B and the reference strain NY05. The goal was to exclude redundant bacterial strains in order to reduce time spent on phenotypic, genetic, and metabolic testing. Rep-PCR is a genomic based technique for the identification and classification of bacteria that takes advantage of naturally occurring, repetitive DNA sequences found in multiple copies within the genome. Degenerate primers are utilized in PCR which anneal to the

repetitive sequences. The resulting PCR products are resolved by gel electrophoresis generating profiles similar to bar codes (Rademaker *et al.*, 1997). The results from the procedure are presented in the form of an electrophoretogram (Fig. 7). Visual examination of the gel revealed that isolated strains O1A and O1B, P2A and P2B, P3A and P3B, and D3 and D4 manifest similar banding patterns and therefore appear to be related strains. This reduced the number of unique putative PCB degrading strains to ten: D1, D2, D4, O1, P1, P2, P3, T1, T2A, and T2B.

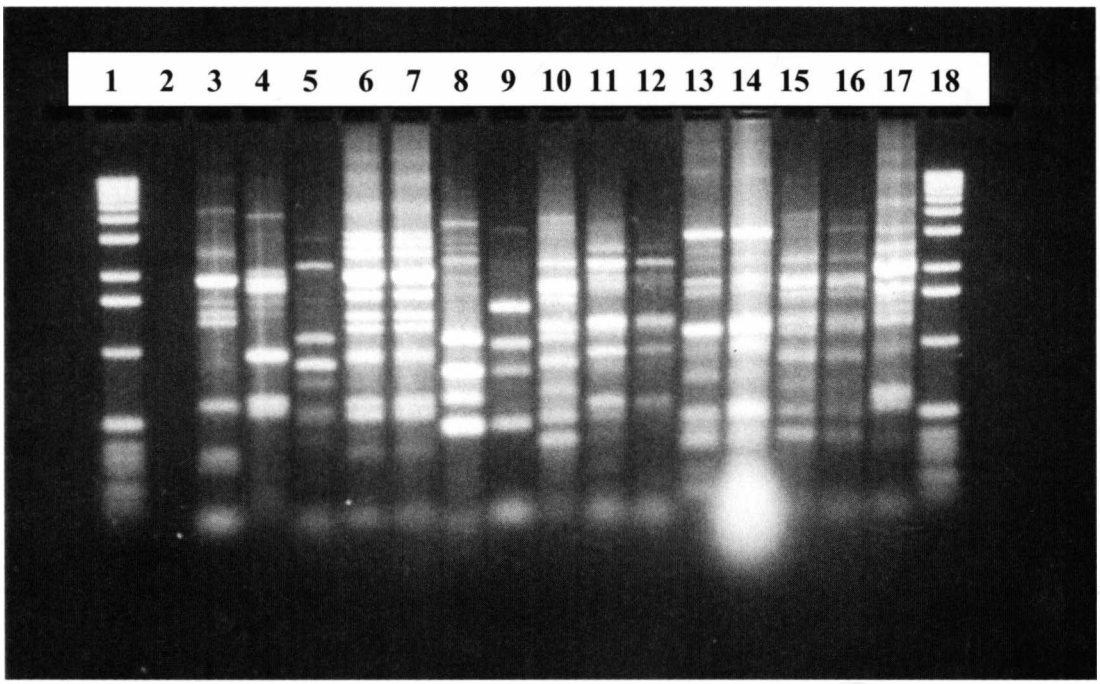

Figure 7. Electrophoretogram of rep-PCR Products. Banding profile of each isolated strain and the reference strain *Rhodococcus* NY05. Lane 1-1 kb marker, lane 2- negative control (no DNA), lane 3 - *R. erythreus* NY05, lane 4 - D1, lane 5 - D2, lane 6 - O1A, lane 7 - O1B, lane 8 - T1, lane 9 - T2A, lane 10 - T2B, lane 11 - P2A, lane 12 - P2B, lane 13 - P3A, lane 14 - P3B, lane 15 - D3, lane 16 - D4, lane 17 - P1, and lane 18 - 1 kb marker.

Phenotypic Analysis

The initial phenotypic characterization of ten of the isolated strains (D1, D2, D4, O1, P1, P2, P3, T1, T2A, and T2B) was carried out by performing Gram- and spore-stains. Six out of ten strains tested were determined to be Gram-negative rods (D4, P2, P3, T1, T2A, and T2B), whereas D1 (pleomorphic rod), D2 (coccoid), O1 (rod), and P1 (rod) tested Gram-positive (Table 2).

Spore stains were performed on all Gram-positive strains. Isolated strains P1 and O1 tested positive for spore formation. A cytochrome oxidase test was performed on all ten isolated strains. All Gram-negative strains tested positive for the presence of oxidase, while all gram-positive strains tested negative (Table 2). All Gram-negative, oxidase-positive strains were further characterized with an Oxi/Ferm™ tube II, which is designed to assist in identifying oxidative-fermentative, gram-negative bacterial strains. All Gram-negative strains tested were identified as species of *Pseudomonas* by the Oxi/Ferm™ II identification system, which is in poor agreement with results obtained from GenBank (Table 3).

Table 2

Phenotypic Characterization of Isolated Strains. Phenotypic characteristics of biphenyl/PCB degrading bacterial strains isolated from Kalamazoo River sediments.

Strain	**Gram stain**	**Spore stain**	**Cell Size (µm)**	**Cell Morphology**	**Oxidase**	**Oxi/Ferm™**
D1	+	–	4.5	Pleomorphic rod	–	*
D2	+	–	3.0	Coccoid	–	*
D4	–	*	2.5	Rod	+	*Pseudomonas cepacia*
O1	+	+	2.5	Rod	–	*
P1	+	+	3.0	Rod	–	*
P2	–	*	2.5	Rod	+	*Pseudomonas putida*
P3	–	*	2.0	Rod	+	*Pseudomonas putida*
T1	–	*	3.5	Rod	+	*Pseudomonas aeruginosa*
T2A	–	*	2.0	Rod	+	*Pseudomonas diminuta*
T2B	–	*	2.0	Rod	+	*Pseudomonas cepacia*

*Not done.

Further Genetic Analysis

The 16S rRNA gene analysis of putative PCB degrading strains (D1, D2, D4, O1, P1, P2, P3, T1, T2A, and T2B) was accomplished by performing PCR and DNA sequence analysis. Sequences were compared to those deposited in GenBank's database. RDB II's Classifier identified strain D1 as an unknown species of *Rhodococcus* (Table 3). BLAST determined D1 shared the highest identity (100%) with *Rhodococcus* sp. CHNTR32 which was isolated from swine effluent applied soil at the University of Illinois (Maxwell *et al.*, 2005). Strain D2 was identified as an unknown species of *Cellulomonas* and shared the highest identity (99%) with *Cellulosimicrobium cellulans* strain AS 4.1333 which was isolated at the Institute of Microbiology in Beijing, China (Zhang *et al.*, 2002). Strains D4, P3, T2A, and T2B were all identified as unknown species of *Acidovorax* and shared the highest homologies with bacterium N5511 (99%), uncultured bacterium SX2-10 (99%), uncultured beta proteobacterium S15A-MN11 (100%), and *Acidovorax* sp. c303 (100%), respectively. Bacterium N5511 was isolated from a river aquifer in Canada (Haveman *et al.*, 2005), the uncultured bacterium SX2-10 from mine water samples in China (Xie *et al.*, 2006), the beta proteobacterium S15A-MN11 from ground water contaminated by a deep-well injection disposal site for radioactive wastes in Siberia, Russia (Nedelkova, 2002), and *Acidovorax* sp. c303 was isolated on phenol from soil obtained from a aquifer in Japan (Futumata *et al.*, 2005). Classifier identified isolated strains O1 and P1 as unknown species of *Paenibacillus* which shared the highest identities with *Paenibacillus* sp. KBC101 (98%) and *P. validus* strain PR-P9 (94%),

respectively. *Paenibacillus* KBC101 was isolated in Japan (Sakai *et al.*, 2004), while *P. validus* PR-P9 was isolated from contaminated estuarine sediment (Danne *et al.*, 2001).

Table 3

Genetic Based Identification of Isolated Strains.

Strain	**RBD II Classifier**	**NCBI BLAST**	**% Identity**
D1	*Rhodococcus* unknown species	*Rhodococcus* CHNTR32 (#DQ337561)	562/562 100%
D2	*Cellulosimicrobium* unknown species	*C. cellulans* AS 4.1333 (#AY114178)	635/638 99%
D4	*Acidovorax* unknown species	Bacterium N5511 (#AY928245)	709/711 99%
O1	*Paenibacillus* unknown species	*Paenibacillus* sp. KBC101 (#AB186915)	565/574 98%
P1	*Paenibacillus* unknown species	*P. validus* PR-P9 AF353697	399/423 94%
P2	*Achromobacter* unknown species	*Alcaligenes* sp. YEN (#AY744384)	608/609 99%
P3	*Acidovorax* unknown species	Uncultured bacterium SX2-10 (#DQ469201)	686/687 99%
T1	*Pseudomonas* unknown species	*Pseudomonas* sp. SCD-4 (#AF448040)	676/677 99%
T2A	*Acidovorax* unknown species	Uncultured beta proteobacterium S15A-MN11 (#AJ534662)	662/662 100%
T2B	*Acidovorax* unknown species	*Acidovorax* sp. c303 (#AB167175)	600/600 100%

Strain P2 was identified as an unknown species of *Achromobacter* sharing the highest identity (99%) with *Alcaligenes* sp. YEN which was isolated from an unknown origin

in Taiwan (Lee *et al.*, 2004). Strain T1 was identified as an unknown species of *Pseudomonas* and shared the highest identity (99%) with *Pseudomonas* sp. SCD-4. Strain SCD-4 was isolated from tall grass prairie soil contaminated with crude oil at the University of Oklahoma (Duncan *et al.*, 2001).

Literature reviews resulted in no record of biphenyl/PCB degradation by any of the isolated strain's closest relatives identified above, except for *Paenibacillus* sp. KBC101 which was found to degrade coplanar PCBs (Sakai *et al.*, 2004), and *Pseudomonas* sp. SCD-4 which is known to degrade naphthalene (Duncan *et al.*, 2001). Most naphthalene degrading strains are also capable of degrading biphenyl (Pellizari *et al.*, 1996).

Isolates D2 and P1 were eliminated from further testing due to poor growth in liquid K1 medium and/or a general lack of vigor when grown with biphenyl as the sole source of carbon. Isolate T2B was also eliminated because it was believed to be closely related to strain T2A. Seven of the ten strains identified were selected to undergo biochemical characterization as evidenced by prolific growth in liquid K1 medium with biphenyl as the sole source of carbon.

Growth Curves

It has been demonstrated that cells in late log phase degrade PCBs more efficiently than cells in early to mid-log phase (Joon Park, MSU, personal communication). As a result, growth curves were performed for seven of the isolated strains, and the reference strain NY05, prior to the resting cell assays. This was done in order to identify strains that were potentially weak PCB degraders based on their

ability to catabolize biphenyl, to establish optimal conditions (late log phase) for the remaining strains being assayed, and to establish time frames for starting cultures so that they reached late log phase concurrently. Growth curves for each organism were determined by culturing the isolates in liquid K1 medium with biphenyl, taking periodic readings with a photoelectric colorimeter, and plotting the data in a spreadsheet and graphing (Fig. 8). A summary of the results was based on lag times, the time it took to attain late log phase, and turbidity measurements. Strains T1 and T2A proved to be the most potent biphenyl degraders based on lag times of 10 and 20 hrs, respectively, and by attaining late log phase in approximately 45 hrs (Fig. 8A and 8B). Strains P2 and D1 had lag times of 25 and 30 hrs and attained late log phase by approximately 70 and 110 hrs, respectively (Fig. 8C and 8D). The reference strain NY05 had a lag time of 35 hrs and reached late log phase in approximately 60 hrs (Fig. 8E), while isolated strain D4 had a lag time of 55 hrs and reached late log phase by approximately 110 hrs (Fig. 8F). Strain P3 had a lag time of 70 hrs, but late log phase could not be determined because it never entered stationary phase in the time allotted (Fig. 8G). Strain O1 exhibited an extremely long lag phase of approximately 90 hrs and never attained late log phase in our experiment (Fig. 8H). As a result, O1 was eliminated from the resting cell assays and replaced by strain T2B. Strain T2B had been previously rejected from further testing because it was thought to be closely related to strain T2A, and may not yield novel data.

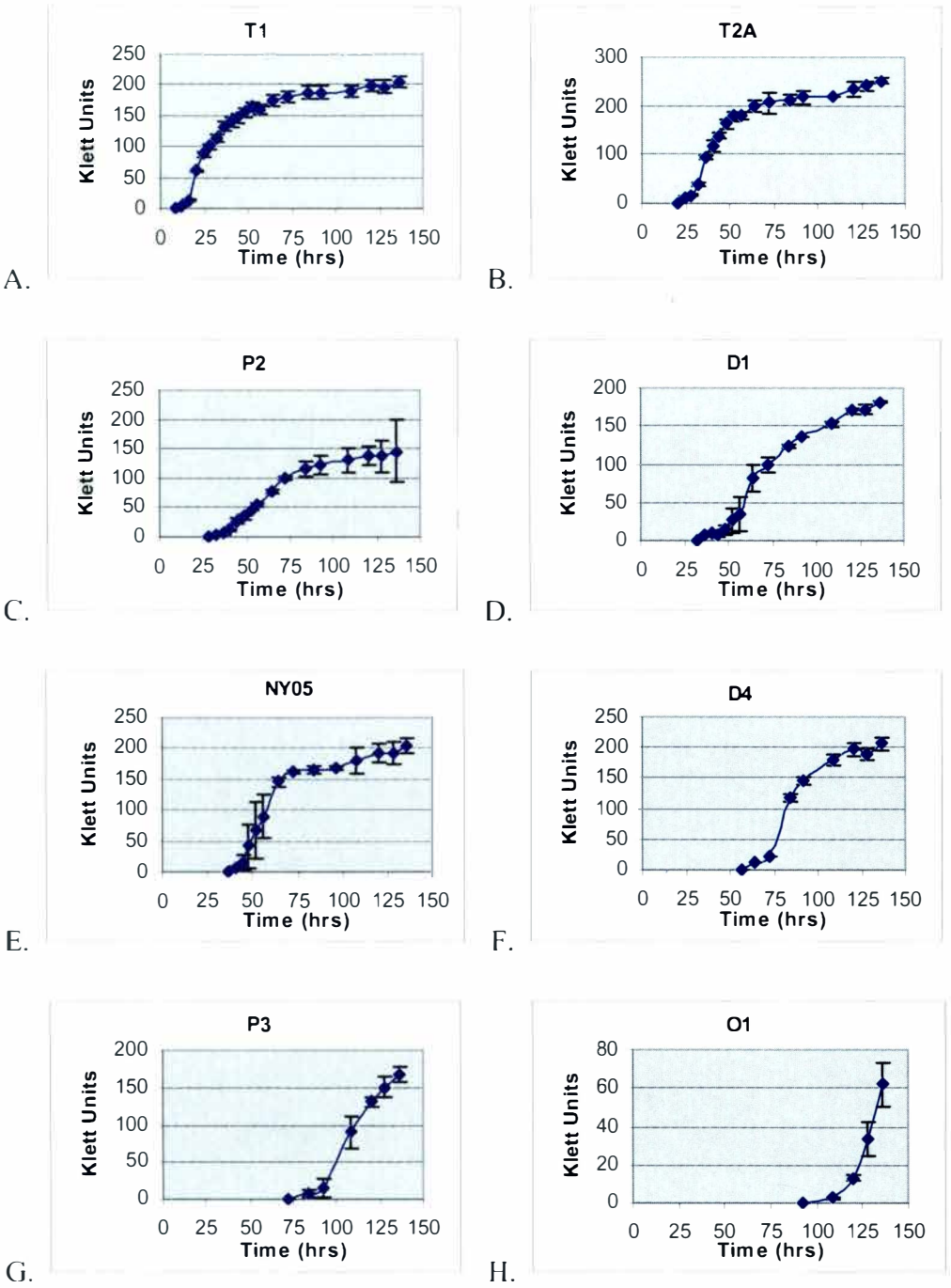

Figure 8. Graphical Representation of Growth Curves. Growth curves were performed on isolated bacterial strains and the reference strain NY05 grown in K1 mineral medium with 0.1% biphenyl as the sole carbon source. Error bars denote standard deviation.

Biochemical Analysis

Rapid Screening Assay

In order to rapidly detect bacterial strains with the ability to oxidize (attack) the *ortho*-substituted ring of 2,4'-CB, a simple screening assay was developed by Maltseva *et al.* (1999), and was performed on seven of the isolated bacterial strains (D1, D4, P2, P3, T1, T2A, and T2B) and the reference strain NY05. Negative results were evidenced by the accumulation of the yellow-colored *meta*-cleavage product HOPDA (Fig. 9), and were indicative of attack on the *para*-substituted ring of 2,4'-CB (Fig. 4 and 10). Strains mounting a *para*-attack (e.g. *Rhodococcus* NY05) fall into the KF707-like degradation profile for aerobic-PCB-degrading-bacteria and accumulate HOPDA. Conversely, aerobic strains that preferentially attack the *ortho*-chlorinated ring of 2,4'-CB can then be identified by a lack of yellow precipitate in the culture medium. No yellow precipitate actually indicates two possible outcomes: 1) dioxygenase attack occurred at the *ortho*-chlorinated ring and 2,4'-CB was degraded to 4-CBA and pentadienoic acid, or 2) no attack occurred and 2,4'-CB remained unchanged. Strains mounting an *ortho*-attack fall into the LB400-like degradation profile and are known to degrade a wide range of PCB congeners including those with di-*ortho*-substitutions (Maltseva *et al.*, 1999). The results indicated that isolated strains D1, P2, P3, D4, T1, T2A, and T2B attack 2,4'-CB at the *para*-substituted 4'-Cl ring as indicated by a yellow precipitate (Fig. 9). The reference strain *Rhodococcus* NY05, which appears white, would seem to initiate an attack on the *ortho*-substituted 2-Cl ring as indicated by a lack of yellow precipitate (Fig. 9).

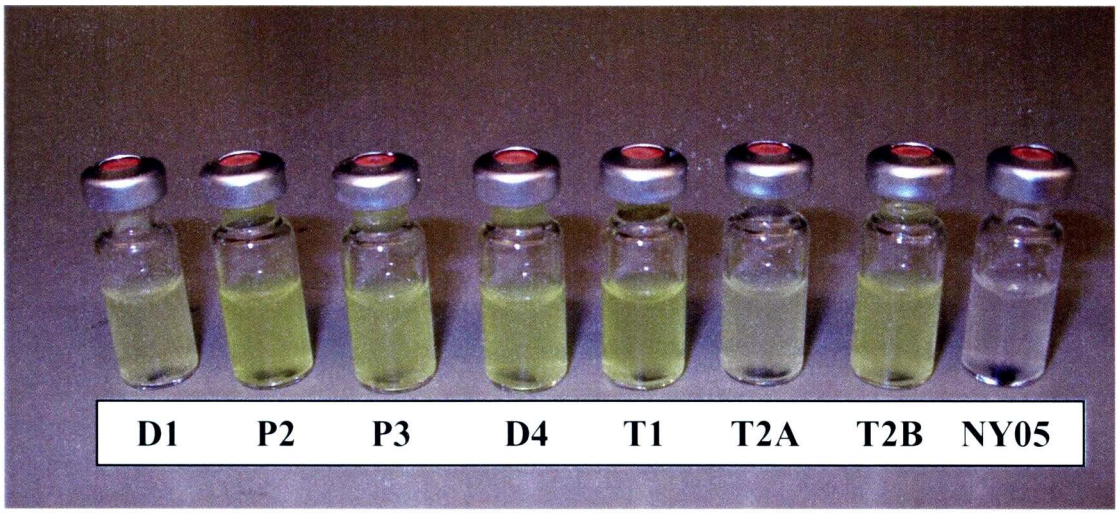

Figure 9. Photograph Showing the Results of the Rapid Screening Assay. Strains isolated in this study and the reference strain *Rhodococcus* NY05 were screened for their ability to oxidize 2,4'-CB at the *ortho*-substituted ring. The yellow precipitate (HOPDA) is indicative of enzymatic attack occurring at the *para*-substituted 4-Cl ring of 2,4'-CB and is considered to be a negative result.

This is contrary to results observed in previous assays conducted in this study with 2,4'-CB (data not reported), the resting cell assay #1 of this study (see below), and reports by Maltseva *et al.* (1999). However, when analyzed spectrophotometrically the supernatant from the NY05 culture had a maximum wavelength (λ_{max}) of 396 nm which is indicative of 3,8-dichloro-HOPDA, the intermediate formed from an attack on the 4-Cl ring. From the rapid assay it can be inferred that all seven isolated strains have a KF707-like degradation profile. This was also confirmed by performing a resting cell assay supplemented with 2,4'-CB (see below; Fig. 11).

Resting Cell Assay Version #1

Seven of the isolated bacterial strains (D1, D4, P2, P3, T1, T2A, and T2B) chosen for their ability to readily degrade biphenyl, and the reference strain NY05 were screened as resting cells to characterize their PCB degrading ability and congener specificity. All cultures used in the assay were synchronized to reach late log phase concurrently. This was made possible based on data generated by the growth curves (Fig. 8). Three replicates originating from the same preculture were tested for each strain. The assay utilized a mix of congeners (1B and 2B) representative of those found in commercial mixtures of Aroclor 1242. Table 4 shows the results of the resting cell assay as determined by GC analysis. The congener mixes 1B and 2B were grouped according to available (open) active sites for analysis. It should be noted that two adjacent unsubstituted carbons are required for the initial dioxygenase attack to occur, and that 5,6-sites are equivalent to 2,3-sites, while 4,5-sites are equivalent to 3,4-sites. In order to simplify the analysis, 5,6-sites are referred to as 2,3-sites, while 4,5-sites are referred to as 3,4-sites. Congeners with open 2,3- and 3,4-sites are evaluated first, followed by congeners with open 2,3-sites only, open 3,4-sites only, and congeners with both sites closed.

All strains tested readily degraded the di-substituted congeners with open 2,3- and 3,4-sites except for the di-*ortho* substituted congener 2,2'-CB. Strains D4 and D1, however, oxidized 68% and 100% of 2,2'-CB, respectively. All of the strains tested were less efficient at oxidizing the tri-substituted congeners of this group including

2,5,4'-CB (two open 2,3-sites and one open 3,4-site), which had degradation rates ranging from 20 to 75%, and 2,5,2'-CB (one open 2,3-site and two open 3,4-sites), which had degradation rates ranging from 3 to 95%. It is noteworthy that strain D1 oxidized 75% and 95% of 2,5,4'-CB and 2,5,2'-CB, respectively. All strains tested encountered difficulties mounting a dioxygenase attack on the tetra-substituted congeners of this group, except for 2,3,2',3'-CB (two open 2,3- and 3,4- sites). Four out of seven of the isolated strains were able to oxidize approximately 50% of this compound, while the reference strain NY05 was able to degrade 86% of 2,3,2',3'-CB. Strains D1 and NY05 were also able to oxidize 51% and 56% of 2,3,2',5'-CB, respectively. The penta-substituted congeners 2,3,4,2',5'-CB and 2,4,5,2',3'-CB (both have one open 2,3- and 3,4,-site) proved to be the most resistant to oxidation in this group with only negligible to nondetectable rates of degradation noted by all strains tested except for D1 and NY05 which oxidized 21% and 65% of 2,4,5,2',3'-CB, respectively.

All strains tested readily degraded the di-*para*-substituted congener 4,4'-CB and the tri-substituted congener 2,4,4'-CB with four and three open 2,3-sites, respectively. All tetra-substituted congeners (two open 2,3-sites) in this group were found to be highly resistant to attack. Four out of the seven isolated strains oxidized approximately 35% of 2,4,3',4'-CB, while NY05 degraded 68% of this congener. None of the strains tested could significantly oxidize 3,4,3',4'-CB except for strain P3 which degraded 22% of this congener. None of the strains tested were able to significantly degrade 2,4,2',4'-CB, which proved to be highly recalcitrant.

The tetra-substituted congener 2,5,2',5'-CB, with no open 2,3-sites, was found to be highly resistant to oxidation. This particular congener is used to test a strain's ability to mount a 3,4-dioxygenase attack. Only strains D1 and P3 were able to oxidize this compound, but with rates of only 21% and 20%, respectively. None of the strains tested were able to substantially oxidize the penta-substituted congener 2,4,5,2',5'-CB (one open 3,4-site), or the hexa-substituted congener 2,4,5,2',4',5'-CB (all sites closed).

All strains tested with congener mix 1B readily accumulated HOPDA which persisted throughout the experiment. This particular mix contains the congeners 2,4'-CB and 2,4,4'-CB, among others. It is not certain whether the accumulation of HOPDA was due to the degradation 2,4'-CB and 2,4,4'-CB or other congeners contained in the mix. Conversely, all strains tested with congener mix 2B did not accumulate HOPDA during the course of the experiment. There was no oxidation observed by the dead cell controls. In summary, the isolated strains and the reference strain's ability to degrade congener mixes 1B and 2B, ranked from strongest to weakest, are NY05 = D1 > P3 > D4 > T2A = T2B > T1 > P2 (Table 4).

Table 4

Results of the Resting Cell Assays for PCB Degradation. PCB metabolizing activities of Kalamazoo River isolates and the reference strain NY05 as determined by gas chromatography analysis.

PCB CONGENERS	BACTERIAL STRAINS							
Open 2,3- and 3,4-sites	**P2**	**T1**	**T2B**	**T2A**	**D4**	**P3**	**D1**	**NY05**
2,3-CB	++++	++++	++++	++++	++++	++++	++++	++++
2,4'-CB	++++	++++	++++	++++	++++	++++	++++	++++
2,2'-CB	+	-	++	++	+++	++	++++	+
2,5,4'-CB	-	++	++	+	++	+	+++	++
2,5,2'-CB	-	-	-	+	+	+	++++	+
2,3,2',3'-CB	-	-	++	++	++	++	+	++++
2,3,2',5'-CB	-	-	-	-	-	+	++	++
2,5,3',4'-CB	-	-	-	-	-	-	+	+
2,3,4,2',5'-CB	-	-	-	-	-	-	-	-
2,4,5,2',3'-CB	-	-	-	-	-	-	+	+++
2,6-CB*	-	-	-	-	-	-	-	-
Open 2,3-sites								
4,4'-CB	++++	++++	++++	++++	++++	++++	++++	++++
2,4,4'-CB	++++	+++	++++	++++	++++	++++	+++	++++
2,4,3',4'-CB	-	-	+	+	+	+	+	+++
2,4,2',4'-CB	-	-	-	-	-	-	-	-
3,4,3',4'-CB	-	-	-	-	-	+	-	-
Open 3,4-sites								
2,5,2',5'-CB	-	-	-	-	-	+	+	-
2,4,5,2',5'-CB	-	-	-	-	-	-	-	-
Closed 2,3- and 3,4-sites								
2,4,5,2',4',5'-CB	-	-	-	-	-	-	-	-

The data reported were obtained from resting cell cultures after 24 hrs of incubation, and are represented as ranges of percentage of depletion: ++++ = 80 – 100%, +++ = 60 – 79%, ++ = 40 – 59%, + = 20 – 39%, - = 0 – 19%. *Resting cell assay version #2.

Resting Cell Assay Version #2

The di-*ortho*-substituted congener 2,6-CB is known to accumulate during the reductive dechlorination process of the sequential anaerobic-aerobic treatment scheme. This particular congener is also highly resistant to attack and degradation by aerobic strains. As a result, seven of the Kalamazoo River isolates and the reference strain NY05 were tested as resting cells for their ability to mount an attack on 2,6-CB. Not unexpectedly, all strains tested were unable to significantly degrade 2,6-CB (Table 4).

Determining the Site of Initial Dioxygenase Attack on 2,4'-CB

In order to more accurately determine the site of attack on 2,4'-CB by the *bphA* encoded 2,3-dioxygenase, seven of the isolated strains (D1, D4, P2, P3, T1, T2A, and T2B) and the reference strain NY05 were tested as resting cells for their ability to oxidize 2,4'-CB. The site of attack can be determined by visible spectral scanning of the intermediate catabolite HOPDA (Fig. 10A). HOPDA and chlorinated HOPDAs have characteristic absorbance spectra or maximum wavelength (λ_{max}) values. Seeger *et al.* (1995) found that a strict correlation exists between the presence of an *ortho*-chlorinated HOPDA and a low λ_{max} (390-400 nm) and a non- *ortho*-chlorinated HOPDA and a high λ_{max} (430-440 nm). Attack on the *para*-substituted ring of 2,4'-CB results in the formation of the *ortho*-chlorinated 3,8-dichloro-HOPDA (Fig. 10C). Maltseva *et al.* (1999) had previously determined the λ_{max} for 3,8-dichloro-HOPDA to be 396 nm, which is considered low and indicative of *ortho*-chlorinated HOPDAs. Attack on the *ortho*-substituted ring of 2,4'-CB results in the

formation of 5,10-dichloro-HOPDA with a λ_{max} of 434 nm. However, if dehalogenation occurs at the *ortho*-position, as is the case with *Burkholderia* LB400 and *Rhodococcus* RHA1, the net result would be the production of 10-chloro-HOPDA with a λ_{max} of 438 nm (Seah *et al.*, 2000; Maltseva *et al.*, 1999).

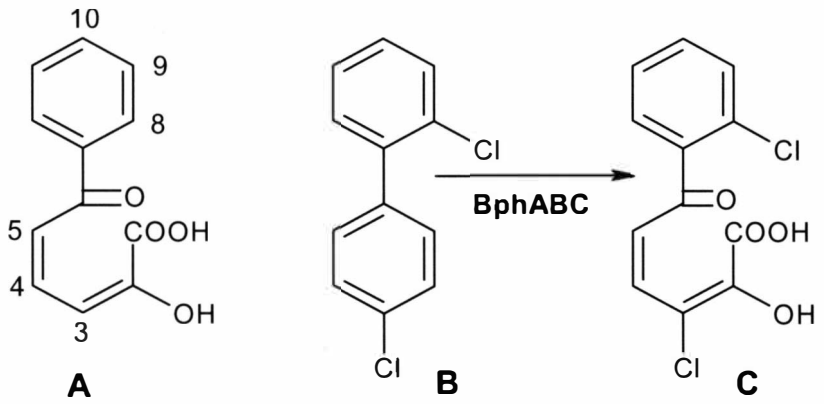

Figure 10. HOPDA Structure and Nomenclature. The structure and numbering system for the *meta*-cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA). Numbering begins on the cleaved ring and continues counterclockwise (A). The congener 2,4'-CB (B) becomes 3,8-dichloro-HOPDA when an enzymatic attack occurs at the *para*-substituted ring (C).

Absorption was measured at wavelengths of 360, 370, 380, 396, 400, 410, 420, and 434 nm on supernatants obtained from resting cell cultures following amendment with of 2,4'-CB and a 12 hr incubation period. Maximum absorption was recorded at 396 nm (Fig. 11) which is indicative of attack on the *para*-substituted ring (Fig. 10C) and a KF707-like degradation profile. Problematic *meta*-cleavage products accumulated heavily by 24 hours, and even though there was visible evidence of

diminished HOPDA concentrations by most strains within 96 hours, it still remained highly persistent (Fig. 11). Again, strain NY05 did not accumulate visually detectable amounts of HOPDA in contrast to expectations. The most prolific producers of HOPDA from weakest to strongest as determined by UV spectral analysis are NYO5 < D1 < T2B < D4 < T2A < T1 < P2 < P3.

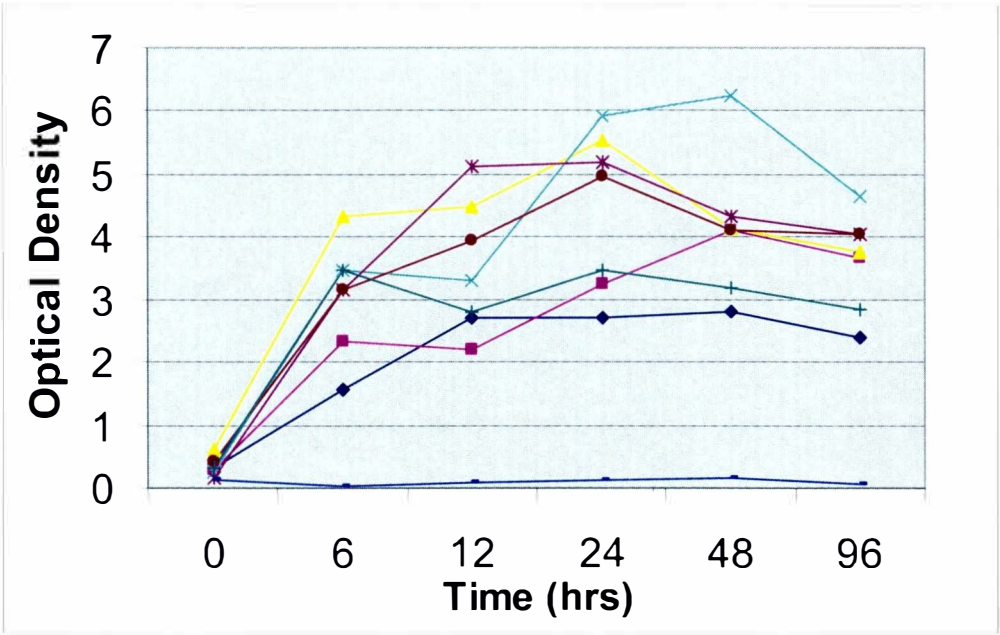

Figure 11. Graphical Representation of the 2,4'-CB Assay (optical density measured at 396 nm). Graph depicts the accumulation of the problematic *meta*-cleavage product HOPDA by seven Kalamazoo River isolates and reference strain *Rhodococcus* NY05 during an incubation period of 96 hr. Strains tested included D1 (dark blue diamonds), D4 (pink squares), P2 (yellow triangles), P3 (light blue X's), T1 (purple X's), T2A (maroon circles), T2B (green crosses), and NY05 (blue rectangles).

CHAPTER V

DISCUSSION

This study accomplished four objectives: 1) PCB concentrations in Kalamazoo River sediments were quantitated, 2) the particular class of Aroclors present in sediments was determined, 3) the reductive dechlorination potential by anaerobic microorganisms was evaluated, and 4) seven novel PCB-degrading-bacterial strains were isolated, identified and characterized in regards to their PCB degrading abilities.

Sediment depths in the Kalamazoo River range from a few inches to approximately 20 feet, with the deeper sediments accumulating higher concentrations of PCBs over time (Blasland *et al.*, 2000). The majority of the core samples taken in this study contained approximately 10 cm (4 in) of superficial silt with the exception of the Trowbridge Dam, D Ave, and Ceresco Reservoir samples which consisted entirely of silt. Not unexpectedly, only two sediment samples (Trowbridge Dam and D Ave) out of eleven showed significant concentrations of PCBs. This was probably due to insufficient concentrations of organic carbon, which PCBs are known to adsorb to, in eight of the nine remaining samples. Low concentrations of PCBs were anticipated from the Ceresco Reservoir sample (background control) because this site has no record of PCB discharges. PCBs are not evenly dispersed throughout Kalamazoo River sediments. Instead, they converged in the slower moving reaches of the river which are known to accumulate silt and are referred to as "hot spots". The

Kalamazoo River has no true "hot spots"; however, focused sampling of river sediments by Blasland *et al.* (2000) resulted in PCB concentrations ranging from 160 ppm to nondetectable levels with an average of 6.9 ppm. The spatial distribution of PCBs was also found to be highly variable over short horizontal distances. Samples collected approximately 30 ft apart had concentrations varying by a factor of 12 (Blasland *et al.*, 2000). This study detected no PCBs in Lake Allegan sediments even though they harbor 70% of the total mass of PCBs in the river system. This was most likely a result of the sampling technique used which was conducted around the edges (shallows) of the lake. Unlike the river, where sediments accumulate along the edges, larger bodies of water like Lake Allegan have negligible currents and tend to accumulate sediments in the deeper regions toward the middle (Blasland *et al.*, 2000). The sampling probe used in this study was not long enough to take samples from the middle of the lake. The two samples taken from Lake Allegan contained approximately 10 cm of superficial silt, while the deeper regions consisted primarily of sand followed by gravel. Blasland *et al.* (2000) determined that coarse Kalamazoo River sediments harbor far less PCBs than fine silt due to a lack of organic carbon.

GC analysis performed in this study of PCB extracts taken from Trowbridge Dam and D Avenue sediments revealed concentrations of 20.5 and 12.5 ppm, respectively. Blasland *et al.* (2000) have detected only four samples over 100 ppm out of 2,428 random samples, 96% of which had concentrations below 10 ppm. PCBs in this study were extracted from the top 20 cm of each sample. If the sediments would have come from deeper within the cores of the Trowbridge Dam, D Ave, and Ceresco

Reservoir samples (all silt) higher concentrations of PCBs may have been detected. Blasland *et al.* (2000) have detected the greatest concentrations of PCBs at depths ranging from 5 to 90 cm.

The chromatographic profile generated from environmental PCBs extracted from Trowbridge Dam sediments (Figure 5B) most resembled that of Aroclor 1242 (Figure 5A), while the profile generated from extracts taken from D Avenue sediments (Figure 6B) most resembled that of Aroclor 1248 (Figure 6A). The detection of Aroclor 1248 in D Avenue sediments was not unexpected since Aroclors other than 1242 were known to have been released both up- and downstream of the paper recycling plants. Blasland *et al.* (2000) have detected Aroclor 1248 in both fish and sediments throughout the river.

One of the goals of this study was to explore the potential for sequential anaerobic-aerobic treatment as a theoretical remediation option for the Kalamazoo River system, most notably PCB degradation by aerobic bacteria. Therefore, our technique involved extracting PCBs from the top 20 cm of each sediment sample, which included both anaerobic and aerobic zones. This allowed each sample to be analyzed for signs of reductive dechlorination, and to be used as an inoculum for the culturing of aerobic bacteria. This study found little evidence of reductive dechlorination by anaerobic microorganisms as indicated by chromatograms. The lack of a dechlorination signature is not unusual at such low PCB concentrations because it takes concentrations in the hundreds of ppm to select for dechlorinating microorganisms (John F. Quensen III, MSU, personal communication). However, GC

analysis of anaerobic microcosms inoculated with microorganisms extracted from Kalamazoo River sediments showed dehalogenation of Aroclor 1242, predominantly from the *meta*-positions (John F. Quensen III, MSU, unpublished data). Therefore, PCB dechlorinating microorganisms have been shown to be present in Kalamazoo River sediments but were not very active, which could be the result of low PCB concentrations.

Blasland *et al.* (2000), however, have reported signs of reductive dechlorination in Lake Allegan sediments (30 – 60 cm), albeit subtle, and Otsego City Impoundment sediments (30 – 60 cm). This was evidenced by increases in chromatographic peaks corresponding to 2,4'-CB and 2,4,3'-CB which are normally small components of Aroclor 1242. The heightened peaks can be attributed to the *meta*-dechlorination of more highly chlorinated congeners by anaerobic microorganisms. This, in some ways, is unfortunate in that all the strains isolated in this study accumulated problematic *meta*-cleavage products when amended with 2,4'-CB.

This study resulted in the isolation of ten biphenyl degrading bacterial strains, seven of which were selected to undergo metabolic testing with a select group of PCB congeners. It was successfully demonstrated that all isolated strains tested preferentially attacked the *para*-substituted ring of 2,4'-CB as evidenced by the accumulation of HOPDA (Fig. 9 and 11). It is not certain why the reference strain *Rhodococcus* NY05 failed to accumulate the problematic *meta*-cleavage product when its presence was observed in resting cell assay #1 of this study, and reported by

others (Maltseva *et al.*, 1999). Strain NY05 may have been more efficient at degrading 2,4'-CB than the other strains tested in this study, but this did not account for the almost complete lack of HOPDA in NY05 cultures. As previously mentioned, certain species of *Rhodococcus* have been found to harbor multiple copies of *bphC*, some of which were located on linear plasmids (Taguchi *et al.*, 2004). A possible explanation for the absence of HOPDA could be that strain NY05 may have lost the plasmid on which *bphC* genes may have resided, because it is at this point in the degradation pathway of 2,4'-CB that HOPDA is known to accumulate (HOPDA is the product of BphC).

Nearly all PCB degrading bacterial strains required unsubstituted 2,3-sites to mount an initial *ortho-meta* dioxygenase attack (Furukawa *et al.*, 1979). There were, however, exceptions. *Burkholderia* LB400, *Alcaligenes* H850, and *Rhodococcus* RHA1 were all capable of carrying out dehalogenation from *ortho*-positions, which means they only required an open *meta*-site to initiate an *ortho-meta* dioxygenase attack. These strains were also capable of mounting a *meta-para* attack but required open 3,4-sites to do so (Bopp, 1986; Bedard *et al.*, 1987; Maltseva *et al.*, 1999). The congeners in mixes utilized in this study (1B and 2B) were representative of structural classes of PCBs found in commercial mixtures of Aroclor 1242 (Fig. 12). The recalcitrant congeners 2,4'-CB and 2,6-CB were tested individually in an attempt to discover strains capable of resolving some of the "bottlenecks" encountered in the aerobic degradation scheme. Not unexpectedly, none of the bacterial strains isolated from river sediments were able to mount an initial dioxygenase attack on 2,6-CB, in

fact, none have been discovered to date at all (Furukawa *et al.*, 1979; Maltseva *et al.*, 1999; Kim and Picardal, 2001). Equally disappointing was the fact that the bacterial strains tested were unable to mount an attack on the *ortho*-substituted ring of 2,4'-CB which would result in the production of CBAs rather than in the accumulation of HOPDAs. PCB degrading bacteria have been extensively studied and characterized over the past three decades (Furukawa and Matsumura, 1976; Furukawa *et al.*, 1979; Bedard *et al.*, 1986a; Bedard and Haberl, 1990) making the possibility of discovering a strain with novel PCB congener specificity and degrading abilities unlikely.

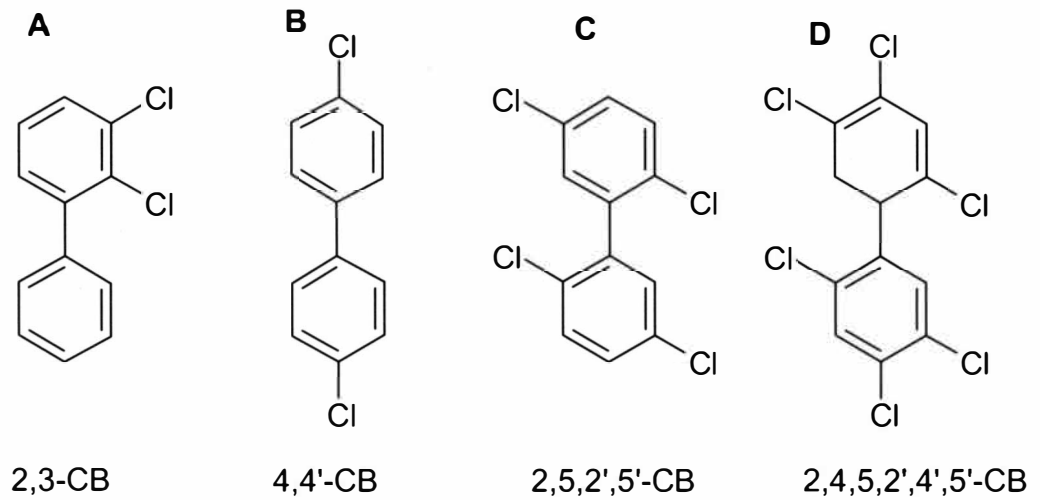

Figure 12. Structural Classes of PCB Congeners. Structures A through D show examples of congeners that represent different structural challenges for PCB-degradative enzymes. Included are congeners with a single ring substitution with open 2,3- and 3,4-sites (A), open 2,3-sites only (B), open 3,4-sites only (C), and both sites closed (D).

The mixes tested in this study included congeners with a single ring substitution (Fig.12A), open 2,3-sites (Fig. 12B), open 3,4-sites (Fig. 12C), open 2,3- and 3,4-sites (Fig. 12A), and both sites closed (Fig. 12D). The PCB congeners were formulated into two mixes due to a tendency of certain congeners to coelute during GC analysis as previously mentioned. When analyzed by gas chromatography the resting cell assay was limited to detecting the bacterial strain's ability to mount an initial dioxygenase attack, and not the complete degradation of the PCB congener. Further spectrophotometric analysis would be required to determine the site of attack (regiospecificity), and analysis by high performance liquid chromatography (HPLC) would be required to determine the extent of degradation sustained which was beyond the scope of this study.

The fact that all strains tested oxidized 80 to 100% of the di-*para* substituted 4,4'-CB, and most of the strains tested were unable to mount an attack on the di-*ortho* substituted 2,2'-CB strongly suggests that the majority of the isolated strains had a KF707-like degradation profile. This is in good agreement with the results obtained from the 2,4'-CB assay. Strains D1 and D4, however, exhibited both LB400 and KF707-like profiles by substantially oxidizing both the di-*ortho* substituted (2,2'-CB) and the di-*para* substituted (4,4'-CB) congeners. Strains D1 and P3 demonstrated 3,4-dioxygenase activity by degrading 21% and 20% of 2,5,2',5'-CB, respectively. It is noteworthy that D1 was able to oxidize 2,5,2',5'-CB (21%), 2,5,2'-CB (95%) and 2,5,4'-CB (75%), which strongly suggests that this particular strain has 3,4-dioxygenase activity and the ability to mount a *meta-para* attack. Upon examination

of the degradation profiles it appears that the preferred mode of oxidation by the all of the isolated strains is via the 2,3-dioxygenase pathway (*ortho-meta* attack), the range of congeners degraded is limited to mono- to tri-substituted, with a couple of minor exceptions (D1 and P3 demonstrated 3,4-dioxygenase activity by significantly degrading 2,5,2',5'-CB). This is also highly characteristic of strains with KF707-like degradation profiles.

Strain D1 was determined to be the most potent PCB degrader isolated from river sediments, and was found to be about as strong of a degrader as strain NY05, in this study (Table 5). *Rhodococcus* NY05 is considered to be an above average degrader of PCBs (Olga V. Maltseva, WMU, personal communication) (Pellizari *et al.*, 1996; Maltseva *et al.*, 1999). These results indicated that most of the isolated strains tested were moderate to average PCB degraders when compared to previously characterized strains (Furukawa and Matsumura, 1976; Furukawa *et al.*, 1979; Bedard *et* al., 1986a; Bedard and Haberl, 1990; Pellizari *et al.*, 1996; Maltseva *et al.*, 1999).

CHAPTER VI

CONCLUSION

All conclusions made in this study were based on the analysis of eleven sediment samples taken from different locations along the Kalamazoo River. It should be noted that this is too few samples to be considered representative of the study site, and for making practical assessments regarding remediation strategies. However, Blasland *et al.* (2000) have clearly demonstrated that the Kalamazoo River system is moderately contaminated with PCBs when compared to sites like the Hudson River (Harkness *et al.*, 1993), and has three significant sources of ongoing PCB loading (the MDNR former impoundments). This study also detected significant concentrations of PCBs in some of the Kalamazoo River sediments tested. As previously mentioned, Blasland's *et al.* (2000) proposed remediation strategy included plans for the containment of PCBs through bank stabilization in critical areas, and the monitoring of natural attenuation processes, most notably the physical process of burial. Their data indicates that physical processes have reduced the bioavailability of PCBs to benthic feeders, fish and other wildlife over the last two decades (Blasland's *et al.*, 2000).

Blasland *et al.* (2000) discounted sequential anaerobic-aerobic treatments as a practical means of remediation due to minimal anaerobic dehalogenating activity, even though PCB dechlorinating anaerobic microorganisms were extracted from river

sediments. This study also failed to detect any significant signs of dechlorinating activity by anaerobic microorganisms as evidenced by gas chromatograms. As previously mentioned, PCB dehalogenating microorganisms require PCB concentrations of several hundred ppm to be selected for. Blasland *et al.* (2000) determined that the Kalamazoo River has no true "hotspots" and as a result minimal anaerobic dehalogenating activity.

The company Envirogen demonstrated that aerobic degradation by bacteria indigenous to Kalamazoo River sediments reduced PCB concentrations 35% when amended with nutrients alone, and 70% when amended with nutrients, biphenyl, and their proprietary PCB-degrading bacteria (Blasland *et al.*, 2000). A 35% reduction is a significant loss. The indigenous PCB degraders isolated in this study were determined to have a *Pseudomonas* KF707-like degradation profile, which means they could degrade a narrow range of congeners with up to four substitutions, including di-*para*-substituted congeners. The addition of nutrients and perhaps bioaugmentation with potent PCB degrading strains with complementary degradation profiles may be a potential remediation option for the Kalamazoo River. Evans *et al.* (1996) found aerobic degradation to be almost as effective as sequential bioremediation in laboratory bench scale models.

At least three major obstacles must be overcome in order for the aerobic degradation of PCBs to become a practical means of remediation: 1) the inability of any known strain to substantially degrade 2,6-CB, 2) the accumulation of toxic intermediate metabolites (HOPDA) by some strains, and 3) the inability of many

strains to degrade the second aromatic ring resulting in the accumulation of chlorobenzoates (Maltseva *et al.*, 1999). There is hope, however, in the form of genetic engineering. Family gene shuffling of the *bphA* region that determined specificity was utilized to evolve novel biphenyl dioxgenases. Hybrids were made by shuffling *bphA* gene segments from species of *Burkholderia*, *Commamonas,* and *Rhodococcus*. The hybrids exhibited enhanced dioxygenase activity towards 2,2'-CB, 3,3'-CB, 4,4'-CB, and most notably 2,6-CB (Barriault *et al.*, 2002). Also, two aromatic-ring-dehalogenase genes (oxygenolytic and hydrolytic) were individually cloned into a species of *Comamonas*. This resulted in two recombinant strains with the ability to utilize the CBAs generated from the degradation of *ortho-* and *para-*CBs. Together the two recombinant strains grew on, and dehalogenated, high concentrations of 2-CB, 4-CB, 2-CBA and 4-CBA. Net protein yields corresponded to complete oxidation of both rings (Hrywna *et al.*, 1999).

Some problems specific to the Kalamazoo River in regards to aerobic degradation include the persistence of the di-*ortho* substituted congener 2,6-CB which was not significantly degraded by any of the strains tested in this study. Also, when liquid cultures were amended with 2,4'-CB all of the isolated strains tested accumulated problematic *meta*-cleavage products (HOPDA). Blasland *et al.* (2000) determined that the concentrations of 2,4'-CB are increasing in the river system due to the preferred *meta*-dechlorination of more highly chlorinated congeners by anaerobic microorganisms. All isolated strains tested in this study oxidized the 4'-Cl ring of 2,4'-CB resulting in the formation of 3,8-dichloro-HOPDA, which is known to inhibit

the *bphD* gene product HOPDA hydrolase (Fig. 4 and 10), which in turn, inhibits the degradation of all congeners (Seah *et al.*, 1998, 2000).

Based on the facts presented above PCB remediation by sequential anaerobic-aerobic treatment may not be the most practical option at the present time. This is primarily due to minimal dechlorinating activity by anaerobic microorganisms. The addition of nutrients and even bioaugmentation with select aerobic-PCB-degrading bacteria as a single treatment appears to be promising. However, continued research is warranted in order to fine-tune the aerobic degradation process. Currently, the most promising avenue appears to be the construction of genetically modified (GM) microorganisms. However, the scientific community is reluctant to release GM bacteria into the environment for fear of unregulated horizontal gene transfer.

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