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# Genetic analyses of microbial polychlorinated biphenyl degradation in natural and engineered systems

Yi Liang
*University of Iowa*

# GENETIC ANALYSES OF MICROBIAL POLYCHLORINATED BIPHENYL DEGRADATION IN NATURAL AND ENGINEERED SYSTEMS

by

Yi Liang

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Thesis Supervisor: Associate Professor Timothy E. Mattes

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

Yi Liang

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Civil and Environmental Engineering at the May 2014 graduation.

Thesis Committee: _____
Timothy E. Mattes, Thesis Supervisor

_____
Keri Hornbuckle

_____
Larry Robertson

_____
Tonya Peeples

_____
Jerald Schnoor

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ABSTRACT

Polychlorinated biphenyls (PCBs) are carcinogenic, persistent, and bioaccumulative contaminants that pose risks to human and environmental health. PCB biodegradation by indigenous microbial communities could be a cost-effective and an environmental-friendly bioremediation strategy for *in situ* PCB removal. A comprehensive understanding of the microbial PCB degradation at the contaminated site is required for the acceptance and optimization of using microbial PCB degradation as the site clean-up strategy. This thesis describes investigations of the aerobic and anaerobic microbial degradation of PCBs under both field and laboratory conditions.

The microbial PCB degradation potential in sediments from Indiana Harbor and Ship Canal (IHSC), a site that was historically contaminated by PCBs, was explored by analyzing the PCB congener distributions and microbial communities in two core sediment samples. PCB congener analysis suggested the possibility of *in situ* dechlorination in deep sediments. Molecular analysis of biomarker genes revealed the potential of both aerobic and anaerobic PCB degradation in sediments. Microbial communities were characterized by the combination use of terminal restriction fragment length polymorphism (T-RFLP), clone library, and pyrosequencing. These methods elucidated the dominant role of *Proteobacteria,* especially *Acidovorax* and *Acinetobacter* in sediments.

To improve the microbial PCB degradation, phytoremediation with switchgrass (*Panicum vigratum*) was employed under laboratory conditions. Congener analysis showed that both phytoextraction and microbial PCB degradation contributed to the enhanced PCB removal in the presence of switchgrass. Bioaugmentation with *Burkholderia xenovorans* LB400 was performed to further promote aerobic PCB degradation. The presence of LB400 was associated with improved degradation of PCB 52, but not PCB77 or PCB 153. Increased abundance of the biphenyl dioxygenase gene,

which is indicative of aerobic PCB degradation, and its transcript were observed after bioaugmentation, suggesting active aerobic PCB degradation.

To promote the anaerobic PCB degradation, redox cycling (alternating flooding and non-flooding) was performed. Redox cycling was found to improve the removal of PCB 153 in unplanted soils and to increase the dechlorinating *Chloroflexi* population. Characterization of the microbial community by T-RFLP and clone library revealed that *Proteobacteria* and *Acidobacteria* were dominant. Species that contain dechlorination potential were identified, including *Geobacter* and *Clostridium*, suggesting that their possible role in PCB dechlorination.

The research described in this thesis provides scientific knowledge and evidence for the feasibility of employing bioremediation including natural attenuation, phytoremediation, and bioaugmentation to clean up PCB contamination. Such information will be critical in selecting and optimizing remediation strategies for PCB contaminated sites.

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Polychlorinated biphenyls (PCBs) are a group of 209 synthetic chemicals that were widely used in industry during the last century and are still in use in closed application today. They pose a threat to environmental and public health because they are persistent, bioaccumulative and carcinogenic. Microbial PCB degradation could be a feasible way to detoxify and remove PCBs in the environment. The main objective of this work was to investigate the microbial PCB biodegradation in the contaminated soils and sediments.

The first part of this work explored the microbial PCB degradation potential in sediments from Indiana Harbor and Ship Canal (IHSC), a site that was historically contaminated by PCBs, by analyzing the PCB congener distributions and microbial communities from two core sediment samples (Chapter III and Chapter IV). The following hypothesis was tested:

*Hypothesis 1*: IHSC sediments contain the potential for aerobic and anaerobic microbial PCB biodegradation.

To test this hypothesis, the specific aims are to:

1. Evaluate the PCB dechlorination potential by sediment congener profile analysis.

2. Enumerate biomarker genes (biphenyl dioxygenase genes and putative dechlorinating *Chloroflexi* 16S rRNA genes) by qPCR.

3. Identify possible PCB degrading populations by T-RFLP, clone library and pyrosequenicng of bacterial 16S rRNA gene.

While the field studies provided valuable information about *in situ* microbial PCB degradation, the lack of knowledge of the complex environment conditions at the contaminated site usually makes it challenging to explain the microbial activities. To better understand microbial PCB biodegradation and to examine how PCB degradation can be improved, soil microcosms spiked with PCB 52, 77 and 153 were investigated under laboratory conditions. Phytoremediation with switchgrass (*Panicum vigratum*) was employed to improve PCB removal efficiency and PCB degrading bacteria *Burkholderia xenovorans* LB400 was introduced into the switchgrass planted rhizosphere to further enhance the aerobic PCB degradation (Chapter V). The following hypotheses were tested:

*Hypothesis 2:* The microbial PCB degradation in soil is improved in the presence of switchgrass (*Panicum virgatum).*

*Hypothesis 3:* Bioaugmentation with biphenyl-grown *Burkholderia xenovorans* LB400 improves the aerobic microbial PCB biodegradation in the switchgrass rhizosphere as compared with the rhizosphere without bioaugmentation treatment.

For these hypotheses, the specific aims are to:

1. Evaluate the PCB removal efficiency by measuring PCB concentrations in unplanted, switchgrass treated, and *Burkholderia xenovorans* LB400 bioaugmented soils.

2. Evaluate the abundance of biphenyl dioxygenase genes and transcripts in unplanted, switchgrass treated, and *Burkholderia xenovorans* LB400 bioaugmented soils.

To enhance the anaerobic microbial PCB degradation, a sequential anaerobic-aerobic environment was created by redox cycling (two weeks of flooding and two weeks of non-flooding) in PCB-spiked soil microcosms (Chapter VI). The following hypothesis was tested:

*Hypothesis* 4: Redox cycling improves anaerobic microbial PCB biodegradation in the switchgrass rhizosphere.

The hypothesis was addressed with the following specific aims:

1. Evaluate the transformation products of PCBs in unplanted, switchgrass treated, and redox cycled soils.

2. Evaluate the abundance of dechlorinating *Chloroflexi* 16S rRNA genes in unplanted, switchgrass treated, and redox cycled soils.

3. Evaluate the microbial community structure variation and identify key members associated with PCB degradation in unplanted, switchgrass treated, and redox cycled soils.

# CHAPTER II. BACKGROUND

## Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are a group of 209 synthetic chemicals consisting of 1-10 chlorine atoms on a biphenyl backbone (Figure 2.1) (Gustafson, 1970, Wiegel & Wu, 2000). PCBs were discovered over 100 years ago and were widely used in industry as transformer dielectric fluids, heat transfer fluids, hydraulic fluids, coolants and plasticizers because of their excellent chemical stability and electrical insulating properties (Erickson and Kaley, 2011). It is estimated that about 1.3 million tons of PCBs were produced worldwide between the 1930s and the 1990s, 48% of which was in US. About 30% of the production has entered the environment (Abraham et al., 2002; Breivik et al., 2002a, 2007).

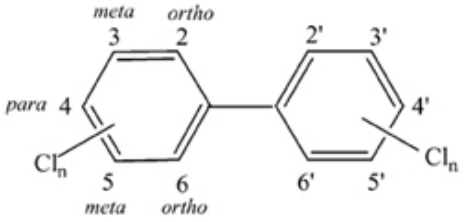


Figure 2.1.Structure formula of PCBs (Wiegel and Wu, 2000).

PCBs are among the 12 worldwide priority persistent organic pollutants (POPs) listed in the Stockholm Convention and are ranked the fifth on the EPA Priority List of Hazardous Compounds (ATSDR, 1994; UNEP, 2001). PCBs has been detected in the atmosphere, water, sediment and soil from urban and rural areas (Jeremiason et al., 1994; Motelay-Massei et al., 2004; Simcik et al., 1997; Zhang et al., 2007) and even from

remote places such as Arctic (Hung *et al.*, 2010). Because of the high hydrophobicity, PCBs, especially highly chlorinated congeners, tend to adsorb to soil and sediment particles. Lightly chlorinated congeners (1-4 chlorine substituents) are more volatile and have long-range atmospheric transport potential (MacDonald *et al.*, 2000). The current total PCB burden in global background soils was estimated to be 21,000 tons (Meijer *et al.*, 2003). The PCB concentrations in contaminated sediments can be as high as several hundred ppm, while the cleanup level is 50 ppm determined by EPA.

The production, processing, distribution and commercial sale of PCBs in US were prohibited by the EPA in 1979 under Toxic Substances Control Act. Nowadays PCBs may be released into the environment by accidental spills, uncontrolled landfills and hazardous waste sites, incineration of PCB-containing wastes, and leakage from old electrical equipment in use (Breivik et al., 2002b). PCBs is resistant to degradation and can accumulate through the food web, posing a potential risk to public health and ecosystem (Campfens and MacKay, 1997). People may be exposed to PCBs mainly by food consumption, including meat, milk and fish, and by inhaling contaminated air (Huwe *et al.*, 2009). Some old building materials such as pigment and caulk contain PCBs which can migrate into air, dust and surrounding material and cause potential exposure (Herrick et al., 2007; Hu and Hornbuckle, 2010).

PCBs are categorized as probably carcinogenic to humans by EPA and as Group I human carcinogen by the International Agency for Research on Cancer (IARC), mainly based on the evidence in animal studies and mechanistic considerations. PCBs are also endocrine disruptors that are capable of interfering with natural hormones in the human body (Grimm *et al.*, 2013). Other adverse health effects include alternation of the immunological system, the neurological system, skin, liver, and the cardiovascular system. PCBs also present a potential reproductive hazard to humans and prenatal exposure to PCBs may result in developmental deficiency in the offspring (Longnecker et al., 1997; Ross, 2004; Van den Berg et al., 2006). The tolerable daily intake (TDI) of

PCB is 1 μg/kg body weight (MinRat-Wirtschaftsministerium et al., 1995). Currently the EPA has established a maximum contaminant level goal of zero and a maximum contaminant level of 0.5 ppb for PCBs in public drinking water supplies (USEPA, 1991).

## Microbial PCB biodegradation

Microorganisms are known to degrade PCBs via two general processes: aerobic oxidative mineralization and anaerobic reductive dechlorination. Aerobic PCB biodegradation is usually restricted to lightly chlorinated congeners (no more than 4 chlorine atoms per molecule), while PCBs with five or more chlorine atoms per molecule are generally resistant to aerobic degradation. Chlorine removal via anaerobic reductive dechlorination processes could make these congeners more amenable to aerobic degradation (Borja *et al.*, 2005).

Under aerobic conditions, the PCB congener biphenyl ring can be opened by an oxidative ring cleavage mechanism (Pieper and Seeger, 2008). A variety of aerobic bacteria that oxidize PCBs in this manner have been identified, including Gram-negative strains of *Pseudomonas, Alcaligenes, Achromobacter, Janibacter, Burkholderia, Acinetobacter, Comamonas, Sphingomonas, Ralstonia,* and *Enterobacter* and Gram-positive strains of *Corynebacterium, Rhodococcus, Bacillus, Paenibacillus, Arthrobacter* and *Micrococcus* (Bedard et al., 1986; Bopp, 1986; Furukawa, 2000; Macková et al., 2010; Pieper and Seeger, 2008).

PCBs can be used as carbon and energy sources by some strains (such as *Burkholderia xenovorans* LB400 and *Burkolderia cepacia* P166) and be degraded by biphenyl-degrading bacteria via co-metabolism (Field and Sierra-Alvarez, 2008; Gibson et al., 1993; Hernandez et al., 1995). Most aerobic PCB-degraders employ an enzyme system called the upper biphenyl degradation pathway. This pathway is catalyzed by a series of enzymes designated BphA, BphB, BphC and BphD. Biphenyl 2,3-dioxygenase

(BphA), a Rieske non-heme iron dioxygenase, catalyzes the incorporation of two oxygen atoms into the aromatic ring to form an arene *cis*-diol (Gibson and Parales, 2000). This initial step in the upper biphenyl pathway transforms susceptible PCB congeners to the corresponding *cis*-dihydrodiol (Furukawa, 1994). Microbes can typically metabolize one of the biphenyl rings to 2-hydroxypenta-2,4-dienoate, which is further degraded to metabolites that can enter the citric acid cycle, a central metabolic pathway. However, this process often leaves behind a chlorobenzoate, which most mediating PCB-degrading microbes cannot degrade further (Pieper and Seeger, 2008). Therefore a population of chlorobenzoate-degraders would be required to achieve mineralization of PCB congeners. Chlorobenzoate-degraders (such as *Pseudomonas. cepacia* and *Pseudomonas. aeruginosa* ) have been previously isolated from PCB-contaminated soil and sediment (Pavlů *et al.*, 1999). PCB-degrading and chlorobenzoate-degrading microorganisms can be genetically modified and thus degrade PCBs without accumulation of chlorobenzoates (Rodrigues et al., 2006; Saavedra et al., 2010).

Biodegradation efficiencies for different PCB congeners vary based on the number and position of the chlorine substituents. PCB congeners with chlorines on one aromatic ring are more easily degraded than those with chlorine substituents on both rings (Pieper, 2005). Highly chlorinated and *ortho*-chlorinated PCB congeners appear to be particularly resistant to biodegradation (Field and Sierra-Alvarez, 2008). Higher chlorination levels result in a highly hydrophobic PCB molecule, a property which reduces their accessibility to enzymes. The bulkiness of the chlorine atoms may also prevent access to the enzyme's active site (Bedard *et al.*, 1986). Chlorine atoms on the *ortho* position may prevent oxygenases from attacking the aromatic ring. It is also reported that *ortho*-chlorinated PCB metabolites strongly inhibit the dioxygenase that catalyzes the third step of the biphenyl degradation pathway, promoting its suicide inactivation and interfering with the degradation of other compounds (Dai *et al.*, 2002). Furthermore, some metabolites of PCB degradation via the upper biphenyl pathway,

including dihydrodiols, dihydroxybiphenyls and chlorinated 2-hydroxy-6-phenyl-6-oxohexa-2,4-dieneoates (HOPDAs), can be very toxic and accumulate in bacteria, adversely affecting cell viability and growth rates even more than the parent PCB congeners (Camara *et al.*, 2004).

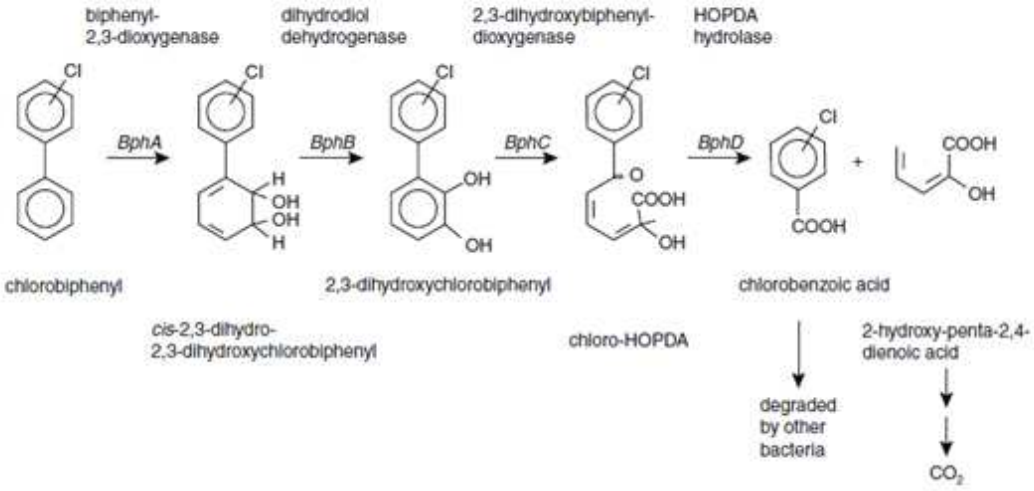


Figure 2.2. Biphenyl pathway of microbial PCB biodegradation under aerobic condition. BphA: biphenyl-2,3-dioxygenase; BphB: *cis*-2,3-dihydroxybiphenyl dehydrogenase; BphC: 2,3-dihydroxybiphenyl 1,2-dioxygenase; BphD: 2-hydroxy-6-phenyl-6-oxohexa-2,4-dieneoate (HOPDA) hydrolase (Pieper and Seeger, 2008).

Under anaerobic conditions, some members of the *Chloroflexi* phylum, including *Dehalococcoides* spp. , and the o-17/DF-1 group, can use certain PCB congeners as electron acceptors and transform them into less chlorinated congeners by reductive dechlorination (Adrian et al., 2009; Cutter et al., 2001; Fagervold et al., 2007; Fagervold et al., 2005; Wiegel and Wu, 2000; Yan et al., 2006). Only several PCB dechlorinating bacteria have been isolated, including *Dehalococcoides maccartyi* strain CBDB1, *Dehalococcoides maccartyi* strain 195 and *Dehalobium chlorocoeria* DF-1 (Adrian et al., 2009; Fennell et al., 2004; May et al., 2008). *Dehalobacter* phylotypes have been found

in an sediment-free culture (KFL culture) which dechlorinated 2,3,4,5-tetrachlorobiphenyl and 2,3,4-trichorobiphenyl where no *Dehalococcoides* or o-17/DF-1 group were detected, which makes *Dehalobacter* a new candidate for anaerobic PCB-degrader (Yoshida *et al.*, 2009).

During microbial PCB dechlorination, chlorines are replaced by hydrogen ions and released as chloride ions, a process catalyzed by membrane-associated reductive dehalogenases (RDases) (Hiraishi, 2008; Holliger et al., 1998; Janssen et al., 2001; Wiegel and Wu, 2000). However, so far no reductive dehalogenase is identified for PCB dechlorination (Sowers and May, 2013). Currently at least eight distinct PCB dechlorination patterns have been identified in sediments and were referred to as M, Q, H', H, P, N, LP and T (Table 2.1) (Wiegel and Wu, 2000). Chlorine atoms at the *meta*- and *para*- positions are preferentially removed in many of these dechlorination patterns, which result in the generation of *ortho*-substituted PCB congeners (Tiedje *et al.*, 1993). Dechlorination of chlorine at *ortho*-position is rare but has been reported (Berkaw et al., 1996; Vandort and Bedard, 1991; Wu et al., 1998). Microbial dechlorination is strongly influenced by various environmental factors such as temperature, pH, carbon sources, and the presence of other electron acceptors (Wiegel and Wu, 2000).

Table 2.1. Positions of chlorines removed by different dechlorination patterns. (Wiegel and Wu, 2000)

| Dechlorination process | Susceptible chlorines |
|---|---|
| M | Flanked and unflanked *meta* |
| Q | Flanked and unflanked *para*, *meta* of 23-group |
| H' | Flanked *para*, *meta* of 23-, and 234-groups |
| H' | Flanked *para*, doubly flanked *meta* |
| P | Flanked *para* |
| N | Flanked *meta* |
| LP | Flanked and unflanked *para* |
| T | Flanked *meta* of 2345-group, in hepta- and octachlorobiphenyls |

## Microbial PCB degradation in sediments and soils

Studies have been conducted to investigate the structure and function of bacterial communities in PCB-contaminated soil and sediment systems. Microcosms are a common approach to investigate both aerobic and anaerobic *in situ* PCB biodegradation potential and PCB degrading communities. Microbial diversity in the surface of an Aroclor 1260-laden sandy soil in Quebec, Canada was analyzed, and sequences of twenty clones were found to be related to low G+C gram-positives, *Cytophaga/Flexibacter/Bacteroides*, and the *α-*, *β-*, *γ-Proteobacteria* (Lloyd-Jones and Lau, 1998). In another study, metabolically active bacteria were identified by 16S rRNA gene clone library analysis in surface samples of a PCB-contaminated sandy soil in Germany. Clone sequences were found to cluster within the *Proteobacteria*, the *Holophage-Acidobacterium* phylum, the *Actinobacteria*, and the *Planctomycetales*. The *β-Proteobacteria* were abundant, mostly the genera *Burkholderia* and *Variovorax*. Sequences similar to known PCB-degraders and other organic pollutant degraders were also identified (Nogales *et al.*, 1999). In a microcosm study using aerobic agitated soil slurries with PCB congener spiking, an increase of *β-Proteobacteria* and *Actinobacteria* was observed in all microcosms exposed to PCBs (Correa *et al.*, 2010). Stable isotope

probing (SIP) was also used to explore the bacterial groups in PCB-contaminated environment. *Burkholderia* species were found dominant and active in aerobic PCB biodegradation in a biofilm community grown directly on PCB droplets, as revealed by DNA-SIP analysis of 16S rRNA (Tillmann *et al.*, 2005). In microcosms containing River Raisin sediment with $^{13}$C-biphenyl addition, genera *Achromobacter*, *Psudomonas*, *Acidovorax*, *Ramlibacter*, *Azoarcus* and *Hydrogenophaga* were detected after 14 days incubation but not at the beginning of incubation. The dominant bacterial groups were found closely related to previously known PCB oxidizers, such as *Achromobacter xylosoxidans* KF701, *Acidovorax* sp strain KKS102, and *Hydrogenophaga taeniospiralis* IA3-A. Analysis of aromatic-ring-hydroxylating dioxygenase genes revealed two sequence groups similar to *bph*A of *Comamonas testosteroni* strain B-356 and *Rhodococcus* sp. RHA1 (Sul *et al.*, 2009). The impact of bioremediation of PCB-contaminated sites on the indigenous microbial community was also studied. Changes in the bacterial community structures were observed, after long-term biostimulation treatment with carvone, soya lecithin and xylose, and bioaugmentation treatments with TSZ7 mixed culture and with a *Rhodococcus* sp Z6 pure strain. *Actinobacteria*, *Bacteriodetes*, α-and γ- *Proteobacteria* were more abundant under all three bioremediation treatments, with *Actinobacteria* representing the dominant phylum (Petrić et al., 2011a).

Experiments were also conducted to describe the anaerobic PCB-degrading communities in PCB-contaminated environment. Microorganisms from sediments of Hudson River, NY, and Silver Lake, MA were found capable of removing *meta*- and *para*-chlorines of four Aroclors (Aroclor 1242, 1248, 1254 and 1260) (Quensen *et al.*, 1990). Similar results were described for enrichment cultures from Housatonic River sediments (Bedard et al., 1996; Bedard et al., 1997). Efforts were also made to characterize the dechlorinating populations. 16S rRNA sequences similar to the theta, low-G+C, Gram-positive, and *Thermotogales* subgroups and one sequence similar to

*Dehalococcoides ethenogenes* were found to be associated with PCB *ortho*-dechlorination in a sediment microbial community from Baltimore Harbor, MD (Holoman *et al.*, 1998). In PCB dechlorinating enrichment cultures that selectively dechlorinated double-flanked chlorines in 2,3,4,5-tetrachlorobiphenyl, a low G+C Gram-positive eubacterium, an organism similar to green non-sulfur bacteria, an *Aminobacterium* sp. and a *Desulfovibrio* sp. were identified (Watts *et al.*, 2001). *Dehalococcoides* and o-17/DF-1 type *Chloroflexi*, *Spirochetes and Bacteroidetes* were identified in enriched Baltimore Harbor sediment microcosms, and a twenty five fold increase of putative dechlorinating *Chloroflexi* members (e.g. *Dehalococcoides* and o-17/DF-1 type *Chloroflexi*) was observed during the dechlorination of Aroclor 1260, which suggests the dechlorination activity of *Chloroflexi* members in Baltimore Harbor sediments (Fagervold et al., 2007; Fagervold et al., 2005; Watts et al., 2005). In another study, active indigenous PCB dechlorinating communities were identified in sediments from three PCB-contaminated sites, Anacostia River, DC, Buffalo River, NY, and Grasse River, NY. Notably, an enriched dechlorinating population consisting exclusively of *Dehalococcoides*-like phylotypes was detected in Grasse River sediment and was found to be associated with a significantly higher dechlorination rate. PCB congener profiles, concentrations, and total organic carbon concentrations (TOC) were found to be associated with *in situ* attenuation rates (Kjellerup *et al.*, 2008). A *Dehalococcoides*-like population was found in three anaerobic cultures capable of reductively dechlorinating 2,3,4,5-tetrachlorobiphenyl but not in PCB-free microcosm. The role of the *Dehalococcoides*-like populations in the removal of 2,3,4,5-tetrachlorobiphenyl was evidenced by their disappearance when dechlorination was inhibited or when PCBs was consumed (Yan *et al.*, 2006). Sequence phylotypes related to the genus *Sulforovum*, and the species *Desulfococcus multivorans* and two *Chloroflexi* were found to be enriched throughout incubation time in PCB-spiked microcosm consisting of marine sediments from Venice Lagoon, suggesting such bacteria were involved in PCB dechlorination in

sediments (Zanaroli *et al.*, 2010). Relatively higher abundance of dechlorinating *Chloroflexi*, *Dehalococcoides*, and o-17/DF-1 populations were observed in deep PCB contaminated sediments (Xu *et al.*, 2012). For the above studies, *Dehalococcoides*-specific 16S rRNA genes were usually targeted for the characterization of dechlorinating microbial population, since no PCB dehalogenase has been identified yet.

Bioaugmentation with both aerobic and anaerobic PCB degraders has been applied to remediate PCB contaminated soils and sediments. Various aerobic PCB degraders, including *Burkholderia xenovorans* LB400, *Ralstonia eutrophus* H850, *Arthrobacter* sp. strain B1B, have been used and achieved significantly greater PCB removal in the bioaugmented soil compared with native soil (Egorova et al., 2013; Luepromchai et al., 2002; Singer et al., 2000). The efficiency of bioaugmentation with dehalorespiring bacteria, such as *Dehalococcoides mccartyi* strain 195 and *Dehalobium chlorocoercia* DF1, were tested in microcosms containing PCB-contaminated sediments, and it was found that bioaugmentation enhanced PCB dechlorination rates (Krumins et al., 2009; Payne et al., 2011). The efficiency of bioaugmentation with both *Dehalobium chlorocoercia* DF1 and *Burkholderia xenovorans* LB400 was also tested and a significantly greater PCB mass loss compared with non-bioaugmented sediments was observed. DF 1 was found to reduce the lag time for PCB degradation, but had no significant effect on the final PCB concentration (Payne *et al.*, 2013).

## Phytoremediation of PCBs

Remediation of PCBs has always been a challenge because of the refractory nature of PCB congeners. Many methods including thermal, oxidative, reductive, microbial approaches have been evaluated to enhance PCB degradation (Gomes *et al.*, 2013). Among those methods, phytoremediation, the use of plants and associated bacteria

to remediate contaminated environment, is more cost effective and sustainable (Van Aken *et al.*, 2010).

Phytoremediation comprises a range of processes including phytoextraction, rhizofiltration, phytotransformation, phytovolatilization, rhizoremediation, and phytostabilization (Dietz and Schnoor, 2001; Schnoor et al., 1995; Van Aken et al., 2010). Plants can uptake PCBs from root systems and translocate them to other plant parts (Macková *et al.*, 2006). Studies of PCB congener fate within whole poplar plants (*Populus deltoids x nigra*) in hydroponic exposures revealed that PCBs initially sorbed to the root system were then translocated to the stem and the woody stem accumulated more PCBs than the shoots (Liu and Schnoor, 2008). The root systems of some plant species such as *Cucurbita pepo* (including pumpkin and zucchini), *Carex normalis*, *Brassica nigra* can accumulate PCBs from soils with bioaccumulation factors (BAFs = PCB concentrations in plant tissues / PCB concentrations in soils) higher than one, while the PCB concentrations in shoots were usually lower than those in the roots (Ficko et al., 2010; Greenwood et al., 2011; White et al., 2006; Zeeb et al., 2006). Shoot BAFs ranged from 0.14 – 0.45 were observed for *Cucurbita pepo* and *Carex normalis* (White et al., 2006; Whitfield Åslund et al., 2007; Zeeb et al., 2006). However, shoot BAFs greater than one were observed for *Polygonum persicarria* and *Vicia cracca* (Ficko *et al.*, 2010), and BAFs of lower parts of plant shoot achieved as high as two were reported for *Cucurbita pepo* (Whitfield Åslund *et al.*, 2008).

PCB metabolism by plants is different from that of microorganisms, and varies among plant species (Van Aken *et al.*, 2010). The substitution pattern and the degree of chlorination of PCB congeners also affect transformation by plants (Macková *et al.*, 2006). PCB transformation in plants was found to result in the formation of hydroxylated PCB metabolites (Liu et al., 2009; Macková et al., 2001; Rezek et al., 2007; Zhai et al., 2010a; Zhai et al., 2010b).

Plant-microbial interactions are known to play important roles in enhancing the degradation of xenobiotics, including PCBs, within the rhizosphere, a dynamic environment for microorganisms, which is defined as the zone of soil under direct influence of plant roots that usually extends a few millimeters from the root surface (Macková *et al.*, 2007). Microorganisms can enhance the plant uptake of certain mineral nutrients and can modify the rhizosphere by producing extracellular enzymes and plant growth factors. Plants play critical roles by supplying organic compounds in exudates that can be utilized by microorganisms as carbon and energy sources and regulating deposits of soil water. Some secondary plant metabolites could induce the genes encoding enzymes involved in the degradation of xenobiotics. Plant released compounds can also solubilize contaminants and make them more available to both plant and microbial degradative enzymes (Leigh et al., 2006; Macková et al., 2009; Singer et al., 2003a).

Several studies have demonstrated that the presence of specific plants enhances PCB biodegradation and increases microbial PCB-degrading populations. Austrian pine (*Pinus nigra*) and goat willow (*Salix caprea*) were found to increase the abundance of microbial PCB-degraders in the root zones (Leigh *et al.*, 2006). Crushed fine roots of felt-leaf willow (*Salix alaxensis*) added to soils spiked with PCB congeners were found to effectively increase PCB biodegradation rate (Slater *et al.*, 2011). A stable isotope probing study (SIP) revealed that seventy-five different biphenyl-degrading genera and twenty seven associated functional genes were active in the root zone of an Austrian pine (*Pinus nigra L.*) grown in PCB-contaminated soil (Leigh *et al.*, 2007). Another SIP study demonstrated that the cultivation of horseradish (*Armoracia rusicana*) may affect the microorganisms that are potentially responsible for PCB degradation (Uhlík et al., 2009). Increased numbers of active PCB-degraders in the rhizosphere of goat willow (*Salix caprea*) and horseradish (*Armoracia rusticana*) have also been observed, indicating these

plant species are promising candidates for rhizoremedation of PCB-contaminated soils (Ionescu *et al.*, 2009).

Some plant-released compounds have the capacity to support the growth of PCB-degraders and thereby improve PCB degradation via co-metabolism (Singer et al., 2003a). For example, flavonoids fostered the growth of PCB degraders (such as *Burkholderia xenovorans* LB400 and *Rhodococcus erythropolis* U23A) and were able to induce the biphenyl catabolic pathway (Donnelly et al., 1994; Leigh et al., 2002; Toussaint et al., 2012). Plant terpenes are also natural substrates that can support PCB oxidation (Hernandez et al., 1997; Park et al., 1999; Tandlich et al., 2001). Transformation of Aroclor 1242 occurred completely in PCB-contaminated soils amended with orange peel, ivy leaves, pine needles, or eucalyptus leaves, but not in the non-amended soil. Also, the abundance of biphenyl-degrading microbes in the amended soils were five orders of magnitude higher than in the non-amended soils, indicating terpenes are used as a natural substrate by PCB degraders (Dudášová et al., 2012; Focht, 1995; Hernandez et al., 1997). In another study, S-carvone, a terpenoid compound, was reported to induce the biotransformation of Aroclor 1242 by *Arthrobacter* sp. strain B1B (Gilbert and Crowley, 1997).

Plant-microbial mutual co-operation within the rhizosphere on the level of the intermediates transformation were also studied. Chlorobenzoates are a common end product of upper biphenyl degradation pathway that usually are not degraded further by aerobic PCB degraders and thus may accumulate to inhibit microbial PCB biodegradation (Pieper and Seeger, 2008). Plant tissues of horseradish and black nightshade were reported to show significant transformation of this bacterial product of PCB degradation (Macková *et al.*, 2007). Similarly, hydroxylated PCBs, the product of PCB degradation by plants, can be transformed by PCB degrading microbes. *Ortho*-chlorinated hydroxybiphenyls were degraded by purified biphenyl dioxygenases from *Burkholderia xenovorans* LB400 and *Comamonas testosteroni* B-356 (Francova *et al.*, 2004). Despite

this, hydroxylated PCBs still appear to exhibit higher toxicity than PCBs to microorganisms (Camara et al., 2004; Purkey et al., 2004).

# CHAPTER III. MICROBIAL PCB DEGRADATION POTENTIAL IN CORE 1 FROM IHSC

## Introduction

Polychlorinated biphenyls (PCBs) are a family of 209 congeners that were produced and used during the last century. They remain a major concern to the public and environmental health because of their high stability and potential toxicity (Bedard, 2004).

Despite their environmental persistence, PCBs can be removed and detoxified by anaerobic and aerobic microorganisms. Microorganisms are known to degrade PCBs via two general processes: anaerobic reductive dechlorination and aerobic oxidative biphenyl ring cleavage (Borja *et al.*, 2005). Under anaerobic conditions, some members of the phylum *Chloroflexi*, including *Dehalococcoides* spp. and the o-17/DF1 group, use certain PCB congeners as electron acceptors and transform them into less chlorinated congeners (Adrian et al., 2009; Wu et al., 2002). Under aerobic conditions, some aerobic PCB-degraders employ the upper biphenyl degradation pathway, and biphenyl 2,3-dioxygenase (BphA) catalyzes the first step in this pathway, transforming susceptible PCB congeners into the corresponding *cis*-dihydrodiol (Furukawa and Fujihara, 2008; Gibson and Parales, 2000).

The Indiana Harbor and Ship Canal (IHSC) in the East Chicago, Indiana, is located on the southern shore of Lake Michigan. IHSC is heavily contaminated with a variety of pollutants, including heavy metals, polycyclic aromatic hydrocarbons (PAHs) and PCBs due to years of industrial operations in the area (Ingersoll et al., 2002; Martinez et al., 2010). It is reported to be a large source of PCBs to Lake Michigan, with the PCB concentration in surficial sediments of IHSC ranging from 53 to 35,000 ng $g^{-1}$ dry weight according to an intensive survey (Martinez *et al.*, 2010). To remain viable for large industrial vessels, a long-term dredging project is carried out by the U.S. Army Corps of Engineers, Chicago District to ensure an adequate navigational depth.

PCB-contaminated IHSC sediments are being dredged and permanently stored in a confined disposal facility (CDF) located north of Lake George Branch (USACE, 2013).

The objective of this study is to assess the *in situ* PCB degradation potential in IHSC sediments. The vertical pattern of a core sediment sample (core 1) was analyzed and suggests the possibility of *in situ* dechlorination. The presence of dechlorinating groups and *bph*A indicates that microbial communities in the sediments had the potential to degrade PCBs.

## Materials and Methods

### Sediment sample collection

In August 2006, surficial sediment samples (Figure 3.1) were collected from IHSC from aboard the U.S. Environmental Protection Agency's research vessel *Mudpuppy*. A standard ponar dredge sampler (top 10 cm layer) was used (Martinez *et al.*, 2010). In May 2009, one 4.57 m core sample (core 1) was collected using a submersible vibro-coring system with a PVC tube (length 457 cm, internal diameter 9.5 cm) (Figure 3.1). To examine microorganisms at different sediment depths, core 1was sectioned every 0.152 m. Samples were placed in plastic bags and kept on ice during transportation. Sediment samples were stored at 4°C in the lab until analysis.

### PCB congener analysis[1]

Preparation, extraction and clean-up steps for measuring PCB sediment concentrations have been described previously (Martinez and Hornbuckle, 2011). Briefly, sediments were extracted using pressurized fluid extraction (Accelerated Solvent

[1] The PCB concentration measurement of sediment samples was performed by Andres Martinez, Ph.D., Department of Civil & Environmental Engineering, The University of Iowa.

Extractor, Dionex ASE-300). The extracts were concentrated and eluted through a multilayer silica gel column. Activated granulate copper was used to remove sulfur in solution. Poly-dimethylsiloxane (PDMS) coated fibers were used as passive samplers to determine the sediment pore water PCB concentration. PCB extraction and quantification procedures were also reported previously (Martinez *et al.*, 2013). PCB identification and quantification were conducted employing a modified US EPA method 1668C (USEPA, 2010). Tandem mass spectrometry GC/MS/MS (Quattro Micro GC, Micromass MS Technologies) in multiple reaction monitoring mode was utilized to quantify all 209 congeners in 161 individual or coeluting congener peaks.

The molar dechlorination product ratio (MDPR) was used to examine possible PCB dechlorination in core sediments. When determining the MDPR, it was assumed that exclusively *ortho*-chlorinated PCB congeners undergo no further dechlorination (TAMS Consultants, 1997). In this study, five exclusively *ortho*-chlorinated PCBs (PCB 1, 4, 10, 19, 54) and PCB 8 were selected as the ultimate dechlorination products. The ratio of the sum of the molar concentrations of selected congener over total PCB molar concentration was calculated and defined as MDPR. PCB 8 was considered as a dechlorination product because the proportions of PCB 8 in core sediments (averaged 1.4% of total PCB) were much higher than that in other Aroclor commercial mixtures ( 0.48%, 0%, 0% of Aroclor 1248, 1242, 1016, respectively), which indicates the production of this congener in IHSC sediments.

### Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis

Total chromosomal DNA from sediments was extracted using the MoBio Ultraclean Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) and stored them at -20°C until samples were analyzed with T-RFLP, pyrosequencing, and functional gene sequencing. To characterize the bacterial community composition, T-RFLP was

performed on PCR-amplified 16S rRNA genes. After initial PCR with primer set 8F/1492R and another round of PCR with a fluorescence-labeled primer set (6-FAM 8F/533R), we purified the PCR products using QIAquick PCR purification kit (Qiagen Inc, Valencia, CA), and digested purified PCR products with the restriction enzyme *Msp*I (New England BioLabs, Inc., Ipswich, MA). Then we precipitated DNA by centrifugation at 17,800 × g for 15 min after 2h incubation at -20°C with Glycogen (Fermentas International Inc., Vilnius, Lithuania), sodium acetate (pH 5.2), and ethanol and resuspended the pellet in distilled water (Invitrogen Corp., Carlsbad, CA). We sent digested DNA to the University of Iowa DNA facility for electrophoresis using an Applied Biosystems 3730 DNA analyzer (Life Technologies Corporation, Carlsbad, CA) with the GeneScan 500 LIZ size standard.

After processing T-RFLP profiles with Peak Scanner software (Applied Biosystems, Carlsbad, CA) and T-REX software (Culman *et al.*, 2009), a TRF size matrix containing 29 samples (rows) and 490 unique 16S rRNA gene TRFs (columns) was generated and was ordered by non-metric multidimensional scaling (NMDS) with the Wisconsin transformation and the Bray-Curtis dissimilarity index. The final stress was 9.93. PCB congener profiles were ordered by principal components analysis (PCA) and the correlation between PCB congener profile and T-RFLP profile was tested by Procrustes test. The Mantel test was also applied to assess the correlation between T-RFLP profiles and PCB profiles, where a Euclidean dissimilarity index was calculated for PCB congener profiles and the Bray-Curtis dissimilarity index was calculated for T-RFLP profiles. Pearson correlation coefficient between each T-RF peak and MDPR values were calculated (González *et al.*, 2000).

## Bacterial tag-encoded amplicon pyrosequencing[2]

To get more detailed sequence information about bacterial communities, pyrosequencing for bacterial 16S rRNA gene was performed for three surficial sediment samples and two sections from core 1 ( 0.15-0.30 m and 1.37-1.52 m). Bacterial 16S rRNA gene fragments were amplified using barcoded primer sets BSF8/USR515 under conditions previously described (Bibby *et al.*, 2010). PCR products were purified with QIAquick PCR purification kit (Qiagen Inc, Valencia, CA) and sent for pyrosequencing on a 454 GS-FLX sequencer (Roche Diagnostics Corporation, Indianapolis, IN) using the Titanium Sequencing Kit (Roche, Branford, CT).

Raw pyrosequencing data was processed on RDP pyrosequencing pipeline (http://pyro.cme.msu.edu/pyro/) (Cardenas *et al.*, 2009). After quality check, trimming of barcodes and filtering of bad reads, sequences were aligned and assigned identities. Operational taxonomic units (OTUs) were generated based on 97% sequence similarity.

## PCR amplification, cloning and sequencing of biphenyl dioxygenase gene (BphA) and *Dehalococcoides*-specific 16S rRNA

BphA genes were amplified using various primer sets, including bphA10, bphA20, bphA30, bphA40, and bphA50 (Hoostal *et al.*, 2002). Each reaction contained 4 μM of each primer and 25-50 ng of DNA template. PCR reaction conditions were as follows: initial denaturing step for 4 min at 94°C, followed by 40 cycles of denaturing for 1 min at 94°C, annealing for 3 min at 40-45°C, and extending for 4 min at 72°C with a final extension step at 72°C for 10 min.

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[2] The pyrosequencing experiment was performed by Joshua Livermore, Ph.D., Department of Civil & Environmental Engineering, The University of Iowa

*Dehalococcoides*-specific 16S rRNA genes were amplified with previously published DHC primer sets (Hendrickson *et al.*, 2002). Each reaction contained 20 ng of DNA template and 20 pM of each primer. Reaction conditions were as follows: 2min denature at 95°C, followed by 40 cycles of 1 min denature at 94°C, 1 min anneal at 55°C, and 1 min extend at 72°C and a final extension of 5 min at 72°C. All PCR experiments were conducted using the Qiagen Master Mix PCR Kit (Qiagen Inc, Valencia, CA).

PCR products were purified by Qiaquick Gel Extraction Kit (Qiagen Inc, Valencia, CA), cloned into the pDRIVE vector using the Qiagen PCR Cloning Kit (Qiagen Inc, Valencia, CA), and transformed into NEB 5-alpha F'$I^q$ competent *E.coli* cells (New England BioLabs, Inc., Ipswich, MA). Recombinant *E.coli* were plated on Luria Broth agar with kanamycin (30 mg/L), X-gal (0.4 mg/plate) and IPTG ($10^{-4}$ mmol/plate) and incubated overnight at 37°C. Plasmid DNA was extracted from white colonies using the Qiagen Miniprep Plasmid Extraction Kit (Qiagen Inc, Valencia, CA) and the inserts were sequenced at the University of Iowa DNA facility. We then analyzed sequence data using Ribosomal Database Project (RDP) pipeline (Cole *et al.*, 2007) and Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997) and performed multiple sequence alignments and phylogenetic tree construction using ClustalX2 programme (Thompson *et al.*, 1997). MEGA 4 was used to graphic present the results (Tamura *et al.*, 2007).

## Results and Discussion

### PCB congener analysis

The vertical pattern of PCB congener distribution of core 1 was examined to search for the evidence of *in situ* PCB dechlorination. Changes of PCB concentration and congener distribution along sediment depth could be the result of the discharge of different commercial mixtures, sediment resuspension and transport, and PCB

biodegradation (Li *et al.*, 2009). Anaerobic PCB biodegradation usually leads to congener profile shift from higher chlorinated congeners to lower chlorinated ones. Furthermore, anaerobic PCB degraders preferentially remove chlorine atoms at *meta*- and *para*-position, resulting in the increase of *ortho* chlorine fraction. Sediment suspension and transportation may lead to accumulation of highly chlorinated congeners because lightly chlorinated PCBs are relatively less hydrophobic and more volatile, and tend to migrate from sediment to water and atmosphere.

For core 1, the fraction of lesser chlorinated PCBs (1-3 chlorine substituents) increased along sediment depth till 2.4 m, and then decreased. Sediment depth 0-2.4 m is where the total PCB concentrations were high (averaged 52,000 $ng/g^{-1}$ d.w. ). Sediment deeper than 2.4 m had much lower PCB concentrations (averaged 2,500 $ng/g^{-1}$ d.w.), which section corresponded to the time period before PCBs were widely used (before 1930s) (Martinez and Hornbuckle, 2011). Thus the accumulation of lesser chlorinated PCBs in sediments 0-2.4 m may suggest the microbial dechlorination. At the same sediment depth, the fraction of *ortho*-Cl kept relative stable, but MDPR values increased, which suggests that PCB dechlorination has occurred (Figure 3.2, Figure 3.3). Furthermore, although the sediment PCB profile was reported to resemble the Aroclor 1248, the average MDPR value for sediments of 0-2.4 m (0.031) was much greater than that of Aroclor 1248 (0.009), which also indicates the *in situ* PCB dechlorination (Frame et al., 1996; Martinez and Hornbuckle, 2011).

## Detection of *bph*A and *Dehalococcoides*-specific 16S rRNA genes in IHSC sediments

The signal of *bph*A gene was detected by PCR in core sediment samples water-sediment surface to 2.4 m deep (Table 3.1). In deeper sediments, *bph*A genes were not amplified, possibly because of the intense anaerobic conditions. Eight different partial *bph*A sequences were identified by clone library, six of which were 93-98% identical to

BphA from *Acidovorax* sp. KKS102 (GenBank Acc. No. BAJ72245). These sequences were also 91-97% similar to BphA from *Comamonas testosteroni* TK102 (GenBank Acc. No. BAC01052), also a known PCB degrader. The other two sequences were 98-99% similar to BphA in *Pseudomonas putida* B2-6 (GenBank Acc. No. ACN62349).

We also identified four partial *bph*A sequences from S11, five partial *bph*A sequences from S21 and one sequence from S56. Two sequences of S21 were 95~96% identical to BphA from uncultured bacterium (GenBank Acc. No. ACL99824). The two sequences were 95~96% similar to BphA from *Pseudomonas alcaligenes* B357 (GenBank Acc. No. ABR08355), respectively (Uhlík et al., 2009). Two other sequences were 98~99% identical to ring hydroxylating dioxygenase alpha subunit in *Polaromonas naphthalenivorans* CJ2 (GenBank Acc. No. ABM39439), which is a naphthalene-degrading bacterium (Jeon *et al.*, 2004). Another five sequences were 98~99% identical to BphA in *Pseudomonas putida* B6-2 (GenBank Acc. No. ACN62349). One sequence was 48~54% similar to BphA from *Acidovorax* sp. KKS102 (GenBank Acc. No. BAJ72245), a soil PCB degrading bacterium (Kimbara *et al.*, 1988). A phylogenetic tree of translated *bph*A sequences shows the relationship among various aerobic PCB-degrading organisms and the *bph*A sequences retrieved from the sediment samples, revealing the diversity of those obtained *bph*A sequences (Figure 3.5).The successful identification of *bph*A genes from S11, S21, S56 and core 1 (0.16-0.32 m) indicates possible occurrence of aerobic PCB degradation.

The signal for *Dehalococcoides*-specific 16S rRNA gene was negative till 0.91 m, and was positive between 0.91 - 3.05 m, then disappeared till the end of the core. Five unique *Dehalococcoides*-specific 16S rRNA sequences were obtained from core sediments at the depth 1.44-1.60 m. Three sequences were 99-100% identical to 16S rRNA gene from *Dehalococcoides* sp. enrichment culture clone CT3-2 (GenBank Acc. No. JF698644), which was isolated from a 2,4,6-trichlorophenol dechlorinating microcosm. The other two sequences were 96% similar to uncultured *Chloroflexi*

bacterium 16S rRNA gene (GenBank Acc. No.CU923702). These *Dehalococcoides* sequences were closely related to known PCB degraders and were within the dechlorinating *Chloroflexi* group (Figure 3.6), indicating the presence of microbial dechlorinating bacteria in contaminated sediments.

## Bacterial community structure assessed by T-RFLP and pyrosequencing

Bacterial communities in upper sediments were different from those in deeper sediments according to the NMDS ordination of T-RFLP profiles, primarily on NMDS axis 1, suggesting depth-associated shifts in bacterial communities (Figure 3.7). A strong correlation between T-RFLP profile and PCB congener profile was detected by Mantel test ($p=0.028$).The correlation was also confirmed by Procrustes test ($p=0.002$). The correlation suggests that sediment sections with similar PCB congener profiles tend to have similar bacterial community structures.

Several T-RFs that co-varied with the PCB congener patters along depths were identified (Pearson correlation, $p < 0.001$). T-RFs 235 bp, 250 bp, 258 bp, 273 bp, 278 bp, 302 bp, and 398 bp were found to have strong correlation with sediment MDPR values, while T-RFs 252 bp and 515 bp were correlated with pore water MDPR values. Those T-RF peaks were not abundant, usually 0.01~0.1% of total T-RFs, which is not surprising, because microbes associated with PCB degradation do not necessarily dominate the bacterial community (Petrić et al., 2011a).

The bacterial communities were also assessed by pyrosequencing. In total, pyrosequencing generated 144,163 16S rRNA sequences from five sediment samples, among which 9,119 unique OTUs were identified (Table 3.2). At least twelve bacterial phyla were detected in all the five sediment samples, among which, *Proteobacteria* was found to be the most abundant taxa in all the samples, accounting for 95~99% of each sample's membership. The *Betaproteobacteria* were the dominant class among the

*Proteobacteria* in all the samples except for S56, where *Alphaproteobacteria* were most abundant. Overall, each sample had different community composition (Figure 3.8).

Many known aerobic PCB degraders belong to genera within the gamma- and beta- subclasses of *Proteobacteria*, such as *Burkholderia, Pseudomonas*, and *Sphingomonas* (Furukawa and Fujihara, 2008). In this study, the *Betaproteobacteria* were the dominant class in all the samples except for S56. Even in S56, *Betaproteobacteria* occupied 36% of total sequences. *Gammaproteobacteria* also took considerable percentage, ranging from 1.38~19.1% of total sequences. This finding was consistent with Correa and colleagues' microcosm results, where the addition of PCB congeners into soil samples resulted in statistically significant increase of *Betaproteobacteria* (Correa et al., 2010).

Genera that include possible aerobic PCB degraders (such as *Acidovorax, Acinetobacter, Burkholderia, Comamonas, Polaromonas, Pseudomonas*) were detected in all five sediment samples, but they were not abundant. *Chloroflexi* phylum was detected, 94% of which were found in at depth of 1.44-1.60 m of core 1 (C10). However, BLAST results show that the sequences belong to Class *Anaerolineae* rather than *Dehalococcoides*. The *Dehalococcoides* was not detected probably because of the low abundance of *Dehalococcoides* species in the sediments.

## Conclusions

The microbial PCB degradation potential was evaluated for a 4.57 m core sediment sample collected from IHSC by exploring the congener profiles and microbial communities. The vertical pattern of the congener profile suggested some extent of *in situ* dechlorination. The successful identification of *bph*A and *Dehalococcoides*-specific 16S rRNA genes in sediments indicated both aerobic and anaerobic PCB degradation

potential. The presence of microorganisms that are known to degrade PCBs may also take part in the microbial PCB metabolism.

Table 3.1. PCR analysis of DNA extracted from different depths of PCB-contaminated core 1 sediment sample at IHSC.

| Depth, m | PCR test results | |
|---|---|---|
| | bphA 10/30 | dhc 946/1212 |
| 0-0.16 | + | - |
| 0.16-0.32 | + | - |
| 0.32-0.48 | + | + |
| 0.48-0.64 | + | - |
| 0.64-0.80 | + | - |
| 0.80-0.96 | + | - |
| 0.96-1.12 | + | + |
| 1.12-1.28 | + | + |
| 1.28-1.44 | + | + |
| 1.44-1.60 | + | + |
| 1.60-1.76 | + | + |
| 1.76-1.92 | + | - |
| 1.92-2.08 | + | + |
| 2.08-2.24 | + | + |
| 2.24-2.40 | + | + |
| 2.40-2.56 | + | + |
| 2.56-2.72 | + | + |
| 2.72-2.88 | + | + |
| 2.88-3.04 | - | + |
| 3.04-3.20 | + | + |
| 3.20-3.36 | - | + |
| 3.36-3.52 | - | - |
| 3.52-3.68 | - | + |
| 3.68-3.84 | - | - |
| 3.84-4.00 | - | - |
| 4.00-4.16 | - | - |
| 4.16-4.32 | - | - |
| 4.32-4.48 | + | - |
| 4.48-4.57 | + | - |

Table 3.2. Summary of 454 sequence library sizes, operational taxonomic unites (OTUs).

| Sample | 454 sequence libraries | |
|---|---|---|
| | Sequence library size | Number of OTUs |
| S11 | 4192 | 3413 |
| S21 | 11584 | 10019 |
| S56 | 4109 | 3756 |
| Core 1 (0.16-0.32m) | 3806 | 3282 |
| Core 1 (1.44-1.60m) | 120472 | 110402 |


Figure 3.1. Aerial view of Indiana Harbor depicting the location of sediment samples analyzed in this study (Blue open square: core sediment samples; Red open circle: surficial sediment samples).

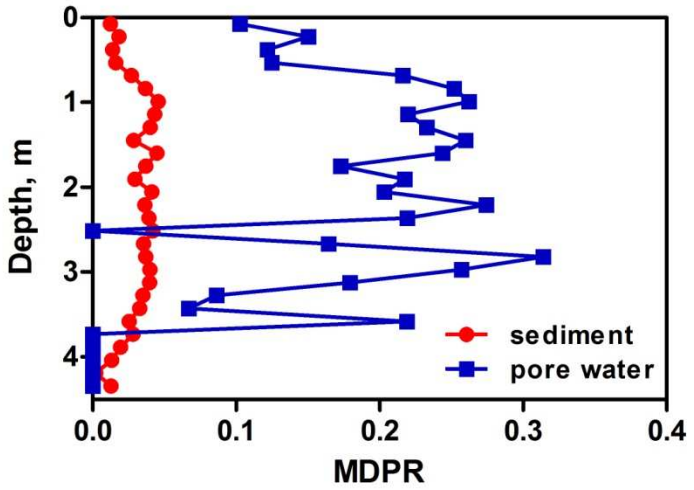


Figure 3.2. Sediment and pore water MDPR values against sediment depth for core 1.

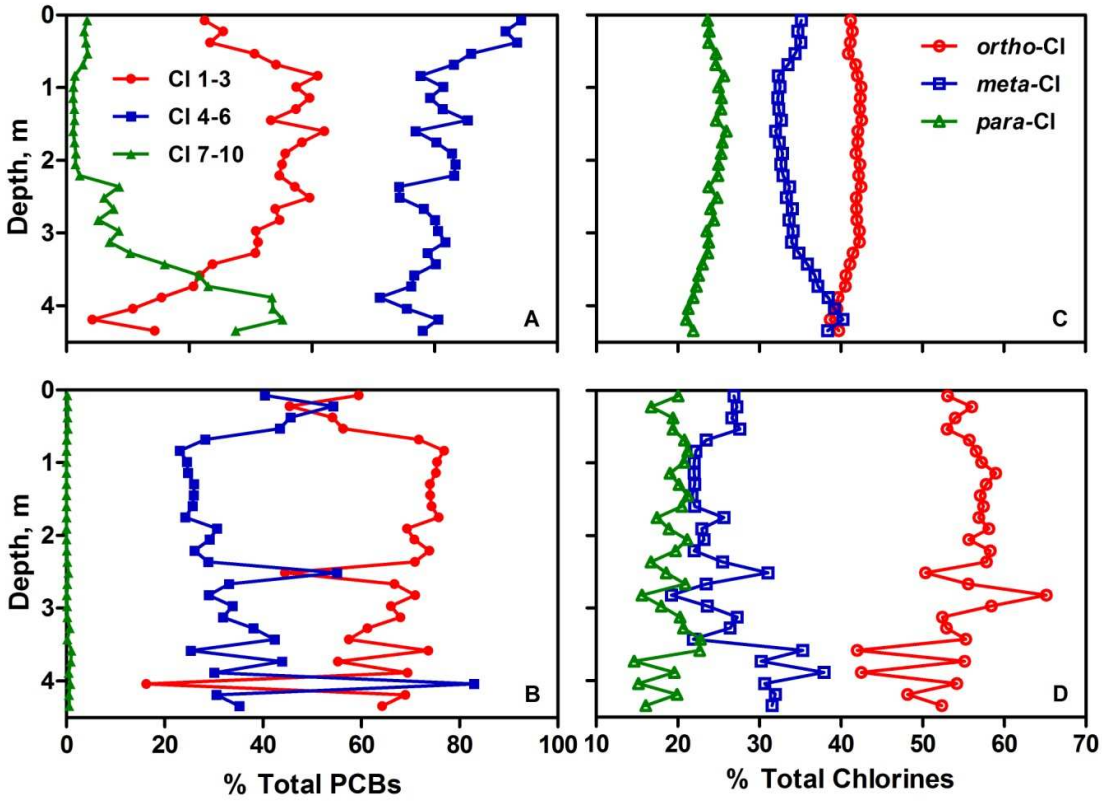


Figure 3.3. Fraction of light, medium and heavy PCBs versus sediment depth in core sediment (A) and pore water (B), and fraction of *ortho*-, *meta*-, *para*-chlorines versus sediment depth in core sediment (C) and pore water(D).

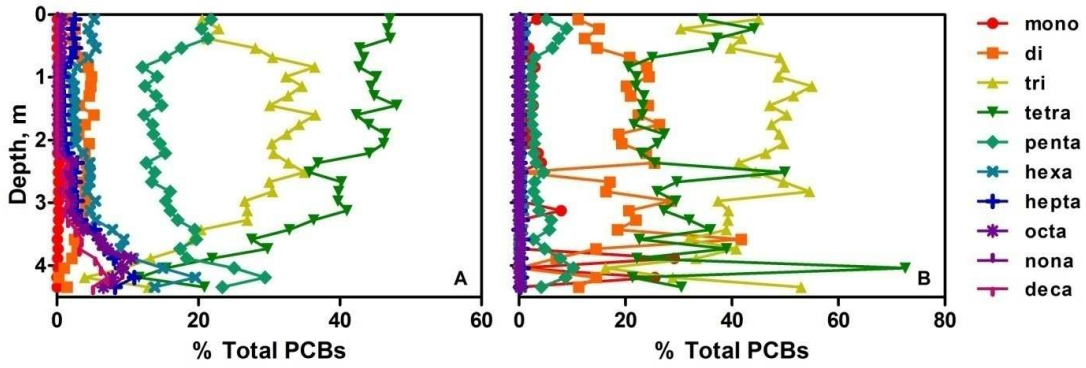


Figure 3.4. Fraction of each PCB homolog group in sediment (A) and pore water (B) against sediment depth for core 1.

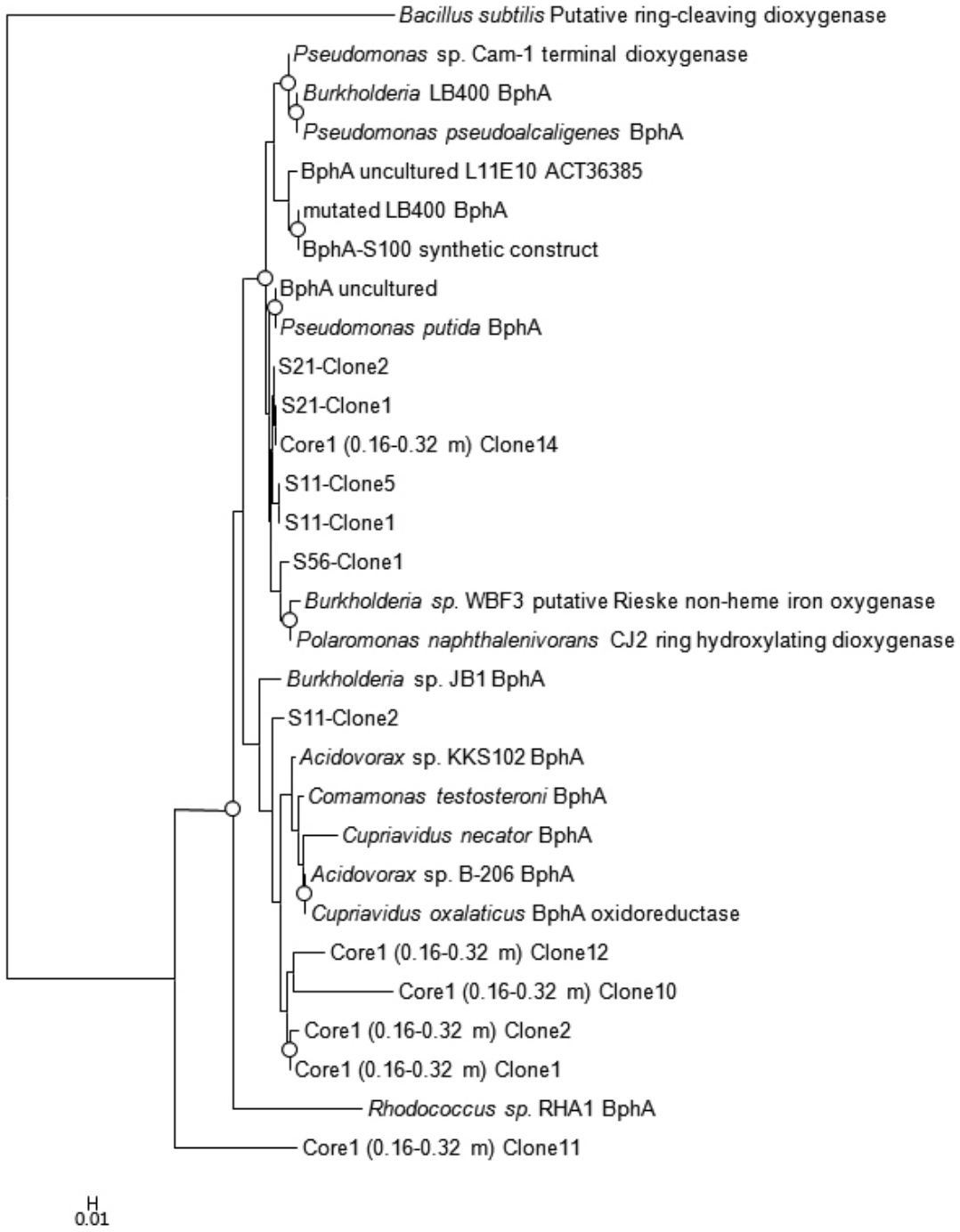


Figure 3.5. Phylogenetic analysis of twelve amino acids of translated sequences of BphA genes retrieved from PCB-contaminated surficial sediment sample 11, 21, 56 and core 1 sediment sample (0.16-0.32 m) at IHSC. The alignment was made with ClustalX and converted into a neighboring-joining tree, which was visualized with MEGA4 with *Bacillus subtilis* Putative ring-cleaving dioxygenase as an outgroup. The filled circles at nodes indicate bootstrap values higher than 95%, while the open circles indicate bootstrap values of 75-95%. Bootstrap values lower than 75% are not shown. The bar represents 1% sequence difference.

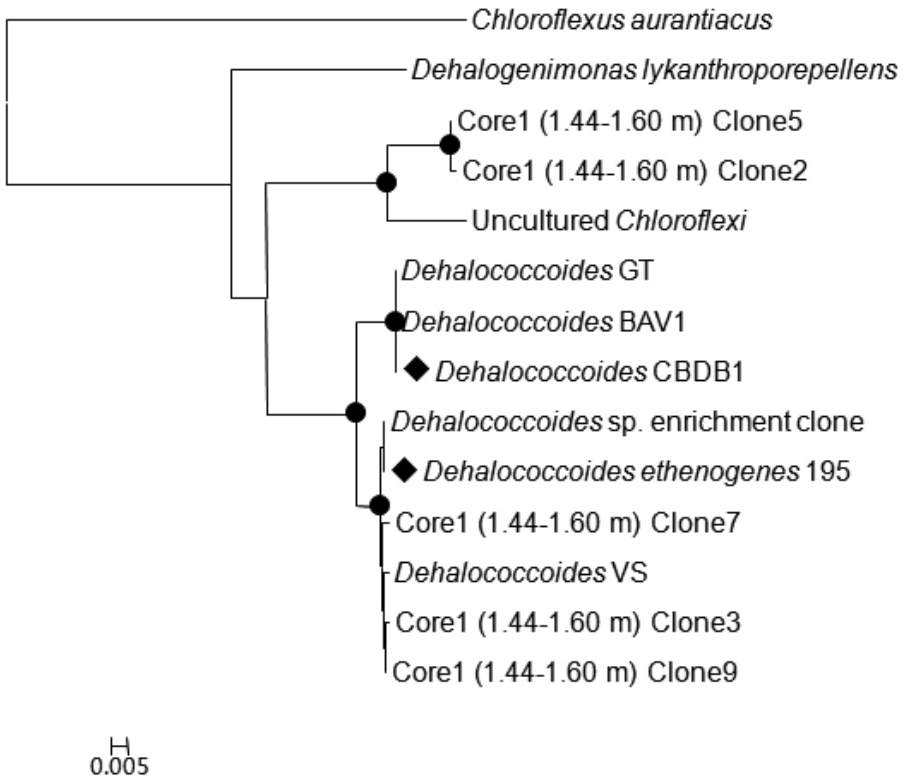


Figure 3.6. Phylogenetic analysis of 16S rRNA genes retrieved from surficial and core sediment samples at IHSC. The alignment was made with ClustalX and converted into a neighboring-joining tree, which was visualized with MEGA4 with *Chlorofexus aurantiacus* J-10-fl as an outgroup. The filled diamonds represent known PCB degraders. The filled circles at nodes indicate bootstrap values of 95%, while open circles indicate bootstrap values of 75~95%. Bootstrap values lower than 75% are not shown. The bar represents 0.5% sequence difference.

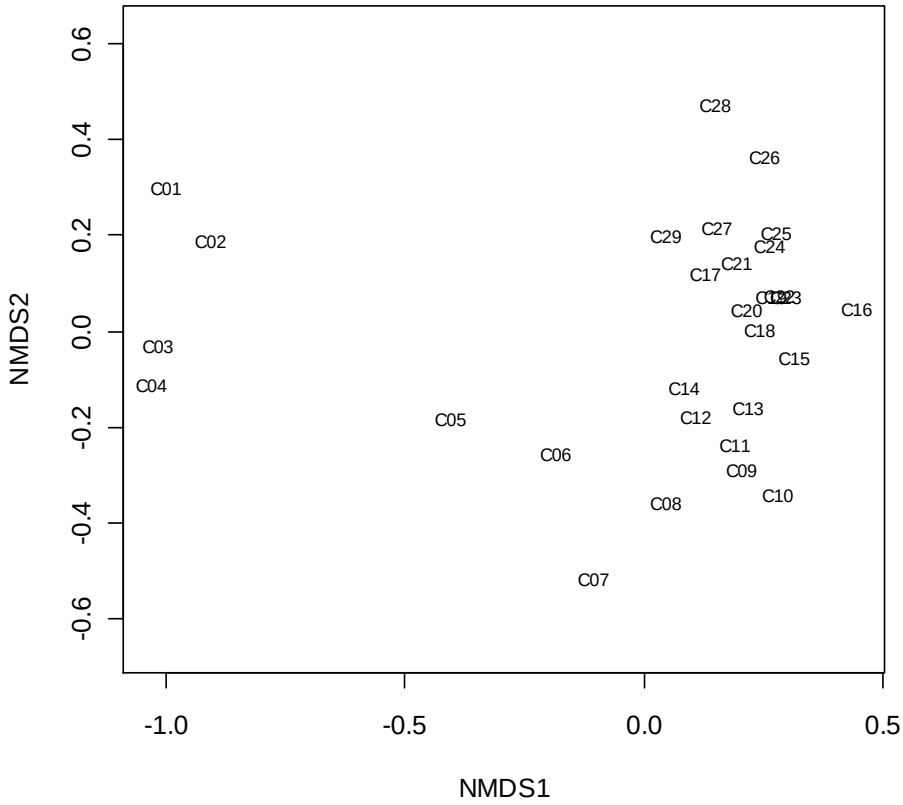


Figure 3.7. NMDS ordination of Wisconsin transformed T-RFLP profiles of core 1 (C01: 0-0.16 m, C02: 0.16-0.32 m…).

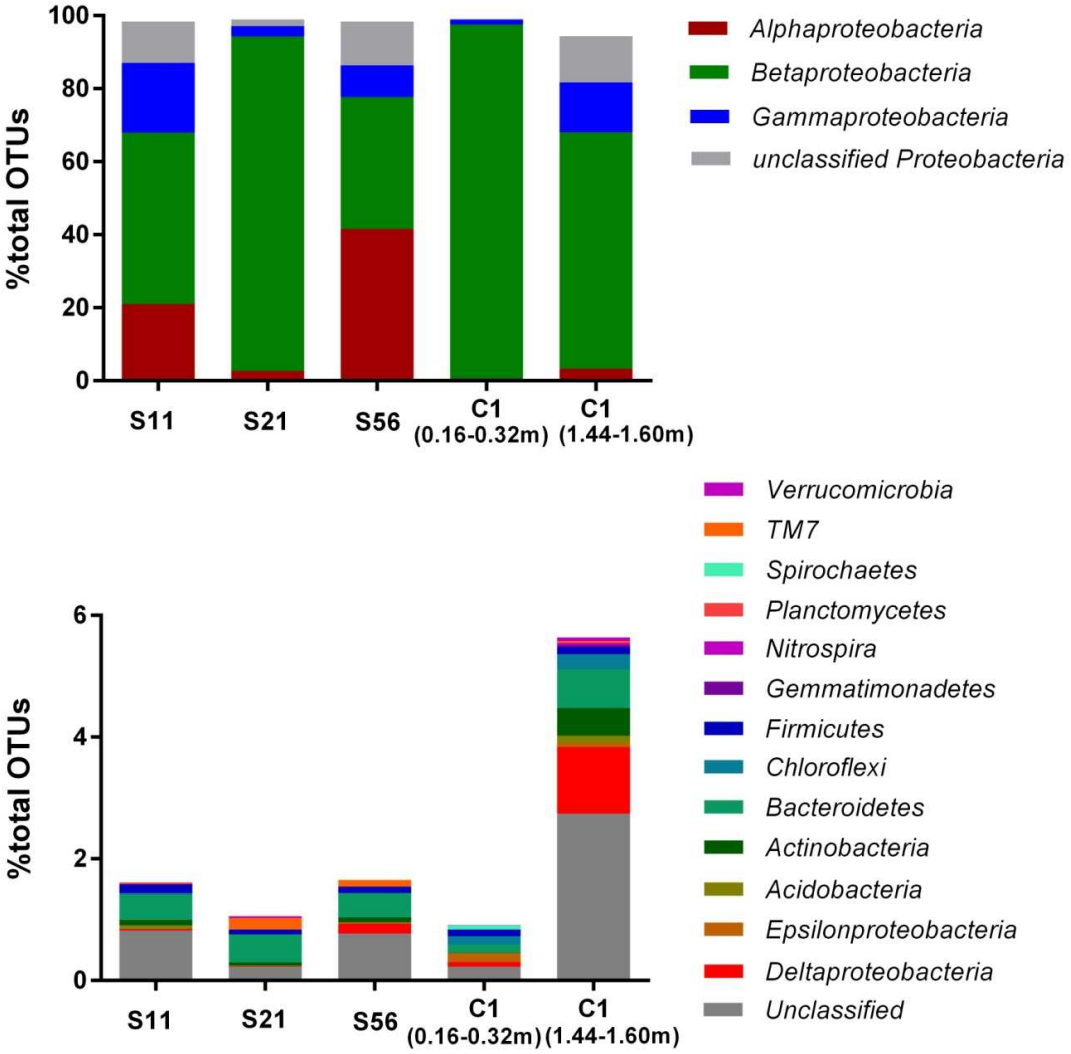


Figure 3.8. Relative abundance of phyla and proteobacterial classes for surficial sediment samples (S11, S21 and S56) and core 1 sediment samples (depth 0.16-0.32 m and 1.44-1.60 m).

# CHAPTER IV. MICROBIAL PCB DEGRADATION POTENTIAL IN CORE 2 FROM IHSC

## Introduction

Polychlorinated biphenyls (PCBs) are a group of 209 synthetic chemicals that were widely used in industry during the last century. The production and commercial use of PCBs were banned by the EPA in 1979 because they were found to be toxic, carcinogenic, and bioaccumulative in the food web (Ross, 2004). Nevertheless, due to their high stability, PCBs still exist in the environment, and pose a potential risk to public health more than 30 years later (Bedard, 2004). Despite their environmental persistence, PCBs can be transformed to less toxic forms or even mineralized by microorganisms. Field and laboratory studies indicate two predominant processes: anaerobic dechlorination and aerobic biphenyl ring cleavage (Borja *et al.*, 2005).

Under anaerobic conditions, PCBs are used as electron acceptors, resulting in the removal of chlorine atoms. The less chlorinated congeners are more amenable to aerobic degradation (Alder *et al.*, 1993). Anaerobic PCB-degraders include *Dehalococcoides* spp., the o-17/DF-1 group, as well as some other members of the *Chloroflexi* (Adrian et al., 2009; Wiegel and Wu, 2000; Wu et al., 2002). Reductive dehalogenase (RDase) is considered to be the key enzyme catalyzing the PCB dechlorination process (Hiraishi, 2008; Pieper and Seeger, 2008). Nonidentical RDase genes were found in PCB degrading strains such as *Dehalococcoides mccartyi* strain 195 and CBDB1, but no RDase gene was identified to dechlorinate PCBs (Fung et al., 2007; Hölscher et al., 2004; Wagner et al., 2009).

Under aerobic conditions, PCBs can be used as electron donors, or fortuitously oxidized by oxygenase enzymes. A variety of PCB-oxidizing bacteria have been identified, including Gram-negative strains of *Pseudomonas, Burkholderia, Acinetobacter,*and *Comamonas* and Gram-positive strains of *Corynebacterium*,

*Rhodococcu* and *Bacillus* (Furukawa and Fujihara, 2008; Pieper and Seeger, 2008). These microbes harbor biphenyl dioxygenase (Bph), the key enzyme catalyzing the first step of aerobic biphenyl ring cleavage, generating a *cis*-dihydrodiol intermediate, which is further degraded to a chlorobenzoate (Gibson and Parales, 2000). PCB-oxidizing microbes usually cannot metabolize the chlorobenzoate, thus a consortium of chlorobenzoate-degraders are required for complete PCB mineralization (Pavlů *et al.*, 1999).

The site of this study is the Indiana Harbor and Ship Canal (IHSC), a heavily industrial area of southern Lake Michigan contaminated with a variety of pollutants, including heavy metals, polycyclic aromatic hydrocarbons (PAHs) and PCBs. It is reportedly a major source of PCBs to Lake Michigan, with PCB concentrations in IHSC surficial sediments ranging from 53 to 35,000 ng $g^{-1}$ dry weight (Martinez *et al.*, 2010). PCB-contaminated IHSC sediments are currently being dredged and permanently stored in a confined disposal facility (CDF) (USACE, 2013).

The purpose of this study is to evaluate both anaerobic and aerobic PCB biodegradation potential in IHSC sediments. We characterized PCB congener profiles and bacterial communities in a 4.57-m long core sediment sample, which revealed evidence of *in situ* aerobic and anaerobic PCB degradation in upper 1.83 m sediments. We also explored the correlation between the bacterial communities and PCB congener profiles and found that sediment sections with similar PCB congener profiles tend to have similar bacterial community structure. We conclude that microbial communities in IHSC sediments have the potential for aerobic and anaerobic PCB biodegradation. This suggests that natural attenuation of PCBs could continue in IHSC sediments after they are transferred to the CDF.

## Materials and Methods

### Site description and sampling

In May 2009, a 4.57-m core sediment sample from IHSC was collected using a submersible vibro-coring system with a PVC tube (length 457 cm, internal diameter 9.5 cm) from aboard the U.S. EPA's research vessel, *Mudpuppy* (Figure 3.1). The core was sectioned every 0.305 m, and each section was homogenized, placed in plastic bags and kept on ice during transportation. Sediment samples were stored at 4°C in the lab until analysis.

### PCB congener analysis[3]

Preparation, extraction and clean-up steps for measuring PCB sediment concentrations were performed as previously described (Martinez and Hornbuckle, 2011). Briefly, sediments were extracted using pressurized fluid extraction (Accelerated Solvent Extractor, Dionex ASE-300). The extracts were concentrated and eluted through a multilayer silica gel column. Sulfur in solution was removed by activated granulate copper. Poly-dimethylsiloxane (PDMS) coated fibers were used as passive sampler to measure PCB concentration of the sediment pore water. PCB extraction and quantification procedures were also reported previously (Martinez *et al.*, 2013). PCB identification and quantification were conducted employing a modified US EPA method 1668C (USEPA, 2010). Tandem mass spectrometry GC/MS/MS (Quattro Micro GC, Micromass MS Technologies) in multiple reaction monitoring mode was utilized to quantify all 209 congeners in 161 individual or coeluting congener peaks.

The molar dechlorination product ratio (MDPR) was used to examine possible PCB dechlorination in core sediments. When determining the MDPR, it is assumed that

[3] The PCB concentration measurement of sediment samples was performed by Andres Martinez, Ph.D., Department of Civil & Environmental Engineering, The University of Iowa.

exclusively *ortho*-chlorinated PCB congeners undergo no further dechlorination (TAMS Consultants, 1997). In this study, five exclusively *ortho*-chlorinated PCBs (PCB 1, 4, 10, 19, 54) and PCB 8 were selected as the ultimate dechlorination products. The ratio of the sum of the molar concentrations of selected congener over total PCB molar concentration was calculated and defined as MDPR. PCB 8 was considered as a dechlorination product because the proportions of PCB 8 in core sediments (averaged 2.7% of total PCB) were much higher than that in other Aroclor commercial mixtures ( 0.48%, 0%, 0% of Aroclor 1248, 1242, 1016, respectively), which indicates the production of this congener in IHSC sediments.

## Quantitative PCR

Total DNA from sediments was isolated with the MoBio Ultraclean Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) and storedat -20°C until analysis. The abundance of total bacteria, aerobic PCB-degrading bacteria, and putative PCB-dechlorinating bacteria were estimated using qPCR targeting bacterial 16S rRNA gene (primer set 16SU f/r) (Nadkarni *et al.*, 2002), *bph*A (primer set *bph*A 463f/674r) (Petrić et al., 2011b), putative dechlorinating *Chloroflexi* 16S rRNA genes (primer set chl348f/dehal884r) (Fagervold *et al.*, 2005), and *Dehalococcoides*-like 16S rRNA genes (primer set dhc793f/946r) (Yoshida *et al.*, 2005) (Table 4.1). PCR conditions were as follows: 10 min at 95°C, 40 cycles of 15s at 95°C and 1 min at 60°C followed by a dissociation step. Each 25 μl reaction contained 12.5 μl Power SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA), 2.5 pmol primers and variable amounts of primers and template (Table 4.2). Bovine serum albumin(0.5 μg) was added to relieve possible PCR inhibition (Kreader, 1996).

For total bacterial 16S rRNA gene qPCR, the standard DNA template was PCR products amplified from *Burkholderia xenovorans* LB400 with primer set 8F/1492R (Grabowski *et al.*, 2005). For *bph*A, the standard DNA template was the LB400 *bphA*

(amplified with the 463f/674r primer set) cloned into the pCR 2.1-TOPO vector. For putative dechlorinating *Chloroflexi* 16S rRNA genes and *Dehalococcoides*-like 16S rRNA gene, standard curves were prepared from pCR 2.1-TOPO vector containing the target PCR products with primer chl3487f/dehal884r and dhc793f/946r, respectively. All qPCRs were performed with an ABI 7000 Sequence Detection System (Applied Biosystem, Grand Island, NY) and fluorescence data was analyzed by ABI 7000 System SDS Software (Applied Biosystems, Grand Island, NY). With each primer set, the target gene was not detected in no template (DI water) controls (Ct value > 35).Additional qPCR information, such as primer concentrations, template concentrations, qPCR linear range, qPCR efficiency range of the standard curves, and Y-intercepts are provided in Table 4.2, in accordance with MIQE guidelines (Bustin *et al.*, 2009).

### qPCR quality assurance

To verify the specificity of qPCR primer sets chl348f/dehal884and dhc793f/946r, two clone libraries were constructed from the amplification products in DNA extracted from sediments at 0-0.30m and 0.91- 1.22 m. Clone libraries were prepared as described in section 2.6. From the chl348f/dehal884r PCR product clone library, 21 unique sequences were obtained from 22 clones and from the dhc793f/946r PCR product clone library, 10 unique sequences were obtained from 10 clones. All of the sequences were determined to be from *Chloroflexi*, using RDP classifier (Cole *et al.*, 2007), with 91% classified as *Dehalogenimonas* (Table 4.3).The specificity of the SYBR green based qPCR is also validated by dissociation curve analysis, which shows similar melting temperature for each primer set.

### Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis

PCR with fluorescently-labeled 6-FAM 8Fm and 533R was performed with 1 ng

DNA (Schütte *et al.*, 2008). PCR conditions were: 4 min at 94°C, 30 cycles of 1 min at 94°C, 2 min at 55°C and 1 min at 72°C followed by a final extension step of 10 min at 72°C.PCR product (12µl) was digested with the restriction enzyme *Hae*III (New England BioLabs, Inc., Ipswich, MA), and then precipitated with glycogen (Fermentas International Inc., Vilnius, Lithuania), sodium acetate (pH 5.2), and ethanol at -20°C for 2h. DNA was recovered by centrifugation at 17,800 × g for 15 min and resuspended in distilled water (Invitrogen Corp., Carlsbad, CA). The digested DNA was sent to the University of Iowa DNA facility for electrophoresis for sizing using an Applied Biosystems 3730 DNA analyzer (Life Technologies Corporation, Carlsbad, CA) with the GeneScan 500 LIZ size standard.

Terminal restriction fragment (T-RF) sizes were estimated with Peak Scanner software (Applied Biosystems, Carlsbad, CA).The TRF size matrix containing15 samples (rows) and 354 unique 16S rRNA gene TRFs (columns)was generated by T-REX software after filtering background peak noise and rounding fragment sizes to the nearest whole number (Culman *et al.*, 2009).TRF profiles were analyzed by non-metric multidimensional scaling (NMDS) in R (da C Jesus *et al.*, 2009). The Bray-Curtis dissimilarity index was calculated with a random starting configuration, and a two-dimensional solution was reached after seven runs. The final stress was 7.42.

Correlations between PCB congener profiles and T-RFLP profiles were assessed with the Procrustes test, where PCB congener profiles were analyzed by principal components analysis (PCA) and T-RFLP profiles were ordinated by NMDS. The Mantel test was applied to assess the correlation between T-RFLP profiles and PCB profiles, where a Euclidean dissimilarity index was calculated for PCB congener profiles and the Bray-Curtis dissimilarity index was calculated for T-RFLP profiles. Pearson correlation coefficients between each TRF and molar dechlorination product ratio (MDPR) values were also calculated (González *et al.*, 2000).

### PCR amplification, cloning and sequencing of bacterial 16S rRNA

A clone library was constructed with bacterial 16S rRNA genes amplified from sediment at the depth 3.35 – 3.66m.Relatively high MDPR values (0.08 for sediment and 0.37 for pore water) were observed at this depth. Bacterial 16S rRNA genes were amplified using primer set 8F/1492R (Grabowski *et al.*, 2005).PCR conditions were as described in the T-RFLP analysis section.PCR products were purified with the Qiaquick PCR purification Kit (QiagenInc., Valencia, CA), cloned into the pCR 2.1-TOPO vector using the TOPO TA cloning Kit (Invitrogen Corp., Carlsbad, CA), and transformed into One Shot TOP10 chemically competent *E.coli* cells (Invitrogen Corp., Carlsbad, CA). The transformation efficiency was checked by plating recombinant *E.coli* on Luria Broth agar with kanamycin (50 mg/L), and X-gal (0.4 mg/plate) and incubated overnight at 37°C. Clones were Sanger sequenced at HTSeq.org (Seattle, WA) with M13F (5'-TGTAAAACGACGGCCAGT-3') and M13R (5'-CAGGAAACAGCTATGAC-3') primers. Sequence identification and classification were performed using RDP Classifier(Cole *et al.*, 2007)and Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997).Predicted TRFs were generated with TRiFLE (Junier *et al.*, 2008).

## Results and discussion

### Evidence for PCB dechlorination in IHSC sediment congener profiles

Changes in PCB congener distribution patterns with sediment depth can be explained by changes in production, use and discharge of different commercial mixtures, sediment resuspension and transport, and *in situ* microbial PCB degradation(Li *et al.*, 2009). Sediment resuspension and PCB desorption would be expected to result in accumulation of highly chlorinated PCBs in deeper sediments. However, anaerobic PCB

dechlorination in sediments would lead to decreased abundance of higher chlorinated congeners and increased abundance of less chlorinated ones. With this in mind, we examined PCB congener profiles in sediment samples to search for evidence of PCB dechlorination. Both sediment and pore water PCB profiles showed the accumulation of lesser chlorinated PCBs (1-3 chlorine substituents) in the deeper sediment of the core, along with a decrease of medium chlorinated congener abundance (4-6 chlorine substituents) (Figure 4.1A, 4.1B). There was also an increasing trend of *tri*-chlorinated PCBs and a corresponding decreasing fraction of *tetra*-and *penta*-chlorinated congeners with increasing depth (Figure 4.2A, 4.2B).

Anaerobic PCB dechlorination in sediments preferentially removes chlorines at *para*- and *meta*- positions, resulting in the enrichment of *ortho*-chlorinated congeners (Alder et al., 1993; Bedard et al., 1997; Fava et al., 2003). Therefore, if PCB dechlorination had occurred, we would expect to see an increase in the abundance of PCB congeners with *ortho*-chlorine substituents. Indeed, analysis of the relative abundance of *ortho*-, *meta*-, and *para*-chlorines against core depth revealed an accumulation of *ortho*-chlorines in deep sediments, in accordance with a decrease in *meta*- and *para*-chlorinated PCBs (Figure 4.1C). In pore water, the enrichment of *ortho*-chlorinated congeners was also observed with depth (Figure 4.1D).

MDPR values were estimated to examine potential PCB dechlorination in the core sediment. Accumulation of the six selected *ortho*-chlorinated congeners in the core sediments was observed, which further supports that PCB dechlorination has occurred (Figure 4.3).Although sediment PCB profiles in the core reportedly mainly resemble the Aroclor1248 mixture, the average sediment MDPR value (0.052) was much greater than that of Aroclor 1248 (0.009) (Frame et al., 1996; Martinez and Hornbuckle, 2011), which is also a sign of *in situ* dechlorination. MDPR values of pore water were higher than those of corresponding sediment sections (Figure 4.3).This can be explained by the tendency for *ortho*-chlorine substituents to increase the aqueous solubility of the PCB

molecule(Huang and Hong, 2002), and that the lower chlorinated congeners as the six selected ones are more soluble (Van Noort *et al.*, 2010). Another possible reason is that PCBs dissolved in pore water are more bioavailable than those sorbed to sediment particles and thus are more vulnerable to *in situ* dechlorination (Reid *et al.*, 2000).

## Aerobic and anaerobic PCB biodegradation potential in sediment

With the PCB congener-based evidence for reductive dechlorination in IHSC sediments, we aimed next to characterize the PCB biodegradation potential by estimating the abundance of total 16S rRNA genes, putative *Chloroflexi*16S rRNA genes, and *bph*A, a functional gene associated with aerobic PCB oxidation. The total bacterial 16S gene abundance was relative stable with sediment depth ($2.37\times10^{9} \pm 2.41\times10^{9}$ 16S rRNA genes per g sediment) (Figure 4.4). The lowest 16S rRNA gene abundance was from 0-0.30 m sediment, possibly because this section was at the water-sediment surface and had relatively high water content compared with the other sediment samples.

The abundance of *bph*A averaged $7.39\times10^{6} \pm 3.96\times10^{6}$ genes per g sediment (0-1.83m depth), and $3.20\times10^{5} \pm 4.58\times10^{5}$ gene copy per g sediment(1.83 - 4.57m depth). Especially, *bph*A gene abundances were significantly correlated with total sediment PCB concentrations in the upper 1.83 m, which could indicate a microbial response to PCB contamination in the sediments (Pearson's R, $p<0.05$).

Although the upper 1.83m was anaerobic as suggested by negative ORP data (-13- -200mv) (Martinez *et al.*, 2013),micro-aerobic conditions could possibly exist in some portions of the sediment. The sharp decrease in *bph*A abundance below 1.83m could be explained by the presence of a stricter anaerobic environment. A strong positive correlation was observed between the *bph*A abundance and the ORP along depth (Pearson's R, $p<0.01$).

Two primer sets (chl384f/884r and dhc793f/946r) were used to quantify the

dechlorinating *Chloroflexi* group in sediments. According to the clone library results, both of the two primer sets primarily amplified *Dehalogenimonas* sequences. (Table S3). Some isolates of *Dehalogenimonas* strains can reductively dehalogenate polychlorinated alkanes (Maness et al., 2012; Yan et al., 2009). Also, recent study found that *Dehalogenimonas* can couple their growth with PCB dechlorination in a sediment free culture, indicating their role in PCB dechlorination (Wang and He, 2013).

Putative dechlorinating *Chloroflexi* 16S rRNA gene abundances were $2.53 \times 10^5 \pm 2.45 \times 10^5$ genes per g sediment (0-1.83m depth) and $1.53 \times 10^4 \pm 2.80 \times 10^4$ genes per g sediment (1.83-4.57 m depth) as assessed by chl384f/884r, while the gene abundance averaged $2.15 \times 10^5 \pm 2.07 \times 10^5$ gene copy per g sediment (0-1.83m depth) and $7.64 \times 10^3 \pm 1.30 \times 10^4$ gene copy per g sediment (1.83-4.57 m depth) as assessed by dhc793f/946r. These gene abundances are lower than observed in sediments from other PCB-contaminated sites, which were on the order of $10^6$-$10^8$genes per g sediment ,possibly because these other sites contained higher PCB concentrations (Kjellerup et al., 2008; Xu et al., 2012).

The co-occurrence of putative dechlorinating *Chloroflexi* 16S rRNA genes and *bph*A indicate the potential for simultaneous aerobic and anaerobic PCB degradation in the upper 1.83 m of the sediment. However, the pattern of putative dechlorinating *Chloroflexi* population does not appear to be consistent with the congener profile, which displayed greater dechlorination potential in sediments deeper than 1.83 m. A possible explanation is that some other *Chloroflexi* members were degrading PCBs in deep sediments and were not detected by the primers used in this study. It is also possible that the congener profiles in the deep sediments were the record of dechlorination from the past. In addition, the 16S rRNA gene based phylogenetic analysis of *Chloroflexi* doesn't directly identify the dechlorinating capability of the microbial community. Reductive dehalogenase gene would be a better indicator of microbial dechlorination, but due to the lack of knowledge about the PCB dehalogenase, no primer targeting PCB dehalogenase

is currently available.

Relationships between *bph*A, putative dechlorinating *Chloroflexi* 16S rRNA genes, and total bacterial 16S rRNA gene abundance with environmental variables were explored by calculating Pearson correlations. No positive correlations were observed between any of these gene abundances and the total PCB concentration, or with total organic carbon.

## Relationships between microbial community structure and PCB congener profiles

To explore the possibility that other microbial community members could be relevant to PCB biodegradation potential in IHSC sediments, we evaluated microbial community structure with depth using T-RFLP analysis. A significant correlation between the overall sediment T-RFLP profile and PCB congener profile was noted when using the Mantel test ($p=0.001$ for sediment, $p=0.001$ for pore water). This correlation was confirmed with the Procrustes test ($p=0.002$ for sediment, $p=0.001$ for pore water). This suggests that sediment sections with similar PCB congener profiles tend to have similar bacterial community structures.

NMDS ordination of the T-RFLP profiles revealed that bacterial community structures in the shallow sediment sections (0-1.83 m depth) were distinct from the structure in deeper sediments (1.83-4.57 m depth) (Figure 4.5A). The disparity between shallow and deep sediments was also found by qPCR, which show much less abundant *bph*A, putative *Chloroflexi*16S rRNA genes, and *Dehalococcoides*-like 16S rRNA genes in sediments of1.83-4.57 m deep.

The microbial community composition in sediments at 3.35-3.66 m depth was assessed by sequencing 91 clones from a 16S rRNA gene clone library. This indicated that the community was dominated by *Proteobacteria* (11.0% *Alphaproteobacteria*, 34.1% *Betaproteobacteria* and 54.9% *Gammaproteobacteria*). At the genus level, *Acinetobacter*

spp. and *Acidovorax* spp. were dominant, comprising 45.1% and 20.9%of the clones, respectively.

A 196bp T-RF, which was abundant in sediments deeper than 1.22 m (26.9% ± 13.0%) (Figure 4.5B), was significantly correlated with sediment and pore water MDPR values (Pearson's R, $p<0.001$). This T-RF was identified to be from an *Acidovorax* sp. when compared with virtually digested T-RFs from a 16S rRNA gene clone library (91 clones) from sediments of 3.35-3.66 m. The corresponding T-RFs of *Acinetobacter* were 198bp, 199bp and 252bp (Table 4.4). A 199 bp T-RF comprised 20.0% of all T-RFs in 3.35-3.66 m section, but was only 0-2.5% in other sections. Clones identified as *Methylotenera* and unclassified *Comamonadaceae* also generated a 199 bpT-RF, but the majority of the 199 bpT-RFs in the clone library were classified as *Acinetobacter*. Two clones from the clone library generated a 198 bp T-RF. This T-RF was not found in T-RFLP profiles of 3.35-3.66 m sediments, but was found in 0-1.52m sediments (1.97-9.51% relative abundance) and had a relative abundance of 18.74% in sediments at 4.27-4.57 m depth. A 252 bp T-RF was also identified as coming from an *Acinetobacter* sp., and had a relative abundance range of 0.62-8.10% along the core. Overall, this analysis revealed that although *Acinetobacter* was an abundant (and thus potentially important) community member in the core sediments. However, there was no correlation between TRFs identified as *Acinetobacter* and sediment or pore water MDPR values.

*Proteobacteria* are often found abundant in soils and sediments (de Cárcer et al., 2007; Petrić et al., 2011a; Spain et al., 2009). However, an increase in *Betaproteobacteria* and *Alphaproteobacteria* abundance was observed in PCB-exposed samples in a microcosm study (Correa *et al.*, 2010). Many known PCB-degraders are *Proteobacteria* (e.g.*Pseudomonas, Acidovorax, Acinetobacter,Comamonas* and *Burkholderia* (Bedard et al., 1986; Furukawa and Fujihara, 2008)). Both *Acidovorax* and *Acinetobacter* are known to degrade PCBs and were commonly found in PCB-contaminated sites (de Cárcer et al., 2007; Ionescu et al., 2009; Shuai et al., 2010; Slater

et al., 2011). Aerobic PCB biodegradation was likely not active in sediments deeper than 1.83 m, as indicated by negative ORP data and the low abundance of *bph*A. So it is unlikely that *Acinetobacter*, *Acidovorax* or other possible PCB degraders were carrying out aerobic PCB degradation in deep sediments.

Some *Acidovorax* species are anaerobic and are involved in nitrate reduction, but are not known to be associated with dechlorination (Byrne-Bailey et al., 2010; Hohmann et al., 2010). *Acinetobacter* has been detected in sediments deeper than 100 m (Breuker *et al.*, 2011), and studies have reported that some *Acinetobacter* species are capable of dechlorinating and degrading chlorobenzoate, chlorophenols and chloroanilines (Adriaens and Focht, 1991; Copley and Crooks, 1992; Hongsawat and Vangnai, 2011; Kim and Hao, 1999). *Acinetobacter*has been found in PCB-dechlorinating JN cultures (Bedard *et al.*, 2007), although no evidence yet suggests that *Acinetobacter* can dechlorinate PCBs.

*Chloroflexi* was not detected in the 16S rRNA gene clone library, probably because of its relatively low abundance in the sediment sample with respect to the total 16S rRNA gene abundance. This was verified by our qPCR results. Yet PCB congener profile analysis suggests extensive PCB dechlorination in deep sediments, indicating that there might be unknown dechlorinating species other than *Chloroflexi* were degrading PCBs.

## Conclusions

In general, both aerobic and anaerobic PCB degradation potential at IHSC were assessed by the combination of the PCB congener analysis and the microbial analysis. PCB congener profiles of the core sediment revealed that *in situ* dechlorination has occurred. The presence of microbial dechlorinating groups and *bph*A in the upper 2-m sediments suggested that the indigenous microbial communities contained the potential

for both aerobic and anaerobic PCB degradation. In deeper sediments, the microbial communities were different from those in upper sediments as suggested by T-RFLP profiles. *Acinetobacter* was highly abundant in deep sediments, suggesting their possible role in PCB dechlorination. Overall our results provide a comprehensive understanding about the *in situ* PCB degradation potential at the IHSC sediment, and suggest that natural attention of PCBs could continue in IHSC sediments stored at the CDF after being dredged.

Table 4.1. Primer sets used for qPCR analysis in Chapter IV.

| Target gene | Primer name | Sequences | Expected product size | Source |
|---|---|---|---|---|
| total bacterial 16S rRNA | 16SU f | 5'-TCCTACGGGAGGCAGCAGT-3' | 466 bp | (Nadkarni *et al.*, 2002) |
| | 16SU r | 5'-GGACTACCAGGGTATCTAATCCTGTT-3' | | |
| *bph*A | *bph*A 463f | 5'-CGCGTSGMVACCTACAARG-3' | 211 bp | (Petrić et al., 2011b) |
| | *bph*A 674r | 5'-GGTACATGTCRCTGCAGAAYTGC-3' | | |
| putative dechlorinating *Chloroflexi* 16S rRNA | chl348f | 5'-GAGGCAGCAGCAAGGAA-3' | 514 bp | (Fagervold *et al.*, 2005) |
| | dehal884r | 5'-GGCGGGACACTTAAAGCG-3' | | |
| *Dehalococcoides*-like 16S rRNA | dhc 793f | 5'-GGGAGTATCGACCCTCTCTG-3' | 193 bp | (Yoshida *et al.*, 2005) |
| | dhc 946r | 5'-CGTTYCCCTTTCRGTTCACT-3' | | |

Table 4.2. Pertinent qPCR parameters (primer concentration, template mass, linear range, PCR efficiency, and y-intercept of the qPCR standard curve) with core 2 sediment DNA as template.

| Target gene | primer concentration, μM | template | qPCR linear range, gene copies/reaction | qPCR efficiency | Y-intercept |
|---|---|---|---|---|---|
| total bacterial 16S rRNA | 0.1 | 1 ng DNA | 30 - 30 × $10^7$ | 90 - 100% | 37.07 |
| *bph*A | 1 | 10 ng DNA | 40 - 40 × $10^7$ | 95 - 100% | 37.55 |
| putative dechlorinating *Chloroflexi* 16S rRNA | 0.4 | 10 ng DNA | 40 - 40 × $10^5$ | 90 - 100% | 38.02 |
| *Dehalococcoides*-like 16S rRNA | 0.8 | 10 ng DNA | 30 - 30 × $10^7$ | 95 - 100% | 36.55 |

Table 4.3. RDP classification of clone libraries for qPCR primer quality assurance with core 2 sediment DNA as template.

| Primer set | Closest classified relative (% certainty) | No. of clones detected |
|---|---|---|
| chl348f/dehal884r | *Dehalogenimonas* (80-100) | 21/22 |
| | Unclassified *Chloroflexi* (80) | 1/22 |
| dhc793f/946r | *Dehalogenimonas* (83-100) | 8/10 |
| | Unclassified *Chloroflexi* (83-85) | 2/10 |

Table 4.4. 16S rRNA gene clones recovered from sediment at the depth of 3.35-3.66 m. Clone identification was made by RDP classifier.

| Observed T-RF (bp) | Predicted T-RF (bp) | Closest classified relative (% certainty) | No. of clones | NMDS axis 1 | NMDS axis 2 |
|---|---|---|---|---|---|
| 196 | 198 | *Acidovorax* (93-100) | 19 | 0.717 | -0.052 |
| 196 | 198 | Unclassified *Comamonadaceae* (99) | 1 | 0.717 | -0.052 |
| 197 | 198 | *Methylotenera* (100) | 1 | -0.241 | 1.070 |
| 198 | 199 | *Acinetobacter* (100) | 2 | -0.430 | 0.276 |
| 199 | 200 | *Acinetobacter* (100) | 27 | 0.316 | -0.393 |
| 199 | 200 | Unclassified *Comamonadaceae* (90) | 1 | 0.316 | -0.393 |
| 199 | 200 | *Methylotenera* (100) | 2 | 0.316 | -0.393 |
| 217 | 219 | *Janthinobacterium* (97) | 1 | -0.138 | 0.358 |
| 223 | 225 | *Rhizobium*(95-97) | 2 | -0.176 | -0.444 |
| 226 | 227 | *Novosphingobium* (100) | 1 | -0.644 | -0.056 |
| 252 | 253 | *Acinetobacter* (99-100) | 10 | 0.276 | -0.151 |
| 316 | 317 | *Comamonas* (99-100) | 5 | 0.625 | -0.301 |

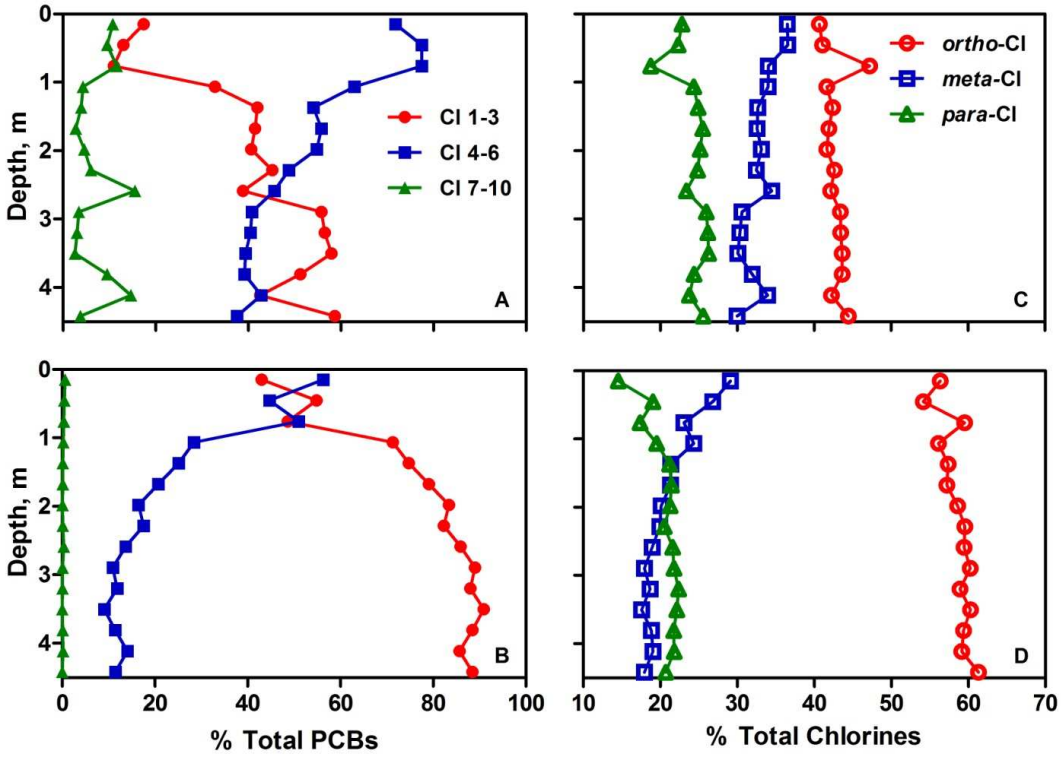


Figure 4.1. Fraction of light, medium and heavy PCBs versus sediment depth in core 2 (A) and pore water (B), and fraction of *ortho*-, *meta*-, *para*-chlorines versus sediment depth in core sediment (C) and pore water(D).

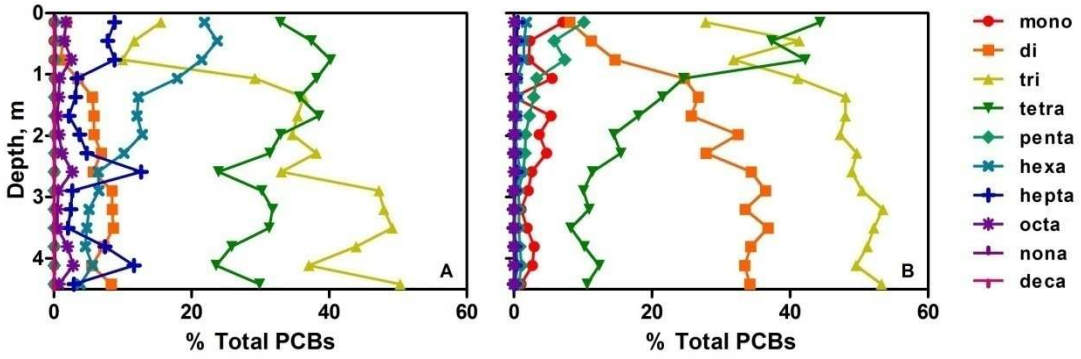


Figure 4.2. Fraction of each PCB homolog group in sediment (A) and pore water (B) against sediment depth for core 2.

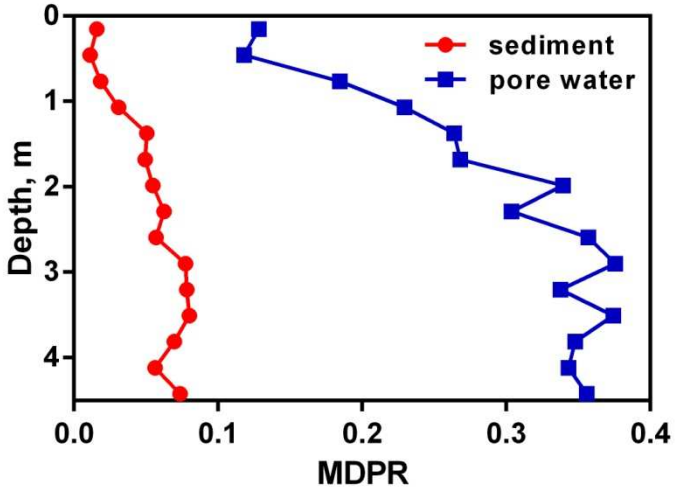


Figure 4.3. Sediment and pore water MDPR values against sediment depth for core 2.

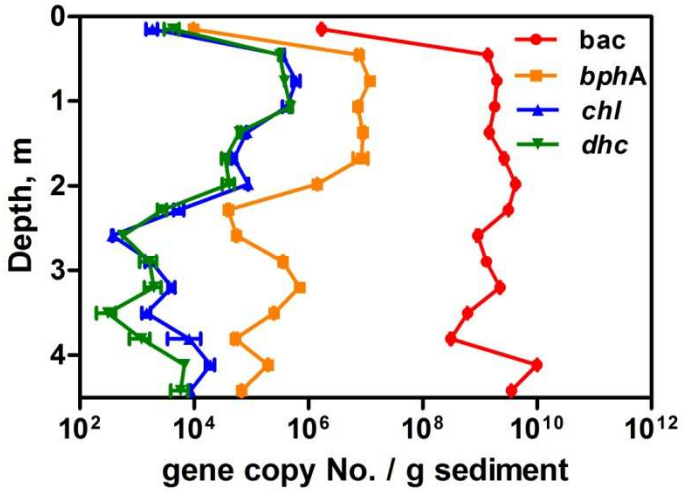


Figure 4.4. Abundances of total bacteria (bac), *bph*A, putative *Chloroflexi* 16S rRNA genes (chl) and *Dehalococcoides*-like 16S rRNA (dhc) against sediment depth by gene copy No. per g sediment for core 2.

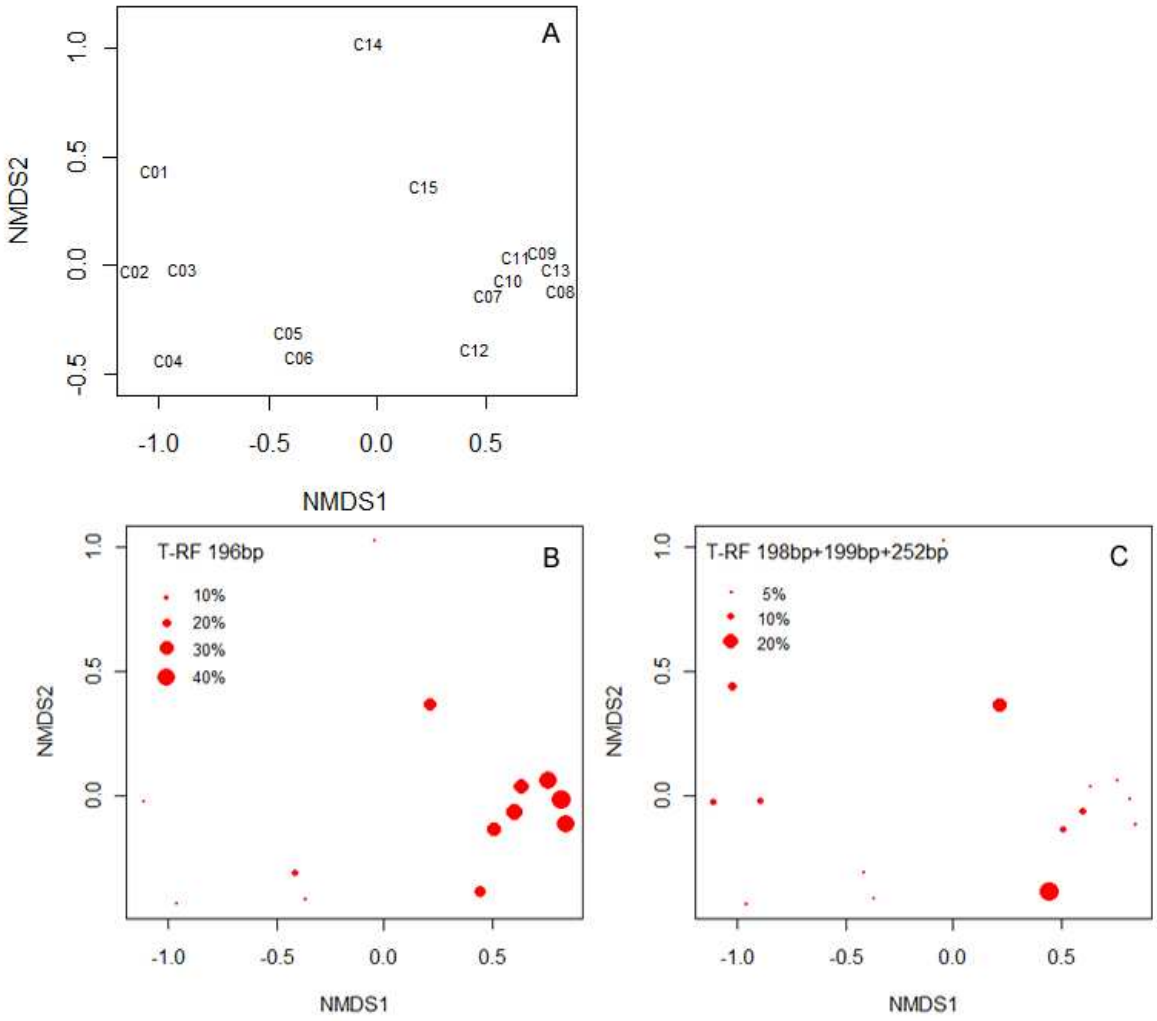


Figure 4.5. NMDS ordination and cluster analysis of T-RFLP profiles of core 2 sediment (C01: 0-0.30 m, C02: 0.30-0.61 m…) (A), the abundance of T-RF 196 bp (B) and 198 bp+199 bp + 252 bp (C) along sediment depth.

# CHAPTER V. ENHANCED POLYCHLORINATED BIPHENYL REMOVAL IN A SWITCHGRASS PLANTED RHIZOSPHERE BY BIOAUGMENTATION WITH *BURKHOLDERIA XENOVORANS* LB400

## Introduction

Conventional remediation strategies such as soil excavation and the following incineration or landfilling are usually expensive and unsustainable (Amend and Lederman, 1992). Phytoremediation, the use of plants and associated microbes to remove and detoxify PCBs, represents a potentially cost effective and less disruptive approach to clean up PCB contaminated soils and sediments (Van Aken *et al.*, 2010).

The presence of specific plant species is known to enhance the PCB removal efficiency in soils and sediments (Campanella et al., 2002; Chekol et al., 2004; Ionescu et al., 2009; Slater et al., 2011; Smith et al., 2007). Plants can uptake PCBs from soil and convert lower chlorinated congeners into hydroxylated products (Ficko et al., 2010; Greenwood et al., 2011; Kučerová et al., 2000; Lee and Fletcher, 1992; Rezek et al., 2007; Zhai et al., 2010b). Also, plants contribute to PCB biodegradation by providing a favorable environment for PCB-degrading microorganisms and can thus increase PCB-degrading populations (de Cárcer et al., 2007; Leigh et al., 2006; Slater et al., 2011; Uhlík et al., 2009). Plants not only increase soil permeability and oxygen diffusion in the rhizosphere, but also release organic exudates as inducers, surfactants and microbial growth factors thereby stimulating and facilitating microbial PCB biodegradation in the root zone (Gilbert and Crowley, 1997; Hernandez et al., 1997; Leigh et al., 2002; Van Aken et al., 2010). For example, augmentation of soil with plant-derived compounds such as carvone and salicylic acid assisted aerobic PCB degraders and increased PCB removal (Singer et al., 2000; Singer et al., 2003b).

Microorganisms are known to degrade PCBs via two general processes: aerobic oxidative biphenyl ring cleavage and anaerobic reductive dechlorination (Adrian et al., 2009; Borja et al., 2005; Gibson and Parales, 2000; Pieper, 2005; Wiegel and Wu, 2000). Most aerobic PCB-degraders employ the upper biphenyl degradation pathway, and biphenyl 2,3-dioxygenase (BphA) catalyzes the first step in this pathway, transforming susceptible PCB congeners into the corresponding *cis*-dihydrodiol (Furukawa, 1994). Under anaerobic conditions, some members of the phylum *Chloroflexi* use certain PCB congeners as electron acceptors and transform them into less chlorinated congeners (Adrian et al., 2009; Fagervold et al., 2007; Fagervold et al., 2005; Wiegel and Wu, 2000). Some efficient microbial PCB degraders, such as *Burkholderia xenovorans* strain LB400, *Arthrobacter* sp. strain B1B, *Ralstonia eutropha* H850, *Dehalococcoides chlorocoercia* DF1 have been isolated and introduced to contaminated soils and sediments to improve PCB removal (Payne et al., 2013; Payne et al., 2011; Petrić et al., 2011b; Singer et al., 2000).

In this study, we monitor the removal of three selected PCB congeners (PCB 52, PCB 77 and PCB 153) in switchgrass-planted soil microcosms and attempt to improve PCB degradation by introducing biphenyl-grown *Burkholderia xenovorans* strain LB400 into the rhizosphere of selected microcosms. The PCB congeners in this study were chosen because they represent bottlenecks in environmental PCB degradation (Bedard *et al.*, 1986). PCB 52 and 153 are both *ortho*-chlorinated, while PCB 77 has a dioxin-like structure, and is considered one of the most toxic congeners (Van den Berg *et al.*, 2006). All three congeners are quite ubiquitous in the environment (Meggo et al., 2013). Enhanced degradation of these congeners was observed in switchgrass treated soil, and following bioaugmentation with LB400 PCB 52 was significantly improved. The combined use of switchgrass and LB400 bioaugmentation was found to be most efficient for specific PCB congener removal in this experiment.

## Materials and Methods

### Soil reactor set up[4]

The soil microcosm set-up procedure has been described previously (Meggo and Schnoor, 2013). Briefly, PCB-free soil from the village of Middle Amana in Iowa, USA, was passed through a 60 mesh sieve before experiments. The soil was spiked with a mixture of PCB 52, PCB 77 and PCB 153 (99% pure) (Accustandard Inc., New Haven, CT) at the concentration of 500 ng $g^{-1}$ each. PCB congeners were dissolved in hexane before adding to the soil. A quartering technique was used to homogenize soil. The soil was divided into four quadrants on a quartering canvas, and 20 diagonal trajectories were used to mix the soil components. The contaminated soil was aged for two months at $25^0$C in sealed tubs to allow PCBs sequestration into the soil matrix and thereby better represent field conditions. To establish the initial PCB concentration, 20 subsets of soil collected from different locations were homogenized and measurements were performed in triplicates (Meggo and Schnoor, 2013).

Plastic containers (33.8cm × 21.6cm × 211.9cm) and lids with aluminum foil covers were used for soil reactor set-up. Each reactor was filled with 2500g sieved and homogenized soil. The spiked soil was planted with switchgrass (*Panicum virgatum)* seeds (Adams-Briscoe Seed Co., Jackson, GA). Switchgrass in all six reactors was grown at 25°C in a plant growth chamber under a 16 hour light/8 hour dark photo period (light intensity of 200 mmol $m^{-2}s^{-1}$ and 60% humidity). Soil samples were taken for PCB congener analysis and microbial analysis. Soil samples were taken at 12 week and 24 week for PCB congener analysis. For microbial analysis, soil samples were taken every four weeks starting at 12 week (Meggo and Schnoor, 2013).

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[4] Soil reactor set-up was performed by Richard Meggo, Ph.D., Department of Civil & Environmental Engineering, The University of Iowa.

## *Burkholderia xenovorans* strain LB400 bioaugmentation

Bioaugmentation with *Burkholderia xenovorans* strain LB400 was conducted with both switchgrass-treated and unplanted microcosms every each month. Prior to bioaugmentationLB400 was grown on solid biphenyl (1 g) as sole carbon and energy source in K1 medium until exponential phase ($OD_{600}$ 0.6~0.8) (Zaitsev and Karasevich, 1985). Cells were harvested by centrifugation (5000 × g, 15 min), washed once with sterileK1 medium, resuspended in K1 medium and inoculated into reactors (approximately $10^9$ CFU per g soil). Autoclaved LB400 was added to a switchgrass planted reactor as control.

Abundance of cultivable aerobic biphenyl-degrading bacteria was estimated by CFU enumeration one day after each bioaugmentation. Soil samples (1 g)were mixed with 9 ml of 0.9% sodium chloride solution and shaken for 1 h at 225 rpm on a platform shaker (New Brunswick Scientific, Pittsburgh, PA) at 25°C. Serially diluted supernatant was spread on K1 agar plates with biphenyl crystals as sole carbon and energy source. CFUs were enumerated after 72h of incubation at 30°C.

## PCB extraction and quantification[5]

The PCB extraction procedure has been previously described (Meggo *et al.*, 2013). Briefly, PCBs in soil and plant material (roots and shoots) was extracted by adding a 1:1 hexane/acetone mixture (3 ml/g) to homogenized soil or plant material (5 g) and sonicated for 1 h. Before sonication, surrogate standards including PCB 14 (3,5-dichlorobiphenyl), deuterated PCB 65 (2,3,5,6-tetrachlorobiphenyl) and PCB 166 (2,3,4,4',5,6-hexachlorobiphenyl) (CambridgeIsotope Laboratories, Inc.) were added into

[5] PCB extraction was performed by Richard Meggo, Ph.D., Department of Civil & Environmental Engineering, The University of Iowa. PCB quantification was performed by Dingfei Hu, Ph.D., Department of Civil & Environmental Engineering, The University of Iowa.

the samples (50 ng of each surrogate) to account for any loss during the extraction. Surrogate recoveries were 103 ±17.2% (PCB 14), 97.9 ± 15.2% (PCB 65) and 97.7 ± 17.7% (PCB 166).

The sonicated material was centrifuged at 1500 × g for 5 min, after which the supernatant was transferred to a fresh vial. The precipitates were subjected to a second extraction. The combined supernatants of first and second extraction were evaporated to dryness using rotary evaporation and the solvent was changed to hexane. Double extraction with concentrated sulfuric acid and hexane was performed to remove of lipids and other polar substances. This hexane extract was concentrated to approximately 0.5 ml under a gentle stream of nitrogen. The concentrate was eluted with 10 ml of hexane through a filter consisting of 0.1 g of silica (70-230 mesh, Fisher Scientific, Inc.), 0.1 g of anhydrous sodium sulfate and 0.9 g silica gel acidified with $H_2SO_4$ (silica:$H_2SO_4$ = 2:1) (Meggo *et al.*, 2013).

The concentrated extracts were spiked with the internal standard containing 100 ng of PCB 204 (2,2',3,4,4',5,6,6'-octachlorobiphenyl). The samples were analyzed for PCB congeners using a gas chromatograph with mass selective detection (GC-MS/MS) modified from the EPA method 1668A (U.S.EPA 1999). The quantification of PCBs was performed by an Agilent 6890N gas chromatograph with an Agilent 7683 series auto sampler coupled to a Waters Micromass Quattro micro GC mass spectrometer (Milford, MA, USA) operating under electron impact (EI) positive mode at 70 eV and multiple reaction monitoring (MRM), and the trap current was 200 μA. The retention windows were defined by PCB parent/daughter ion pairs from mono- to deca- homologs which were 188/152, 222/152.10, 255.96/186, 291.92/222, 325.88/255.90, 359.84/289.90, 393.80/323.90, 427.76/357.80, 461.72/391.83, 497.68/427.70, respectively (Hu *et al.*, 2010).

## DNA and RNA extraction, reverse transcription, and quantitative PCR

Total RNA and DNA were extracted immediately after sampling from 2 g soil using the MoBio RNA PowerSoil Total RNA Isolation Kit (Mobio, Carlsbad, CA) and RNA PowerSoil DNA Elution Accessory Kit (Mobio, Carlsbad, CA) and stored at -80°C prior to further analysis. Before quantitative PCR (qPCR), RNA samples were subjected to contaminating DNA removal, RNA clean-up and reverse transcription. Contaminating DNA was removed by DNase I treatment according to the manufacturer's instructions (Biolab, Ipswich, MA). Each 100 µl DNase I treatment reaction contained 30 µl RNA and 20U DNase I. RNA was then purified with the RNeasy Mini Kit (Qiagen, Germantown, MD) and reversed-transcribed to cDNA by SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Each 80 µl reverse transcription reaction contained 40 µl purified RNA, 10mM dNTP mix, and 3 µg random primers.

To evaluate the RNA recovery efficiency during DNaseI treatment, RNA purification and reverse transcription, 1 ng of luciferase control mRNA (Promega, Madison, WI) was added to RNA sample before DNaseI treatment. After reverse transcription, luciferase cDNA was quantified by qPCR with the ref primer set (Table 5.1) as described previously (Johnson *et al.*, 2005). The RNA recovery efficiency averaged 14.2% ± 11.5%among all RNA samples processed in this study.

The abundances of total bacteria, aerobic PCB-degrading bacteria, and *Burkholderia xenovorans* strain LB400 were estimated by qPCR using the bacterial 16S rRNA gene primer set 16SU f/r (Nadkarni *et al.*, 2002), *bph*A primer set *bph*A 463f/674r (Petrić et al., 2011b), and LB400 16S-23S rRNA internal transcribed spacers(ITS) primer set LB400 84f/278r (Norini *et al.*, 2013), respectively (Table 5.1).Each 25 µl qPCR reaction contained 12.5 µl Power SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA), and variable amounts of primers and templates (Table C.2).Bovine serum

albumin(0.5 μg) was added to relieve possible PCR inhibition (Kreader, 1996). PCR conditions were as follows: 10 min at 95°C, followed by 40 cycles at 95°C (15 s) and 60°C (1 min) followed by a dissociation step. All qPCR was performed with an ABI 7000 Sequence Detection System (Applied Biosystem, Grand Island,NY) and fluorescence data was analyzed by ABI 7000 System SDS Software (Applied Biosystems, Grand Island,NY). With each primer set, the target gene was not detected in no template (DI water) controls (Ct value > 35). Additional qPCR information, such as primer concentrations, template concentrations, qPCR linear range, qPCR efficiency range of the standard curves, and Y-intercepts are provided in Table 5.2, in accordance with MIQE guidelines (Bustin *et al.*, 2009).

For luciferase mRNA qPCR, the standard DNA template was PCR products amplified from luciferase DNA with primer set refST (Table 5.1).For the bacterial 16S rRNA gene, the standard DNA template was prepared from PCR products amplified from *Burkholderia xenovorans* strain LB400 with primer set 8F/1492R (Klappenbach *et al.*, 2000). For *bph*A, the standard DNA templatewastheLB400 *bphA* (amplified with the 463f/674r primer set) cloned into the pCR 2.1-TOPO vector. For LB400 16S-23S rRNA ITS gene, the standard DNA template was the LB400 16S-23S rRNA ITS gene (amplified with the LB400 84f/278r primer set) cloned into the pCR 2.1-TOPO vector.

## Statistical analyses

PCB congener data was analyzed with an independent sample t test using R. Total bacterial 16S rRNA gene abundance, *bph*A gene and transcript abundance and LB400 16S-23S rRNA ITS abundance were analyzed with a two factor analysis of variance after log transformation using R (Rieu and Powers, 2009).

## Results and Discussion

### Enhanced degradation of specific PCB congeners in soil with switchgrass treatment

Significantly higher removal of PCB parent congeners (PCB 52, 77, 153) was achieved in the switchgrass-treated soil than in the unplanted soil after 24 weeks of incubation ($p<0.05$) (Figure 5.1). The presence of switchgrass enhanced PCB 52 removal more than the other two congeners (16.7%), while PCB 77 and PCB 153 removal was improved by 7.9% and 9.6%, respectively. Overall, 39.9 ±0.4% of the total PCB molar mass loss was observed in switchgrass treated soil after 24 weeks of incubation, compared with 29.5 ± 3.4% in unplanted soil (Figure 5.1). The increase of PCB removal efficiency by switchgrass in this study is comparable to some previous studies, although different plants were used (Ding et al., 2009; Ionescu et al., 2009; Secher et al., 2013; Smith et al., 2007).

Switchgrass has been observed previously to enhance PCB removal in soil and promote microbial activity in the rhizosphere (Chekol et al., 2004; Dzantor et al., 2000; Meggo et al., 2013).The enhanced PCB removal could be the result of rhizosphere biostimulation, rhizofiltration, phytoextraction, phytotransformation and phytovolatilization (Van Aken *et al.*, 2010). We investigated this possibility of phytoextraction by quantifying total PCBs in switchgrass roots and shoots. At the end of the 24 week experiment, we found 4.3-7.3% of total PCBs in switchgrass roots, and 0.11-0.24% in shoots, suggesting that plant extraction were active as PCB removal mechanisms (Table 5.3). All three parent congeners and some transformation products were detected in switchgrass roots and shoots. Especially, the detection of PCB 153 in switchgrass shoots demonstrated its capability to take up and translocate highly chlorinated PCB congeners. The uptake of PCBs by switchgrass and other plants has been reported (Ficko et al., 2010; Greenwood et al., 2011; Liu et al., 2009), although in some studies the effect of phytoextraction was considered to be negligible in removing

PCBs from soil because of the low percentage of total PCB mass observed in plant roots and shoots (<1% of total PCBs) (Di Gregorio et al., 2013; Huesemann et al., 2009; Li et al., 2011). In this study, the fraction of total PCB mass in switchgrass was higher than previous studies, probably because of the high plant biomass for each switchgrass treated soil microcosm (560 ± 51 g roots and 360 ± 41 g shoots per soil microcosm). Phytovolitilzation and phytotransformation were not considered to affect the PCB removal much, given the low PCB concentrations in switchgrass shoots. Previous study also showed that hydroxylated metabolite was not detected in switchgrass hydroponically exposed to PCBs, suggesting that PCB transformation in switchgrass was not active (Liu *et al.*, 2009).

Biostimulation of microbial PCB degradation was considered to be another mechanism contributing to enhanced PCB removal in the rhizosphere soil. The occurrence of aerobic microbial PCB degradation in both unplanted and switchgrass treated soils was verified by the decrease in total PCB molar mass (Figure 5.1A).The enhanced microbial activity was estimated to be responsible for about 3.05% total PCB removal from soil (Table 5.3). Different PCB congeners were affected differently by the enhanced microbial PCB degradation. It is estimated that 7.1% of PCB 52 removal was due to the enhanced microbial activity, while this number was 1.8% and 1.3% for PCB 77 and PCB 153, respectively. Microbial degradation of PCB 52 was promoted more than the other two congeners probably because PCB 52 is more susceptible for aerobic degradation by indigenous microorganisms. PCB 77 has a dioxin-like structure and was relatively more toxic than other tetrachlorinated biphenyls. The high chlorination level of PCB 153 results in high hydrophobicity and reduces its accessibility to enzymes. The bulkiness of the chlorine atoms may also prevent access to the enzyme's active site (Bedard *et al.*, 1986).

## Molecular biology analyses of unplanted and switchgrass planted soils

We estimated the abundance of total bacteria, biphenyl dixoygenase gene (*bph*A) and *bph*A transcripts as an independent means of tracking aerobic microbial PCB biodegradation during the experiment. Total bacterial abundance (as estimated by the abundance of bacterial 16S rRNA genes) was significantly higher in switchgrass reactors than in unplanted reactors ($p<0.05$, Figure 5.2A). We suspect that the switchgrass serves to improve oxygen diffusion and soil permeability. Switchgrass root exudates could also increase the microbial population in the rhizosphere by serving as a carbon and energy source.

Detecting bphA in both unplanted and switchgrass-treated soils suggests that the indigenous soil microbial community harbored the potential for aerobic PCB degradation. In the presence of switchgrass, *bph*A abundance was about 20 times higher ($p<0.05$, Figure 5.2B). However, *bph*A transcripts in both switchgrass planted and unplanted reactors were below our quantification limit of RT-qPCR (40 copies/qPCR reaction), suggesting that the *bph*A expression was not promoted much by the switchgrass. It is possible that the presence of other carbon sources in the rhizospehre limited the *bph*A expression (Parnell *et al.*, 2010). The availability of a variety of naturally-occurring organic carbon and plant exudates in the rhizosphere may make PCBs less favorable for indigenous microorganisms (Luo *et al.*, 2008). Soil extract may also inhibit *bph*A expression (Master and Mohn, 2001). The low level of *bph*A expression could also explain why microbial PCB degradation was not improved much by switchgrass as suggested by congener data.

## Enhanced microbial PCB degradation in soil with LB400 bioaugmentation

To further enhance aerobic degradation of PCB congeners in the rhizosphere,

biphenyl-grown *Burkholderia xenovorans* strain LB400 was introduced to the soil (at a loading of approximately $10^9$ CFU per g soil). LB400 is an effective aerobic PCB-degrader, capable of degrading a broad spectrum of PCB congeners including those used in this study (PCB 52, 77, 153) (Bedard et al., 1986; Bopp, 1986). With LB400 bioaugmentation, total PCB molar mass loss increased to 39.4±1.4% and 47.3 ±1.2 % in the unplanted and switchgrass-treated soil, respectively, after 24 weeks of incubation, suggesting enhanced aerobic PCB degradation (Figure 5.1). Removal of PCB 52, 77 and 153 was not significantly improved in the control switchgrass reactor, which was amended with autoclaved LB400 (Figure 5.1).

Specifically, PCB 52 removal appeared to be most improved by LB400 bioaugmentation, achieving 43.7% and 24.9% more removal in unplanted and switchgrass-treated soils, respectively ($p<0.05$).PCB 153 removal in switchgrass-treated soil with bioaugmentation increased by 3.3% as compared with soil with only switchgrass treatment ($p<0.05$). In unplanted soil with bioaugmentation, PCB 77 and 153 removal was not improved (Figure 5.1). This observation is consistent with previous LB400 PCB biodegradation studies, where LB400 displayed a stronger capability to degrade PCB 52 than PCB 77 and 153 (Bedard et al., 1986; Bopp, 1986; Rein et al., 2007). Compared with PCB 52, PCB 77 and 153 have higher $K_{ow}$ values and lower water solubilities. Although LB400 could degrade PCB 77 and 153 in the resting cell assays, in the soil microcosms, the two congeners may be sorbed strongly to soil particles and thus are less bioavailable.

## Molecular biology analyses of unplanted, switchgrass planted, and LB400 bioaugmented soils

The enhanced PCB removal in bioaugmented soil was associated with elevated abundance of *bph*A and its transcripts, suggesting that aerobic PCB degradation was actively occurring (Figure 5.3, Figure 5.4).In bioaugmented soil, *bphA* abundance

increased throughout incubation time, and was two orders of magnitude higher than in reactors without bioaugmentation ($p<0.05$, Figure 5.3B, Figure 5.3D). The transcripts of *bph*A were detected ($3.17\times10^5$ - $8.90\times10^5$ transcripts per g soil in unplanted soil with bioaugmentation, $4.73\times10^4$ - $4.14\times10^6$ transcriptsper g soil in switchgrass treated soil with bioaugmentation), suggesting the successful *bph*A expression (Figure 5.4).

Bioaugmentation did not significantly increase the total microbial abundance ($p>0.05$, Figure 5.3A, Figure 5.3C). Yet the reactor with autoclaved LB400 injection displayed greater total 16S rRNA gene abundance than the reactor with live LB400 bioaugmentation ($p<0.05$, Figure 5.3A), probably because debris from the autoclaved LB400 cells was used as carbon and energy source by indigenous microorganisms.

The survival of bioaugmented bacteria is crucial to the successful bioaugmentation and contaminant removal (Bouchez *et al.*, 2000). To improve LB400 survival and keep a relatively stable LB400 population, we performed bioaugmentation once a month. The survival of LB400 after bioaugmentation was estimated by quantifying biphenyl-degrading bacteria with CFU enumeration. After bioaugmentation, the biphenyl-degrading bacterial abundance increased ($4.7 \times 10^8$ -$1.9 \times 10^9$ CFU per g soil for unplanted reactor, $2.1\times 10^8$ -$2.3 \times 10^9$ CFU per g soil for switchgrass planted reactor). Yet this number decreased to the level of approximately $10^7$ CFU per g soil after four weeks, prior to the next bioaugmentation event (Figure 5.6).The decrease may result from the stressed environment caused by PCB (Kim et al., 2001; Parnell et al., 2006), and the competition with indigenous microbial community. LB400 16-23S ITS gene was quantified approximately two weeks after bioaugmentation, and ranged from $1.16 \times 10^7$to $4.15 \times 10^8$ gene copy per g soil (unplanted soil), and $9.61 \times 10^7$to $7.04 \times 10^8$ gene copy per g soil (switchgrass treated soil)(Figure 5.5). An accumulation of ITS genes was observed throughout the 24 week experiment, suggesting that after each bioaugmentation, a percentage of LB400 adapted to the soil environment and balance with indigenous microorganisms (Figure 5.5).

LB400 16-23S ITS gene quantification shows higher LB400 abundance with the presence of switchgrass (Figure 5.5). Also, the most PCB removal, the highest *bph*A and transcript abundances were observed in switchgrass treated soil with bioaugmentation after 24 weeks of treatment (Figure 5.1, Figure 5.3 and Figure 5.4), indicating switchgrass not only benefited LB400 survival, but also helped LB400 degrade PCBs. The support of plants to bioaugmentation was reported (Juhanson et al., 2009; Secher et al., 2013; Tam and Wong, 2008). Switchgrass helped LB400 survival may be by improving aeration and providing root exudates as extra carbon and energy source, the same way as it benefited indigenous microorganisms. The root system can help spread LB400 through the PCB contaminated soil. Furthermore, switchgrass may increase the bioavailability of PCB molecules by releasing organic compounds as complexing agents.

## Conclusions

In this study, switchgrass was found to be able to improve the removal of PCB 52, 77 and 153 by phytoextraction and biostimulation. PCB 52 was more susceptible to microbial degradation, and its removal was more improved by switchgrass and bioaugmentation with LB400 than PCB 77 and 153. Switchgrass was beneficial for LB400 survival and its ability to perform aerobic degradation. The combination of phytoremediation and bioaugmentation offers an efficient and environmental-friendly strategy to eliminate recalcitrant PCB congeners and remediate PCB contaminated soil.

Table 5.1. Primer sets used in qPCR analysis in Chapter V.

| Target gene | Primer name | Sequences | Expected product size | Source |
|---|---|---|---|---|
| luciferase mRNA | ref f | 5'-TACAACACCCCAACATCTTCGA-3' | 150 bp | (Johnson *et al.*, 2005) |
| | ref r | 5'-GGAAGTTCACCGGCGTCAT-3' | | |
| total bacterial 16S rRNA | 16SU f | 5'-TCCTACGGGAGGCAGCAGT-3' | 466 bp | (Nadkarni *et al.*, 2002) |
| | 16SU r | 5'-GGACTACCAGGGTATCTAATCCTGTT-3' | | |
| *bph*A | *bph*A 463f | 5'-CGCGTSGMVACCTACAARG-3' | 211 bp | (Petrić et al., 2011b) |
| | *bph*A 674r | 5'-GGTACATGTCRCTGCAGAAYTGC-3' | | |
| LB400 16S-23S ITS | LB400 84f | 5'-GAAATTGAAGACAGAAACGCA-3' | 191 bp | (Norini *et al.*, 2013) |
| | LB400 278r | 5'-AGTCATGCACACACCAGAT-3' | | |

Table 5.2. qPCR reaction set up, linear range and efficiency for qPCR reaction in Chapter V.

| Target gene | primer conc, μM | Template | qPCR linear range, gene copies/reaction | qPCR efficiency | Y-intercept |
|---|---|---|---|---|---|
| luciferase mRNA | 0.1 | 2 μl cDNA | 30 - 30 × $10^7$ | 95 - 100% | 37.65 - 38.61 |
| total bacterial 16S rRNA | 0.1 | 1 ng DNA | 30 - 30 × $10^7$ | 95 - 100% | 35.10 - 35.84 |
| *bph*A | 1 | 10 ng DNA or 2μl cDNA | 40 - 40 × $10^7$ | 95 - 100% | 36.43 - 37.64 |
| LB400 16S-23S ITS | 0.2 | 10 ng DNA | 40 - 40 × $10^7$ | 90 - 100% | 36.54 - 37.44 |

Table 5.3. Molar mass balance of PCBs in unplanted soil (UP), switchgrass treated soil (SG), unplanted with bioaugmentaion soil (UP B), switchgrass treated with bioaugmentation soil (SG B), and switchgrass treated with bioaugmentation with dead LB400 soil (SG BD). (n=3).

| | | PCB fraction in soils | PCB fraction in roots | PCB fraction in shoots | Total recovery |
|---|---|---|---|---|---|
| Overall | UP | 70.5 ± 3.4% | - | - | 70.5 ± 3.4% |
| | SG | 60.1 ± 0.4% | 7.3 ± 0.2% | 0.11 ± 0.01% | 67.5 ± 0.6% |
| | UP B | 60.6 ± 1.4% | - | - | 60.6 ± 1.4% |
| | SG B | 52.7 ± 1.2% | 4.3 ± 0.1% | 0.24 ± 0.03% | 57.3 ± 1.4% |
| | SG DB | 57.1 ± 10.4% | - | - | 57.1 ± 10.4% |
| PCB 52 | UP | 98.6 ± 3.9% | - | - | 98.6 ± 3.9% |
| | SG | 81.9 ± 0.6% | 9.4 ± 0.2% | 0.17 ± 0.01% | 91.4 ± 0.8% |
| | UP B | 54.8 ± 0.8% | - | - | 54.8 ± 0.8% |
| | SG B | 57.0 ± 1.7% | 4.7 ± 0.1% | 0.40 ± 0.04% | 62.2 ± 1.9% |
| | SG DB | 76.7 ± 10.7% | - | - | 76.7 ± 10.7% |
| PCB 77 | UP | 61.0 ± 4.0% | - | - | 61.0 ± 4.0% |
| | SG | 53.2 ± 1.2% | 6.0 ± 0.2% | 0.06 ± 0.01% | 59.2 ± 1.4% |
| | UP B | 62.1 ± 1.8% | - | - | 62.1 ± 1.8% |
| | SG B | 53.1 ± 1.4% | 3.8 ± 0.2% | 0.16 ± 0.01% | 57.1 ± 1.6% |
| | SG DB | 51.1 ± 9.4% | - | - | 51.1 ± 9.4% |
| PCB 153 | UP | 66.1 ± 2.4% | - | - | 66.1 ± 2.4% |
| | SG | 56.5 ± 0.0% | 8.2 ± 0.2% | 0.11 ± 0.02% | 64.8 ± 0.25% |
| | UP B | 69.7 ± 1.5% | - | - | 69.7 ± 1.5% |
| | SG B | 53.2 ± 0.6% | 5.0 ± 0.1% | 0.17 ± 0.02% | 58.4 ± 0.7% |
| | SG DB | 53.7 ± 9.0 % | - | - | 53.7 ± 9.0 % |

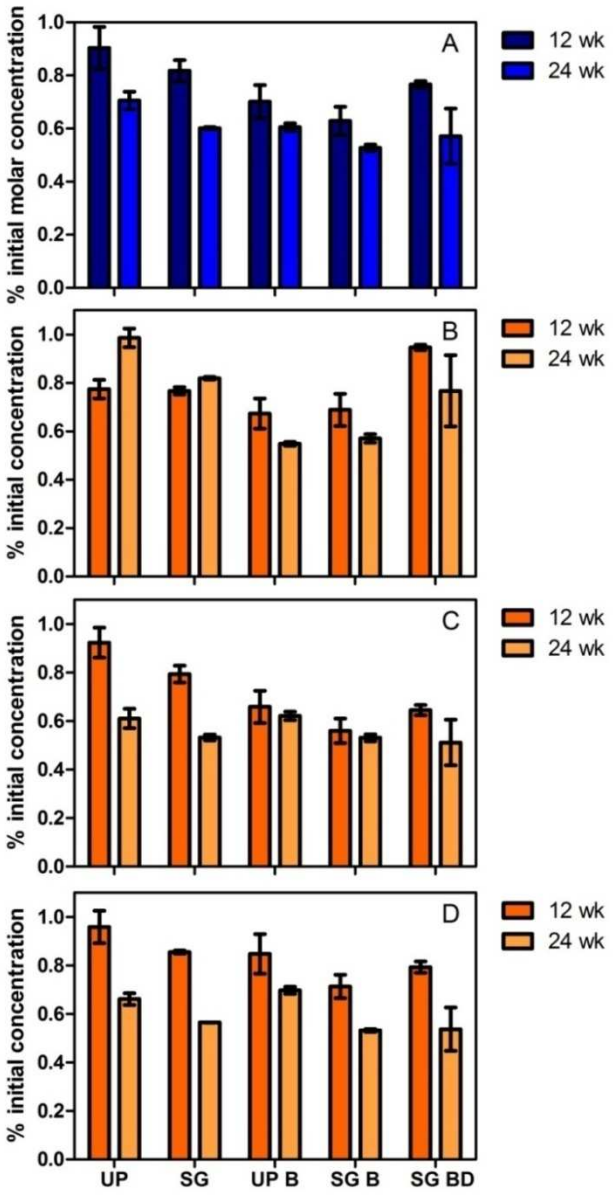


Figure 5.1. The percentage of initial molar concentrations for total PCB (A), PCB 52 (B), PCB 77 (C), and PCB 153(D) after 12 weeks and 24 weeks incubation in unplanted soil (UP), switchgrass treated soil (SG), unplanted with bioaugmentaion soil (UP B), switchgrass treated with bioaugmentation soil (SG B), and switchgrass treated with bioaugmentation with dead LB400 soil (SG BD). Error bars indicate the standard deviation of three soil subsamples from the same reactor.

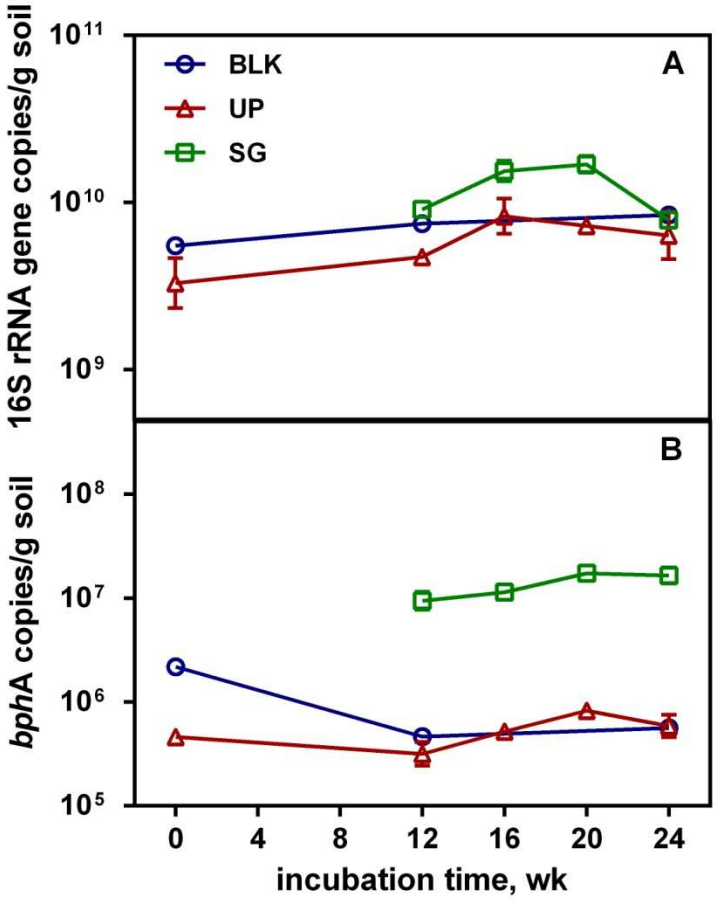


Figure 5.2. qPCR analysis of (A) bacterial 16S rRNA genes and (B) *bph*A in blank (BLK), PCB spiked and unplanted soil (UP), PCB spiked and switchgrass treated soil (SG). Error bars indicate the range of two soil subsamples from the same reactor.

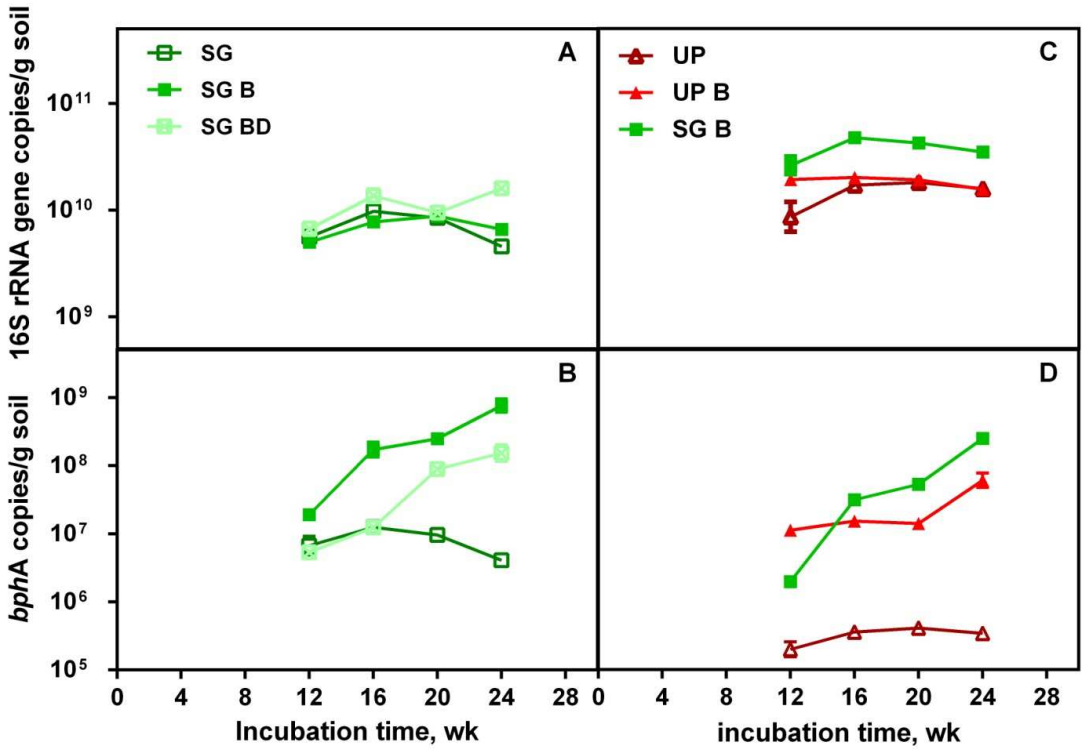


Figure 5.3. qPCR analysis of bacterial 16S rRNA gene (A) and *bph*A (B) in switchgrass treated soil (SG), switchgrass treated soil with bioaugmentation (SG B), switchgrass treated soil with autoclaved LB400 bioaugmention (SG BD); and bacterial 16S rRNA gene (C)and *bph*A (D) inunplanted soil (UP), unplanted soil with LB400 bioaugmentation (UP B), switchgrass treated soil with bioaugmentation (SG B). Error bars indicate the range of two soil subsamples from the same reactor.

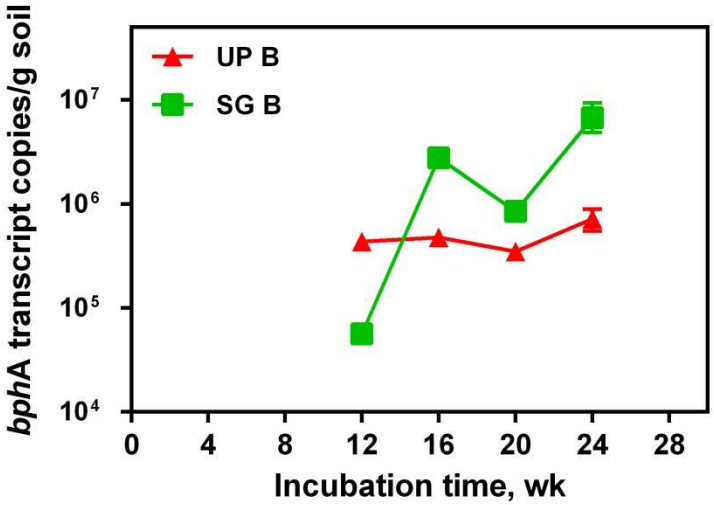


Figure 5.4. qPCR analysis of *bph*A transcripts in unplanted soil with bioaugmentation (UP B), and switchgrass treated soil with bioaugmention (SG B). Error bars indicate the range of two soil subsamples from the same reactor.

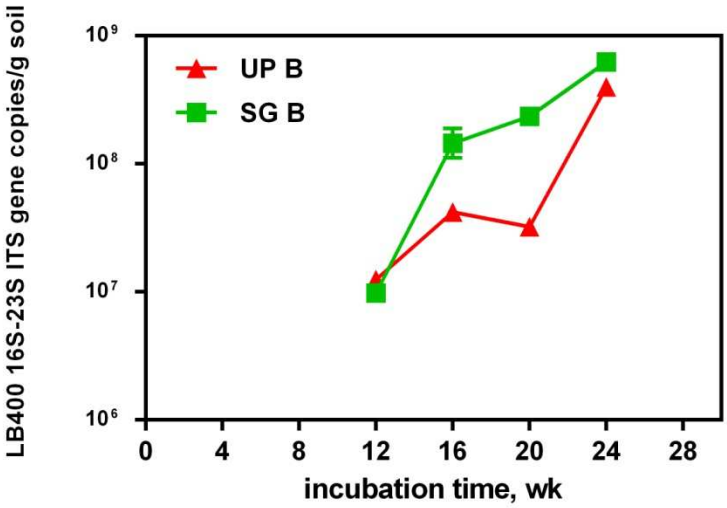


Figure 5.5. qPCR analysis of LB400 16S-23S ITS genes in unplanted soil with bioaugmentation (UP B), and switchgrass treatedsoil with bioaugmention (SG B). Error bars indicate the range of two soil subsamples from the same reactor.

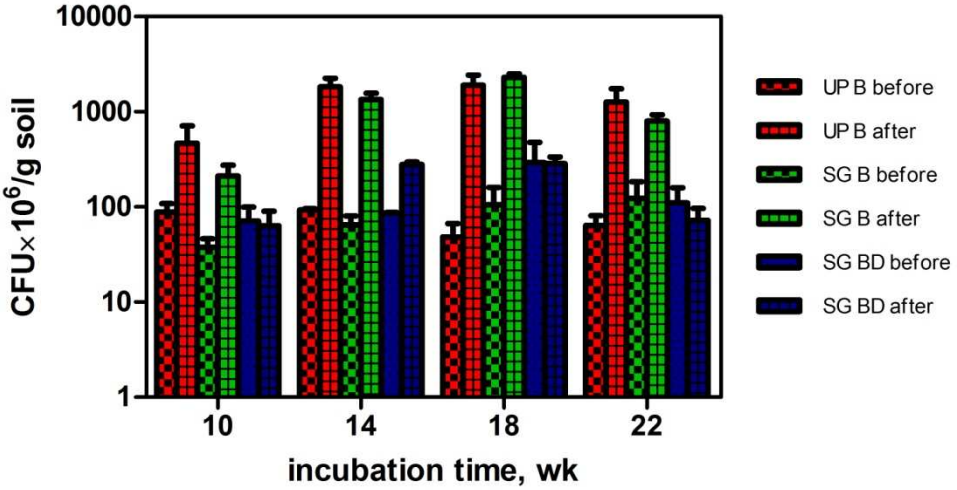


Figure 5.6. CFU counting for unplanted soil with bioaugmentation (UP B), switchgrass treated soil with bioaugmentation (SG B) and switchgrass treated soil with dead LB400 bioaugmentation (SG BD) before and after the application of bioaugmentation. Error bars indicate the standard deviation of three soil subsamples from the same reactor.

# CHAPTER VI. MICROBIAL COMMUNITY ANALYSIS OF A SWITCHGRASS RHIZOSPHERE WITH PCB SPIKING AND REDOX CYCLING

## Introduction

Bioremediation of PCB contaminated soils and sediments has always been challenging because of the hydrophobicity and recalcitrance of PCB congeners (Agarwal et al., 2007; Amend and Lederman, 1992). Phytoremediation of PCBs, the use of plants and associated microorganisms to remediate PCB contaminated soils and sediments, is more cost-effective and less disruptive to the environment (Van Aken *et al.*, 2010).

Microbial PCB degradation in the rhizosphere plays an important part in the phytoremediation of PCBs (Borja et al., 2005; Macková et al., 2010; Pieper and Seeger, 2008). Microorganisms are capable of degrading and detoxifying PCBs via anaerobic PCB dechlorination and anaerobic PCB mineralization (Borja et al., 2005; Gibson and Parales, 2000; Pieper and Seeger, 2008; Wiegel and Wu, 2000). Anaerobic PCB degraders can use PCB congeners as electron acceptors and remove chlorine atoms from the biphenyl ring or dechlorinate PCBs via co-metabolism (Adrian et al., 2009; Wiegel and Wu, 2000; Wu et al., 1998). So far all the known PCB degraders are within the *Chloroflexi* phylum, including the *Dehalococcoides* spp. and the o-17/DF-1 group (Fagervold et al., 2007; Fennell et al., 2004; Yan et al., 2006). Under aerobic conditions, some microorganism can mineralize PCBs via upper biphenyl pathway, leaving behind a chlorobenzoate as product (Gibson and Parales, 2000; Macková et al., 2010).

Many studies have confirmed the effect of plants on enhancing PCB degradation and increasing microbial PCB degrading populations (de Cárcer et al., 2007; Leigh et al., 2006; Macková et al., 2006; Slater et al., 2011). However, most of the current studies focus on the influence of plants on aerobic PCB degradation. The anaerobic dechlorination in the rhizosphere is rarely investigated. Here in this study, we set up soil

reactors with switchgrass treatment and created an anaerobic environment by flooding the soil to facilitate dechlorination. PCB congener analysis confirmed the occurrence of dechlorination in switchgrass treated soils, with or without redox cycling. Putative dechlorinating *Chloroflexi* was more abundant in redox cycled soils, as shown by qPCR. Other bacteria capable of dechlorination such as *Geobacter* and *Clostridium* were detected by clone library analysis, indicating their possible role in PCB dechlorination.

## Materials and Methods

### Soil reactor set up[6]

The soil microcosm set-up procedure was similar as previously described (Meggo and Schnoor, 2013). Briefly, PCB-free soil from the village of Middle Amana in Iowa, USA, was passed through a 60 mesh sieve before experiments. The soil was spiked with a mixture of PCB 52, PCB 77 and PCB 153 (99% pure) (Accustandard Inc., New Haven, CT) at the concentration of 500 ng $g^{-1}$ each. PCB congeners were dissolved in hexane before adding to the soil. A quartering technique was used to homogenize soil. The soil was divided into four quadrants on a quartering canvas, and 20 diagonal trajectories were used to mix the soil components. The contaminated soil was aged for two months at $25^0$C in sealed tubs to allow PCBs sequestration into the soil matrix and thereby better represent field conditions. To establish the initial PCB concentration, 20 subsets of soil collected from different locations were homogenized and measurements were performed in triplicates (Meggo and Schnoor, 2013).

Plastic containers (33.8cm × 21.6cm ×211.9cm) and lids with aluminum foil covers were used for soil reactor set-up. Each reactor was filled with 2500g sieved and homogenized soil. The spiked soil was planted with switchgrass (*Panicum virgatum)*

[6] Soil reactor set-up was performed by Richard Meggo, Ph.D., Department of Civil & Environmental Engineering, The University of Iowa.

seeds (Adams-Briscoe Seed Co., Jackson, GA). Switchgrass in all four reactors was grown at 25°C in a plant growth chamber under a 16 hour light/8 hour dark photo period (light intensity of 200 mmol $m^{-2}s^{-1}$ and 60% humidity). Switchgrass was allowed to establish for 8 weeks. After 8 weeks, alternate cycles (two weeks of flooding and two weeks of non-flooding) were performed to one unplanted soil reactor and one switchgrass treated soil reactor. During the flooding period, soils were submerged by deionized water. After two weeks of flooding, the extra water was withdrawn. Four flooding was performed in total. Another unplanted soil reactor and switchgrass treated soil reactor with normal moisture content and without flooding treatment were used as control. For each soil reactor, redox measurement was performed with a Redox/pH combination meter (Hanna Instruments, Smithfield, RI). Volumetric soil moisture content was monitored with Vernier soil moisture sensors interfaced with the Vernier LabQuest data collection system (Vernier Software & Technology, Beaverton, OR). Soil samples were taken at 12 week and 24 week for PCB congener analysis. For microbial analysis, soil samples were taken every two weeks starting at 8 week (Meggo and Schnoor, 2013).

## PCB congener analysis[7]

The PCB extraction procedure has been described previously (Meggo *et al.*, 2013). PCBs in soil and plant material (roots and shoots) was extracted by adding a 1:1 hexane/acetone mixture (3 ml/g) to homogenized soil or plant material (5 g) and sonicated for 1 h. Before sonication, surrogate standards including PCB 14 (3,5-dichlorobiphenyl), deuterated PCB 65 (2,3,5,6-tetrachlorobiphenyl) and PCB 166 (2,3,4,4',5,6-hexachlorobiphenyl) (CambridgeIsotope Laboratories, Inc.) were added into the samples (50 ng of each surrogate) to account for any loss during the extraction.

[7] PCB extraction was performed by Richard Meggo, Ph.D., Department of Civil & Environmental Engineering, The University of Iowa. PCB quantification was performed by Dingfei Hu, Ph.D., Department of Civil & Environmental Engineering, The University of Iowa.

Surrogate recoveries were 103 ±17.2% (PCB 14), 97.9 ± 15.2% (PCB 65) and 97.7 ± 17.7% (PCB 166).

The sonicated material was centrifuged at 1500 × g for 5 min, after which the supernatant was transferred to a fresh vial. The precipitates were subjected to a second extraction. The combined supernatants of first and second extraction were evaporated to dryness using rotary evaporation and the solvent was changed to hexane. Double extraction with concentrated sulfuric acid and hexane was performed to remove of lipids and other polar substances. This hexane extract was concentrated to approximately 0.5 ml under a gentle stream of nitrogen. The concentrate was eluted with 10 ml of hexane through a filter consisting of 0.1 g of silica (70-230 mesh, Fisher Scientific, Inc.), 0.1 g of anhydrous sodium sulfate and 0.9 g silica gel acidified with $H_2SO_4$ (silica:$H_2SO_4$ = 2:1) (Meggo and Schnoor, 2013).

The concentrated extracts were spiked with the internal standard containing 100 ng of PCB 204 (2,2',3,4,4',5,6,6'-octachlorobiphenyl). The samples were analyzed PCB congeners using a gas chromatograph with mass selective detection (GC-MS/MS) modified from the EPA method 1668A (U.S.EPA 1999). The quantification of PCBs was performed by an Agilent 6890N gas chromatograph with an Agilent 7683 series autosampler coupled to a Waters Micromass Quattro micro GC mass spectrometer (Milford, MA, USA) operating under electron impact (EI) positive mode at 70 eV and multiple reaction monitoring (MRM), and the trap current was 200 μA. The retention windows were defined by PCB parent/daughter ion pairs from mono- to deca- homologs which were 188/152, 222/152.10, 255.96/186, 291.92/222, 325.88/255.90, 359.84/289.90, 393.80/323.90, 427.76/357.80, 461.72/391.83, 497.68/427.70, respectively (Hu *et al.*, 2010).

## Quantitative PCR

DNA was extracted from 2 g soil immediately after sampling using the RNA

PowerSoil DNA Elution Accessory Kit (Mobio, Carlsbad, CA) and stored at -80 C prior to further analysis. The abundances of total bacterial and Putative dechlorinating *Chloroflexi* were estimated using qPCR targeting bacterial 16S rRNA gene (primer set 16SU f/r) (Nadkarni *et al.*, 2002) and putative dechlorinating *Chloroflexi* 16S rRNA genes (primer set dhc793f/946r) (Yoshida *et al.*, 2005), respectively. PCR conditions were as follows: 10 min at 95°C, 40 cycles of 15s at 95°C and 1 min at 60°C followed by a dissociation step. Each 25 µl qPCR reaction contained 12.5 µl Power SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA), 2.5 pmol primers and various amount of primers and templates (Table 6.1). Bovine serum albumin (0.5 µg) was added to relieve possible PCR inhibition (Kreader, 1996).

For total bacterial 16S rRNA gene qPCR, the standard DNA template was a PCR product amplified from *Burkholderia xenovorans* strain LB400 with primer set 8F/1492R (Grabowski *et al.*, 2005). For putative dechlorinating *Chloroflexi* 16S rRNA gene, standard curves were prepared from pCR 2.1-TOPO vector containing the target PCR products with primer dhc793f/946r. All qPCRs were performed with an ABI 7000 Sequence Detection System (Applied Biosystem, Grand Island, NY) and fluorescence data was analyzed by ABI 7000 System SDS Software (Applied Biosystems, Grand Island, NY). With each primer set, the target gene was not detected in no template (DI water) controls (Ct value > 35). Additional qPCR information, such as primer concentrations, template concentrations, qPCR linear range, qPCR efficiency range of the standard curves, and Y-intercepts are provided in Table 6.2, in accordance with MIQE guidelines (Bustin *et al.*, 2009).

## qPCR quality assurance

Several published qPCR primer sets targeting putative dechlorinating *Chloroflexi* 16S rRNA gene and *Dehalococcocides*-like16S rRNA gene were initially tested in this study. Primer sets chl348f/884r (Fagervold *et al.*, 2005), dhc1f/264r (Grostern and

Edwards, 2006) , dhc793f/946r (Yoshida *et al.*, 2005) and dhc1154f/1286r (Krzmarzick *et al.*, 2012) produced single bands of the expected size with DNA template derived from soil. To verify the specificity of these qPCR primer sets, clone libraries were constructed from the amplification products in DNA extracted from soil with redox cycling treatment. From the dhc793f/946r PCR product clone library, 6 unique sequences were obtained from 12 clones and all of these sequences were identified to be *Dehalogenimonas* by RDP classifier (Table 6.3) (Cole *et al.*, 2007). Clone libraries show that primer sets chl348f/884r, dhc1f/264r and dhc1154f/1286r amplified sequences from the class *Anaerolineae* of *Chloroflexi* and also from *Actinobacteria*, both of which are not associated with dechlorination (Table 6.3). Only the primer set dhc793f/946r was used to quantify putative dechlorinating *Chloroflexi* 16S rRNA gene in soil samples. The specificity of the SYBR green based qPCR was also validated by dissociation curve analysis, which shows similar melting temperature for each sample.

## Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis

PCR with a fluorescent-labeled primer set 6-FAM 8Fm/533R was performed with 1 ng DNA as template (Schütte *et al.*, 2008). PCR conditions were as follows: 4 min at 94°C, 30 cycles of 1 min at 94°C, 2 min at 55°C and 1.5 min at 72°C followed by a final extension step of 10 min at 72°C. PCR product (12 μl) was digested with the restriction enzyme HaeIII (New England BioLabs, Inc., Ipswich, MA), and then precipitated with glycogen (Fermentas International Inc., Vilnius, Lithuania), sodium acetate (pH 5.2), and ethanol at -20°C for 2h. DNA was recovered by centrifugation at 17,800 × g for 15 min and resuspended in distilled water (Invitrogen Corp., Carlsbad, CA). The digested DNA was sent to the University of Iowa DNA facility for electrophoresis for sizing using an Applied Biosystems 3730 DNA analyzer (Life Technologies Corporation, Carlsbad, CA) with the GeneScan 500 LIZ size standard.

Terminal restriction fragment (TRF) sizes were estimated with Peak Scanner software (Applied Biosystems, Carlsbad, CA). A terminal restriction fragment (T-RF) size matrix containing 84 samples (rows) and 439 unique 16S rRNA gene TRFs (columns) was generated by T-REX software after filtering background peak noise and rounding fragment sizes to the nearest whole number (Culman *et al.*, 2009). TRF profiles were analyzed by nonmetric multidimensional scaling (NMDS) in R (da C Jesus *et al.*, 2009). The Bray-Curtis dissimilarity index was calculated with a random starting configuration, and a two-dimensional solution was reached after two runs. The final stress was 12.77. The T-RF composition differences among groups were assessed by multi-response permutation procedure (MRPP), which evaluates grouping with real and randomized data based on Bray-Curtis distances and 5000 permutations, and permutational multivariate analyses of variance (PERMANOVA) based on Bray-Curtis distances with 5000 permutations. Random forests classification was used to estimate the importance of the individual T-RFs in the classification (Cutler *et al.*, 2007). An indicator species analysis was performed to assess the treatment preference of T-RFs using multipatt function with R (Cáceres and Legendre, 2009).

## PCR amplification, cloning and sequencing of bacterial 16S rRNA

Clone libraries were constructed with bacterial 16S rRNA genes amplified from unplanted soil, switchgrass treated soil, unplanted and cycled soil, switchgrass treated and cycled soil at 24 wk. Bacterial 16S rRNA genes were amplified using primer set 8F/1492R (Grabowski *et al.*, 2005). PCR conditions were as described in T-RFLP analysis section. PCR products were purified with the Qiaquick PCR purification Kit (Qiagen Inc., Valencia, CA), cloned into the pCR 2.1-TOPO vector using the TOPO TA cloning Kit (Invitrogen Corp., Carlsbad, CA), and transformed into One Shot TOP10 chemically competent *E.coli* cells (Invitrogen Corp., Carlsbad, CA). The transformation

efficiency was checked by plating recombinant *E.coli* on Luria Broth agar with kanamycin (50 mg/L), and X-gal (0.4 mg/plate) and incubated overnight at 37°C. Clones were Sanger sequenced at HTSeq.org (Seattle, WA) with M13F (5'-TGTAAAACGACGGCCAGT-3') and M13R (5'-CAGGAAACAGCTATGAC-3') primers. Sequence identification and classification were performed using RDP Classifier (Cole *et al.*, 2007) and Basic Local Alignment Search Tool (BLAST) . Predicted TRFs were generated with TRiFLE (Junier *et al.*, 2008).

### Statistical analyses

PCB congener data was analyzed with an independent sample t test using R. The total bacterial 16S rRNA gene copy numbers and dechlorinating *Chloroflexi* 16S rRNA gene copy numbers were analyzed with a two factor analysis of variance after log transformation using R (Rieu and Powers, 2009).

## Results and Discussion

### PCB congener analysis

Significantly higher total PCB removal (39.9 ± 0.4 %) was achieved in switchgrass treated soil as compared with unplanted soil (29.5 ± 3.4%) ($p<0.05$, Figure 6.1). PCB 52 removal was enhanced most (16.7%) with the presence of switchgrass, while PCB 77 and PCB 153 removal was improved by 7.9% and 9.6%, respectively ($p<0.05$, Figure 6.1). The enhanced PCB removal in switchgrass rhizosphere could be the result of rhizosphere biostimulation, rhizofiltration, phytoextraction, phytotransformation and phytovolatilization (Van Aken *et al.*, 2010). At the end of the 24 week experiment, we found 4.5-10.8% of total PCBs in switchgrass roots, and 0.11-0.17% in shoots, suggesting the uptake and translocation of PCBs within switchgrass. PCB 153 was less

favorable by switchgrass, which has significantly lower concentration in the roots than PCB 52 and 77 ($p<0.05$), probably because of the higher hydrophobicity.

The experiment design involving redox cycling treatment was intended to create alternating anaerobic-aerobic conditions in the soil reactors by flooding and draining soils. During the non-flooding period, redox potential measurement suggests that anoxic conditions prevailed in both unplanted and switchgrass treated soils ($Eh<350mV$). During the two weeks of flooding period, the redox potential could drop as low as -400mV (Figure 6.4). In redox cycled and unplanted soil, PCB 153 removal was increased by 5.3% after 24 weeks of incubation ($p<0.05$), which could be the result of the enhanced anaerobic dechlorination. The removal of PCB 52 and 77 was not significantly improved. In redox cycled soils with switchgrass treatment, PCB removal was not improved as compared with soil with only switchgrass treatment (Figure 6.1). It is possible that switchgrass roots enhanced the oxygen diffusion in the flooded soils, making it difficult to enhance dechlorination.

The occurrence of dechlorination was verified by the detection transformation products including PCB1, PCB3, PCB 35, PCB 48, and PCB 118 in unplanted, switchgrass treated, and redox cycled soils (Figure 6.2). As suggested by redox measurements, the soils without cycling of flooded and unflooded conditions were under anoxic conditions, which made it possible for microorganisms to use alternative electron acceptors (Figure 6.4). The observed dechlorination is encouraging, because PCB dechlorination was typically observed in anaerobic environments (Bedard et al., 1996; Krumins et al., 2009; Quensen et al., 1990; Rodenburg et al., 2010). However, the transformation products were not very abundant and didn’t accumulate significantly, possibly because of simultaneous aerobic oxidation. PCB 3 was relatively more abundant at 12 weeks, and was at lower level at 24 weeks, indicating the possible PCB 3 removal by aerobic microbial degradation (Figure 6.2).

PCB 101 and PCB 118 could be the dechlorination products of PCB 153 (Figure

6.3). The detection of PCB 118 was notable, which requires *ortho* chlorine removal from PCB 153. PCB 101 could be transformed into PCB 52 by *para* chlorine removal, and PCB 118 can be dechlorinated to PCB 77 by *ortho* chlorine removal (Figure 6.3). Thus PCB 153 can be dechlorinated to PCB 52 and 77. The transformation from PCB 153 to PCB 52 is more likely, which can be achieved by removing two *para*-chlorines, while PCB 77 production requires *ortho* dechlorination and is more difficult (Tiedje *et al.*, 1993). PCB 35 could be the dechlorination product of PCB 77 by *para* chlorine removal, and PCB 26 could be generated from PCB 52 by *ortho* chlorine removal (Figure 6.3).

Some PCB congeners, such as PCB 95 and 110, were also detected. They are not the direct dechlorination product from the three parent PCB congener, and were probably produced by chlorine rearrangement, although the mechanism is unclear. Chlorine rearrangement of PCB congeners have been observed before in poplars, switchgrass and maize (Liu et al., 2009; Wang et al., 2011).

## Quantification of putative dechlorinating *Chloroflexi* 16S rRNA genes

While the detection of transformation products verified the occurrence of dechlorination in the soils, we then aimed to quantify the presence of putative dechlorinating *Chloroflexi*, which phylum includes the only known anaerobic PCB degrader, in the soil samples. The clone library constructed from DNA extracted from switchgrass treated soil with redox cycling treatment shows that the sequences amplified in the qPCR targeting putative dechlorinating *Chloroflexi* were all *Dehalogenimonas* species (Table 6.3). Strains of *Dehalogenimonas* species are able to reductively dechlorinate polychlorinated aliphatic alkanes (Maness et al., 2012; Yan et al., 2009), and a recent study found that *Dehalogenimonas* can couple their growth with PCB dechlorination in a sediment free culture CG3 (Wang and He, 2013). Our sequences share 98-100% similarity with the *Dehalogenimonas* sequence retrieved from the culture CG3

(Figure 6.7). The presence of *Dehalogenimonas* in our soil samples strongly suggests that they may be responsible for the dechlorination in the rhizosphere.

Before the redox cycling treatment, the abundances of putative dechlorinating *Chloroflexi* 16S rRNA genes were low in both unplanted and switchgrass treated soils. After two weeks of flooding, putative dechlorinating *Chloroflexi* 16S rRNA gene abundances increased (Figure 6.8). However, despite of the repeated flooding and draining afterwards, the abundances of putative dechlorinating *Chloroflexi* 16S rRNA genes were stable till the end of the experiment ($2.91\times10^5 \pm 8.64\times10^4$ gene copy per g soil in unplanted soil with redox cycling, $5.63\times10^5 \pm 4.98\times10^5$ gene copy per g soil in switchgrass treated soil with redox cycling), and were not correlated to the redox potential or moisture content change (Pearson's r, $p>0.05$). It appears that the first two weeks of flooding successfully created an environment suitable for the growth of possible PCB-dechlorinating *Chloroflexi* on available electron donors in the soil. In the subsequent non-flooding period, the putative dechlorinating *Chloroflexi* were able to survive in the unplanted soil and the switchgrass rhizosphere. Actually the abundance of putative dechlorinating *Chloroflexi* 16S rRNA genes in switchgrass treated soil was significantly higher than that in unplanted and redox cycled soils ($p<0.05$). It is likely that anaerobic microenvironments existed in the rhizosphere, which could be where dechlorination took place. In the soils without redox recycling treatment, the putative dechlorinating *Chloroflexi* 16S rRNA genes were at or below the detection limit of qPCR (30 gene copies per reaction).

Despite the increase after the first flooding session, putative dechlorinating *Chloroflexi* 16S rRNA genes were still at low abundance with respect to the total bacterial 16S rRNA genes ($0.0028 \pm 0.0008$ % and $0.0029 \pm 0.0025$% of total 16S rRNA genes in unplanted soil with redox cycling and switchgrass treated soil with redox cycling, respectively). The abundance of dechlorinating bacteria in PCB-dechlorinating sediment free culture was higher. For example, *Dehalococcoides* was 3.74% of total bacterial

community in highly enriched sediment-free JN culture (Bedard *et al.,* 2006). *Dehalogenimonas* was 2.16% in a sediment-free culture that dechlorinated Aroclor 1260 (Wang and He, 2013). The total bacterial abundance was not affected by redox cycling ($p>0.05$), while the switchgrass treatment significantly increased the total bacterial abundance ($p<0.05$) (Figure 6.9).

## Microbial community analysis

To explore the effect of switchgrass and redox cycling treatment on microbial communities, the T-RFLP analysis was performed to rapidly assess microbial community structure during the incubation time. NMDS ordination of the T-RFLP profiles revealed that the blank soil (i.e. soil without PCB spiking), unplanted soil, switchgrass treated soil, unplanted soil with redox cycling, and switchgrass treated soil with redox cycling displayed different bacterial community structure. At the beginning of the experiment, unplanted soil and blank soil had similar microbial communities (Figure 6.10). The differences of T-RF compositions in soils with different treatments were confirmed by MRPP and PERMANOVA ($p=0.001$). No significant correlation was found between bacterial communities and the incubation time (Pearson's R, $p>0.05$). Indicator species analysis shows that some T-RFs were statistically associated with different treatments (Table 6.5). Those T-RFs were rare members of the microbial community in the soil samples (< 1% of total T-RFs), and were not identified by clone library analysis.

The microbial community structure in soils with different treatments at 24 weeks were assessed by sequencing in total 187 clones from four 16S rRNA gene clone libraries. All soil communities were dominated by *Proteobacteria* and *Acidobacteria* (comprising 46.9 ± 13.5% and 13.9 ± 5.8% of total clones in the four clone libraries) (Figure 6.11). The rest of phylotypes were affiliated to *Bacteroidetes, Actinobacteria, Chloroflexi, Chloroplast, Firmicutes, Gemmatimonadetes, Nitrospira, OP11, Planctomycetes* and *Verrucomicrobia*. Clone libraries in unplanted and switchgrass treated soils were similar,

while increased abundance of *Proteobacteria* and decreased *Acidobacteria* were observed in redox cycled soils.

*Proteobacteria* are commonly found in soils and sediments (de Cárcer et al., 2007; Petrić et al., 2011a; Spain et al., 2009), and many known aerobic PCB-degraders are *Proteobacteria* (e.g. *Pseudomonas*, *Sphingomonas*, *Acinetobacter*, *Comamonas* and *Burkholderia* (Nováková *et al.*, 2002)), which may be involved in PCB degradation in our soil samples. Possible aerobic PCB degraders other than *Proteobacteria*, such as *Bacillus* and *Rhodococcus*, were also identified (Masai et al., 1995; Shimura et al., 1999). The high abundance of *Acidobacteria* was previously found in other PCB contaminated soils (Nogales et al., 1999; Petrić et al., 2011a), indicating their possible role in PCB biodegradation.

Within *Proteobacteria*, *Delta*-and *Gamma-proteobacteria* include some species that can degrade chlorinated compounds via dehalorespiration or co-metabolic reductive dehalogenation, such as *Desulfuromonas* spp (Krumholz, 1997), *Geobacter lovleyi* (Sung *et al.*, 2006) and *Shewanella putrefaciens(Picardal et al., 1995)*. *Geobacter*, known as sulfate/sulfur and iron reducers, were only detected in the clone library from the unplanted soil with redox cycling treatment. *Geobacter* is able to dechlorinate trichloroacetic acid and tetrachlorethene (de Wever et al., 2000; Strycharz et al., 2008; Sung et al., 2006), and have been observed in PCB dechlorinating enrichment cultures in previous studies (Bedard et al., 2006; Bedard et al., 2007; Holoman et al., 1998). The T-RFs corresponding to *Geobacter* spp. were 203bp and 215bp (Table 6.4). However the 203 bpT-RF is not specific to *Geobacter* sp. – it can also be generated from other *Deltaproteobacteri,* and the 215 bp T-RF could be produced from 16S sequences from other *Betaproteobacteria*, which may explain the relatively high abundances of the two T-RFs in soils with switchgrass treatment (Figure 6.13). Furthermore, T-RF 203 bp was identified as an the important T-RF in the classification of T-RFLP profiles by random

forest algorithm, as well as T-RF 235 bp, another T-RF corresponding to *Deltaproteobacteria* (Figure 6.12).

*Clostridium* is another genera identified in the clone libraries that has the potential to dechlorinate PCBs. For instance, some certain *Clostridium* strains can dechlorinate tetrachloroethylene (Chang et al., 2000; Okeke et al., 2001). *Clostridium* spp. were detected in a PCB-dechlorinating enrichment culture derived from St. Lawrence River sediment (Oh *et al.*, 2008). In another study, *Clostridium* species were abundant in sediments that were experiencing *para*- and *meta*- PCB dechlorination (Hou and Dutta, 2000). *Clostridium* spp. can also produce hydrogen, an important and common electron donor for dechlorination (Bowman et al., 2009; Nandi and Sengupta, 1998). *Clostridium* clones generated 288 bp and 298 bp T-RFs, which comprised 0.25% and 0.54% of the total T-RF peak areas (Figure 6.13). *Geobacter* and *Clostridium* were not abundant in the four clone library, yet the presence of such dehalorespiring species in the soils experiencing PCB dechlorination suggests their possible role in PCB degradation.

*Chloroflexi* detected by clone library in switchgrass treated soils, unplanted soil with redox cycling, and switchgrass treated soil with redox cycling were identified to *Anaerolineae* and *Caldilineae*, which were not associated to dechlorination (Gupta *et al.*, 2013). Putative dechlorinating *Chloroflexi* was not detected by clone library, probably because of their low abundance in the soil samples with respect to total bacteria, which was verified by the qPCR results.

Redox cycling had an effect on the microbial community structure in all, according to the T-RFLP analysis. In clone libraries, higher abundance of *Proteobacteria*, especially *Gammaproteobacteria*, was found in soils with redox cycling. Also, an increase of putative dechlorinating *Chloroflexi* 16S rRNA gene abundances in soils was observed after redox cycling treatment as shown by qPCR. The redox cycling was repeated for in total four times, but the T-RFLP profile didn't show much difference before and after the flooding session, as well as the abundances of putative dechlorinating

*Chloroflexi* 16S rRNA genes, suggesting that the redox cycling resulted in bacterial community change, but the bacterial community was resilient and stable despite of the sequential flooding and non-flooding cycles.

## Conclusions

In this study, we investigated the microbial communities in the PCB spiked and switchgrass treated soils that experienced PCB dechlorination. The occurrence of dechlorination was verified by the detection of transformation products of parent PCB congeners in all soil microcosms, some of which were under anoxic conditions. Redox cycling was found to significantly enhance the removal of PCB 153 in unplanted soil. The first two weeks of flooding of the redox cycling treatment successfully promoted the putative dechlorinating *Chloroflexi* populations, which sequences were confirmed to be *Dehalogenimonas*, a potential PCB-dechlorinating species. Although no putative dechlorinating *Chloroflexi* was detected in soils without redox cycling, the identification of other dehalorespiring bacteria such as *Geobacter* and *Clostridium* suggests the dechlorination potential of the indigenous microbial community and their possible role in PCB dechlorination.

Table 6.1. Primer sets used for qPCR analysis in Chapter VI.

| Target gene | Primer name | Sequences | Expected product size | Source |
|---|---|---|---|---|
| total bacterial 16S rRNA | 16SU f | 5'-TCCTACGGGAGGCAGCAGT-3' | 466 bp | (Nadkarni *et al.*, 2002) |
| | 16SU r | 5'-GGACTACCAGGGTATCTAATCCTGTT-3' | | |
| *Dehalococcoides*-like 16S rRNA | dhc 793f | 5'-GGGAGTATCGACCCTCTCTG-3' | 193 bp | (Yoshida *et al.*, 2005) |
| | dhc 946r | 5'-CGTTYCCCTTTCRGTTCACT-3' | | |

Table 6.2. Pertinent qPCR parameters (primer concentration, template mass, linear range, PCR efficiency, and y-intercept of the qPCR standard curve) in Chapter VI.

| Target gene | primer concentration, μM | template | qPCR linear range, gene copies/reaction | qPCR efficiency | Y-intercept |
|---|---|---|---|---|---|
| total bacterial 16S rRNA | 0.1 | 1 ng DNA | 30 - 30 × $10^7$ | 95 - 100% | 36.11-38.38 |
| *Dehalococcoides*-like 16S rRNA | 0.8 | 10 ng DNA | 30 - 30 × $10^7$ | 95 - 100% | 33.89-35.48 |

Table 6.3. RDP classification of clone libraries for quality assurance of qPCR primers used in Chapter VI.

| Primer set | Target group | RDP classification (% certainty) | No. of clones detected | Sources |
|---|---|---|---|---|
| dhc1f/264r | *Dehalococcoides* spp. | *Rubrobacteridae* (96-100) | 3/9 | (Grostern and Edwards, 2006) |
| | | TM7 (93) | 1/9 | |
| | | unclassified Bacteria (98-100) | 5/9 | |
| chl384f/dehal884r | *Chloroflexi* | *Dehalogenimonas* (84-100) | 5/13 | (Fagervold *et al.*, 2005) |
| | | *Rubrobacter* (100) | 5/13 | |
| | | unclassified Bacteria (89-100) | 3/13 | |
| dhc793f/946r | *Dehalococcoides* spp. | *Dehalogenimonas* (99-100) | 12/12 | (Yoshida *et al.*, 2005) |
| dhc1154f/1286r | *Dehalococcoides*-like *Chloroflexi* | *Anaerolineaceae* (84-89) | 3/12 | (Krzmarzick *et al.*, 2012) |
| | | unclassified *Chloroflexi* (87-92) | 5/12 | |
| | | unclassified Bacteria (86-100) | 3/12 | |
| | | unclassified | 1/12 | |

Table 6.4. 16S rRNA gene clones recovered from unplanted soil (UP), switchgrass treated soil (SG), unplanted soil with redox cycling (UPF), switchgrass treated soil with redox cycling (SG F). Clone identification was made by RDP classifier.

| Observed T-RF (bp) | Predicted T-RF (bp) | Closet classified relative (% certainty) | NMDS axis 1 | NMDS axis 2 |
|---|---|---|---|---|
| 71 | 75 | *Sorangium* (95%) | -0.010 | -0.165 |
| 187 | 194 | unclassified *Betaproteobacteria* (100%) | -0.156 | 0.065 |
| 195 | 196 | *Luteolibacter* (100%) | -0.045 | -0.331 |
| 197 | 200 | *Variovorax* (100%) | 0.267 | -0.229 |
| 196 | 200 | unclassified *Betaproteobacteria* (96%) | -0.004 | 0.307 |
| 203 | 204 | *Geobacter* (100%) | 0.295 | -0.429 |
| 203 | 204 | unclassified *Desulfuromonadales* (100%) | 0.295 | -0.429 |
| 204 | 206 | unclassified *Xanthomonadaceae* (97%) | -0.285 | 0.202 |
| 205 | 206 | *Rhodanobacter* (100%) | -0.198 | -0.014 |
| 210 | 212 | unclassified *Betaproteobacteria* (87%) | 0.567 | -0.109 |
| 210 | 214 | *Dokdonella* (94%) | 0.567 | -0.109 |
| 213 | 214 | *Caldilinea* (100%) | 0.561 | -0.168 |
| 213 | 216 | *Gp3* (100%) | 0.561 | -0.168 |
| 215 | 217 | *Geobacter* (100%) | 1.389 | 0.242 |
| 216 | 217 | unclassified *Comamonadaceae* (100%) | 0.154 | -0.056 |
| 216 | 219 | unclassified *Comamonadaceae* (100%) | 0.154 | -0.056 |
| 225 | 227 | *Novosphingobium* (95%) | -0.176 | 0.230 |
| 228 | 230 | *Gracilibacter* (100%) | -0.109 | 0.178 |
| 229 | 231 | *Bacillus* (100%) | -0.045 | -0.114 |
| 232 | 234 | *Gp6* (100%) | -0.286 | 0.046 |
| 235 | 237 | unclassified *Deltaproteobacteria* (97%) | -0.298 | -0.129 |
| 248 | 250 | unclassified *Ruminococcaceae* (100%) | 0.307 | -0.187 |
| 260 | 262 | *Flavisolibacter* (99%) | 0.085 | 0.047 |
| 279 | 281 | *Planctomyces* (98%) | -0.218 | -0.048 |
| 288 | 290 | *Clostridium XI* (100%) | 0.433 | -0.141 |
| 298 | 300 | *Clostridium sensu stricto* (100%) | -0.142 | -0.028 |
| 314 | 316 | unclassified *Anaerolineaceae* (100%) | 0.247 | -0.051 |
| 328 | 331 | *Nitrospira* (100%) | -0.348 | -0.298 |

Table 6.5. Significant indicator T-RFs determined by indicator species analysis.

| | Indicator T-RF | Statistic | p value |
|---|---|---|---|
| UP | 383 bp | 0.722 | 0.0002 |
| | 286 bp | 0.663 | 0.0008 |
| | 385 bp | 0.632 | 0.0006 |
| | 384 bp | 0.594 | 0.0004 |
| UPF | 128 bp | 0.84 | 0.0002 |
| | 245 bp | 0.836 | 0.0002 |
| | 166 bp | 0.73 | 0.0002 |
| | 396 bp | 0.667 | 0.0002 |
| | 339 bp | 0.663 | 0.0004 |
| | 349 bp | 0.594 | 0.0006 |
| SGF | 108 bp | 0.851 | 0.0002 |
| | 406 bp | 0.804 | 0.0002 |
| | 215 bp | 0.793 | 0.001 |
| | 60 bp | 0.706 | 0.0002 |
| | 514 bp | 0.606 | 0.0008 |
| | 464 bp | 0.557 | 0.001 |
| UPF+SGF | 263 bp | 0.817 | 0.0002 |
| | 231 bp | 0.791 | 0.0002 |
| | 502 bp | 0.778 | 0.0002 |
| SG+SGF | 258 bp | 0.98 | 0.0002 |
| | 265 bp | 0.966 | 0.0002 |
| | 198 bp | 0.918 | 0.0002 |
| | 209 bp | 0.909 | 0.0002 |

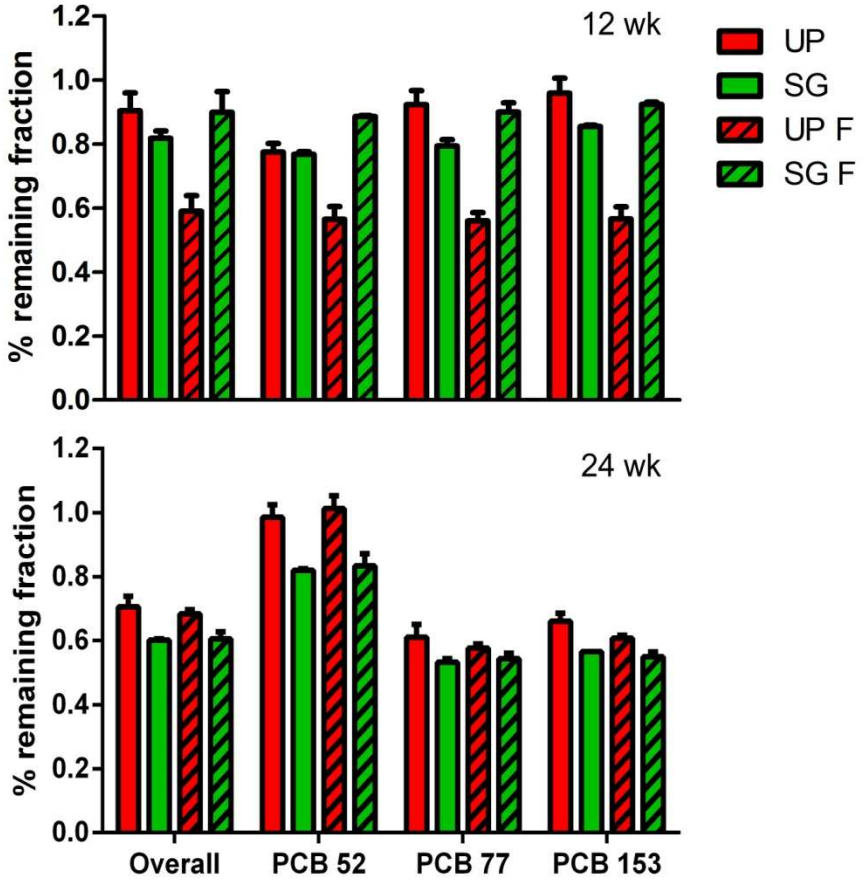


Figure 6.1. The percentage of initial concentration of PCB 52, 77 and 153 after 12 and 24 weeks of incubation in unplanted soil (UP), switchgrass treated soil (SG), unplanted soil with redox cycling (UPF), switchgrass treated soil with redox cycling (SG F). Error bars indicate the standard deviation of three soil subsamples from the same reactor.

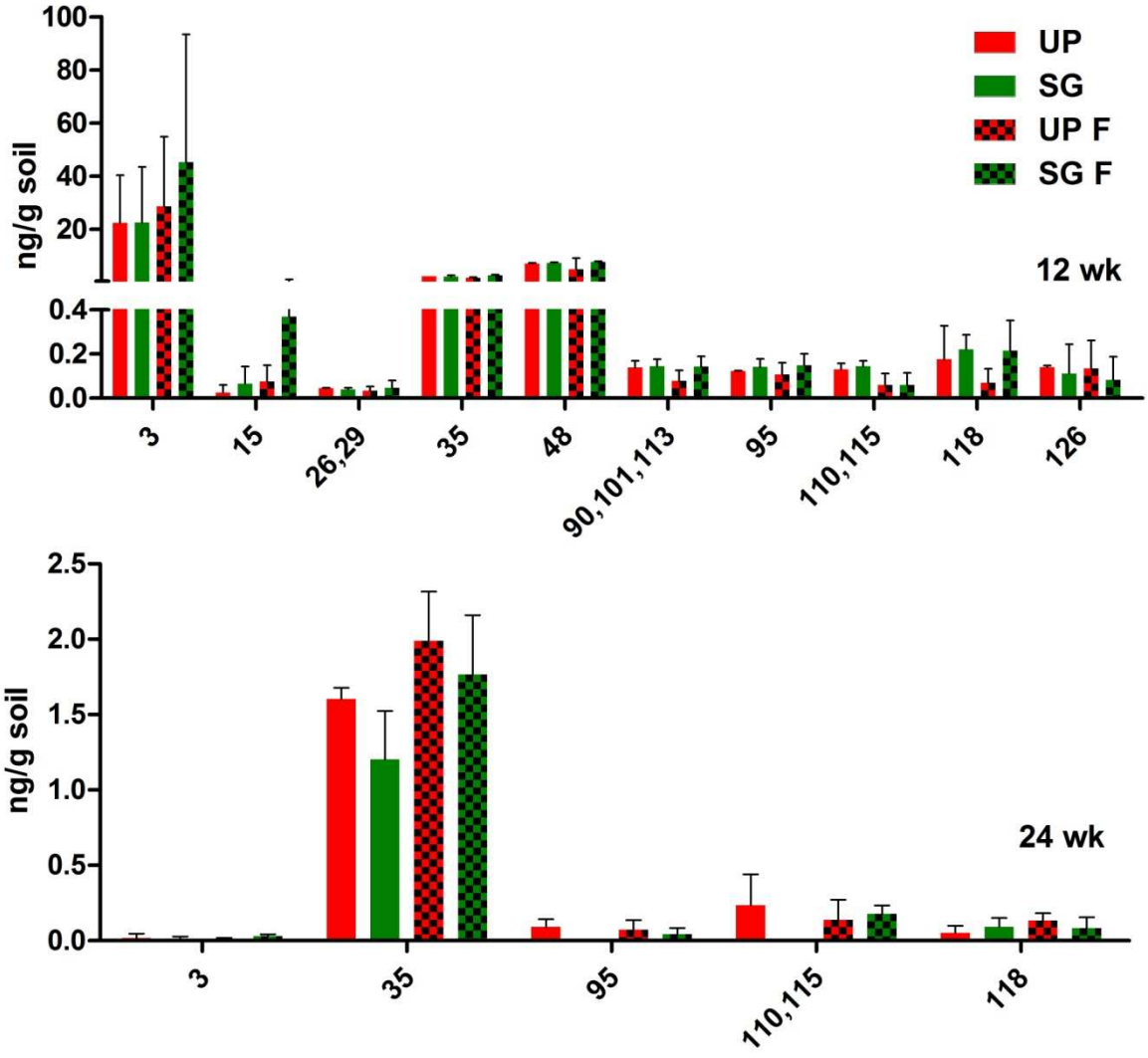


Figure 6.2. Transformation products detected after 12 and 24 weeks of incubation in unplanted soil (UP), switchgrass treated soil (SG), unplanted soil with redox cycling (UPF), switchgrass treated soil with redox cycling (SG F). Error bars indicate the standard deviation of three soil subsamples from the same reactor.

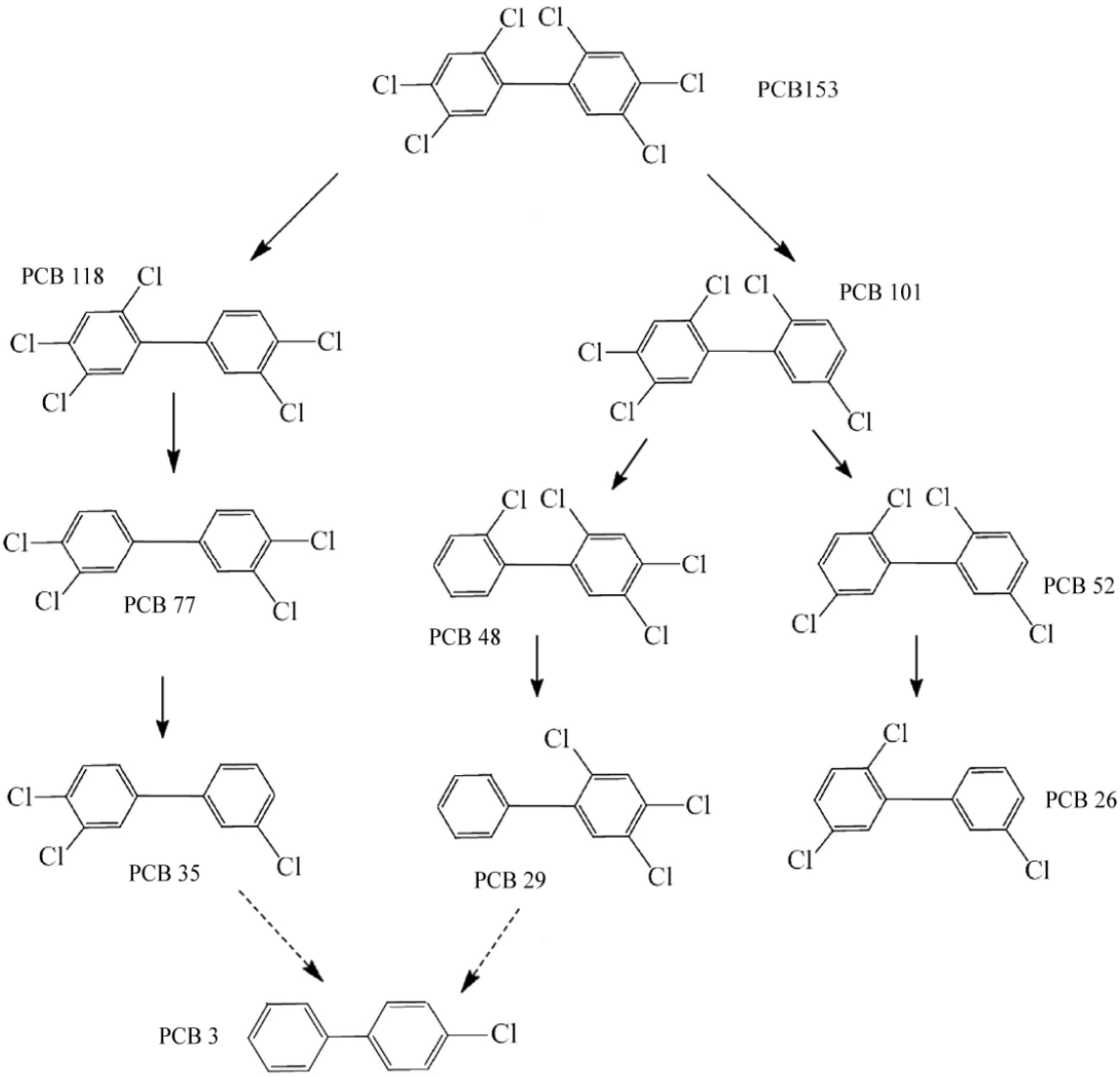


Figure 6.3. Scheme of the possible dechlorination pathways for PCB 52, 77, and 153. Dotted arrows indicate possible pathways but no intermediate was observed.

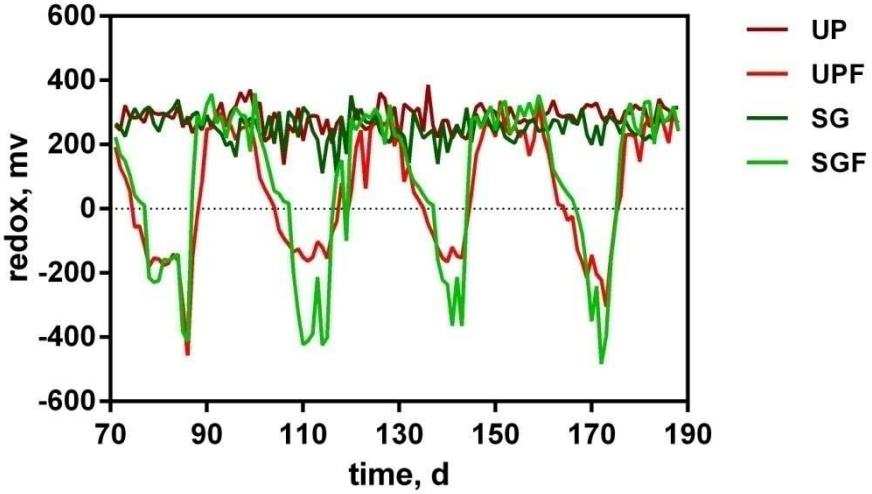


Figure 6.4. Redox potential change in unplanted soil (UP), switchgrass treated soil (SG), unplanted soil with redox cycling (UPF), switchgrass treated soil with redox cycling (SG F).

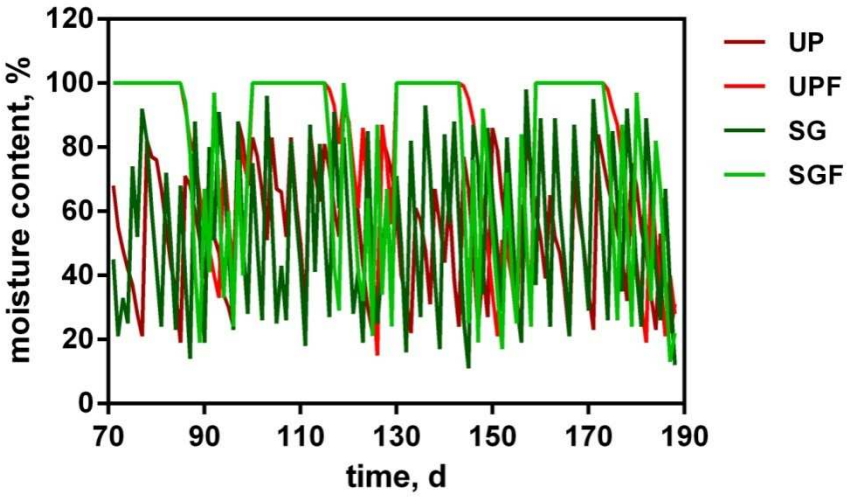


Figure 6.5. Moisture content change in unplanted soil (UP), switchgrass treated soil (SG), unplanted soil with redox cycling (UPF), switchgrass treated soil with redox cycling (SG F).

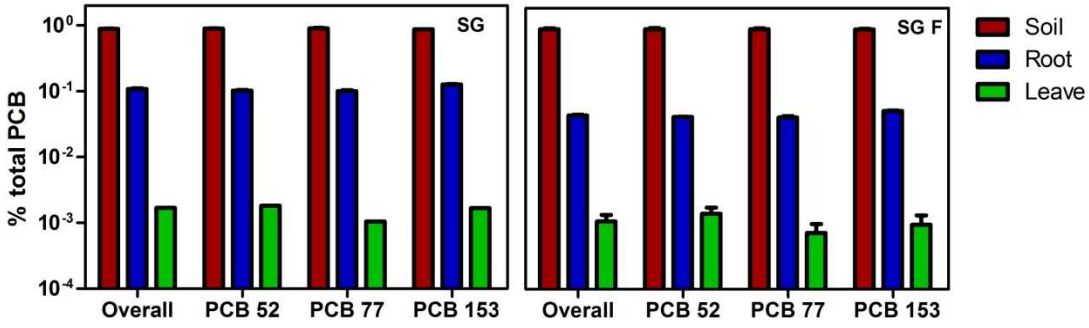


Figure 6.6. Percentage of total PCB mass in soil, switchgrass roots, and leaves after 24 week incubation in switchgrass treated soil (SG) and switchgrass treated soil with redox cycling (SG F). Error bars indicate the standard deviation of three soil subsamples from the same reactor.

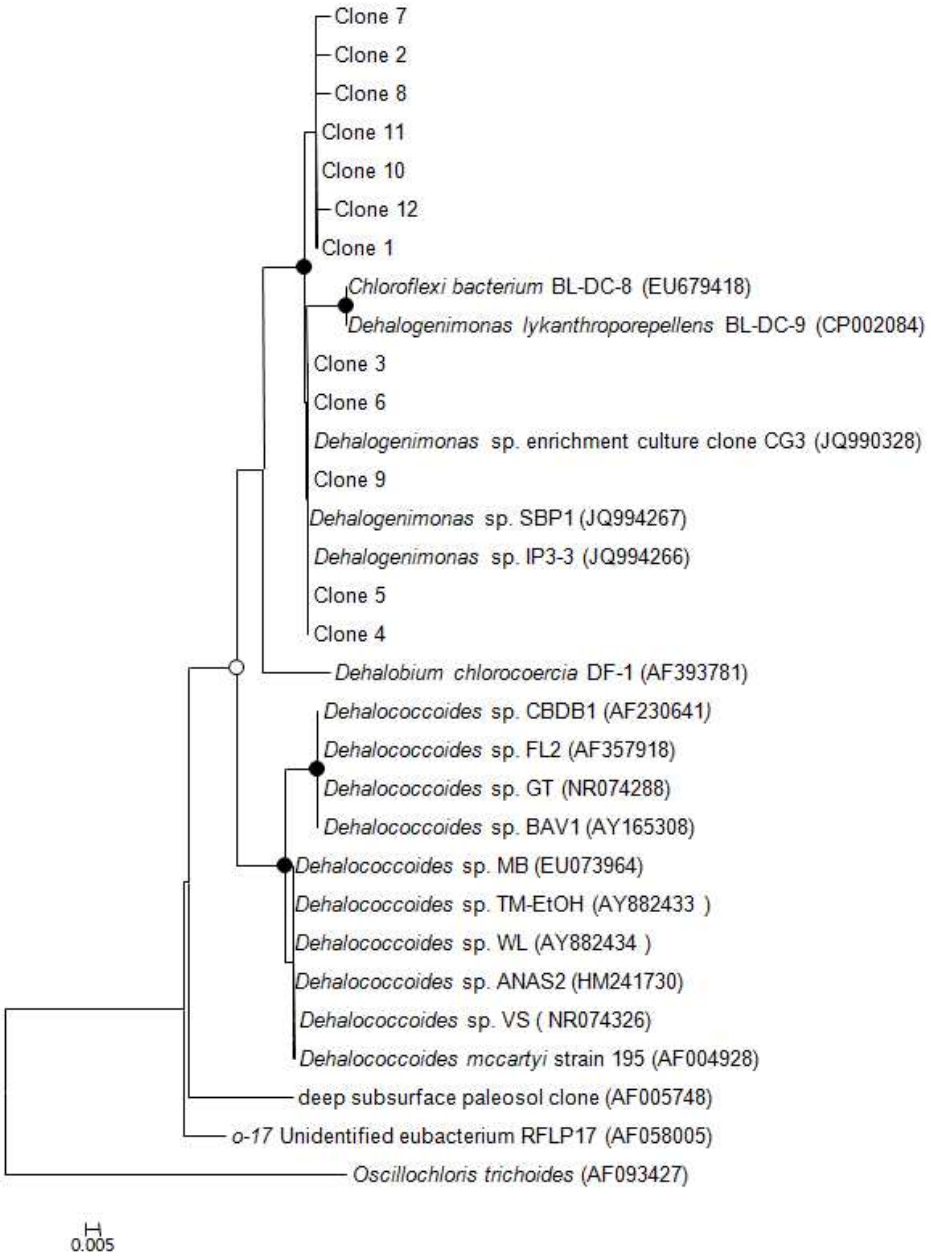


Figure 6.7. Phylogenetic analysis of 16S rRNA genes retrieved from switchgrass treated soils with redox cycling after 24 weeks. The alignment was made with ClustalX and converted into a neighboring-joining tree, which was visualized with MEGA4 with *Oscillochloris trichoides* as an outgroup. The filled circles at nodes indicate bootstrap values of 95%, while open circles indicate bootstrap values of 75~95%. Bootstrap values lower than 75% are not shown. The bar represents 0.5% sequence difference.

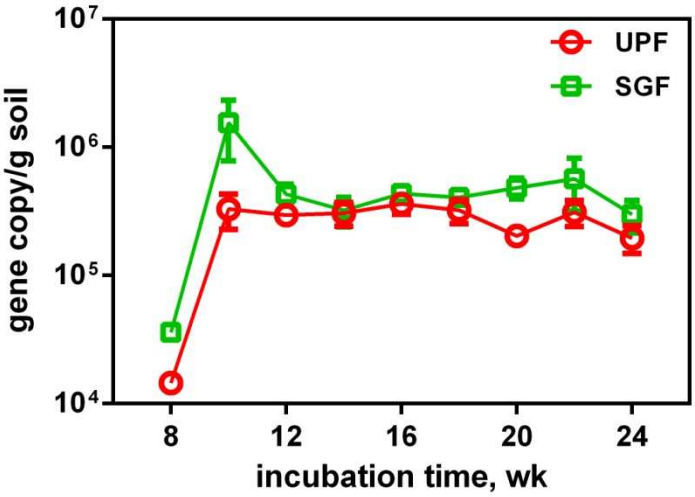


Figure 6.8. qPCR analysis of *Dehalococcoides*-like 16S rRNA gene in unplanted soil with redox cycling (UPF) and switchgrass treated soil with redox cycling (SG F). Error bars indicate the range of two soil subsamples from the same reactor.

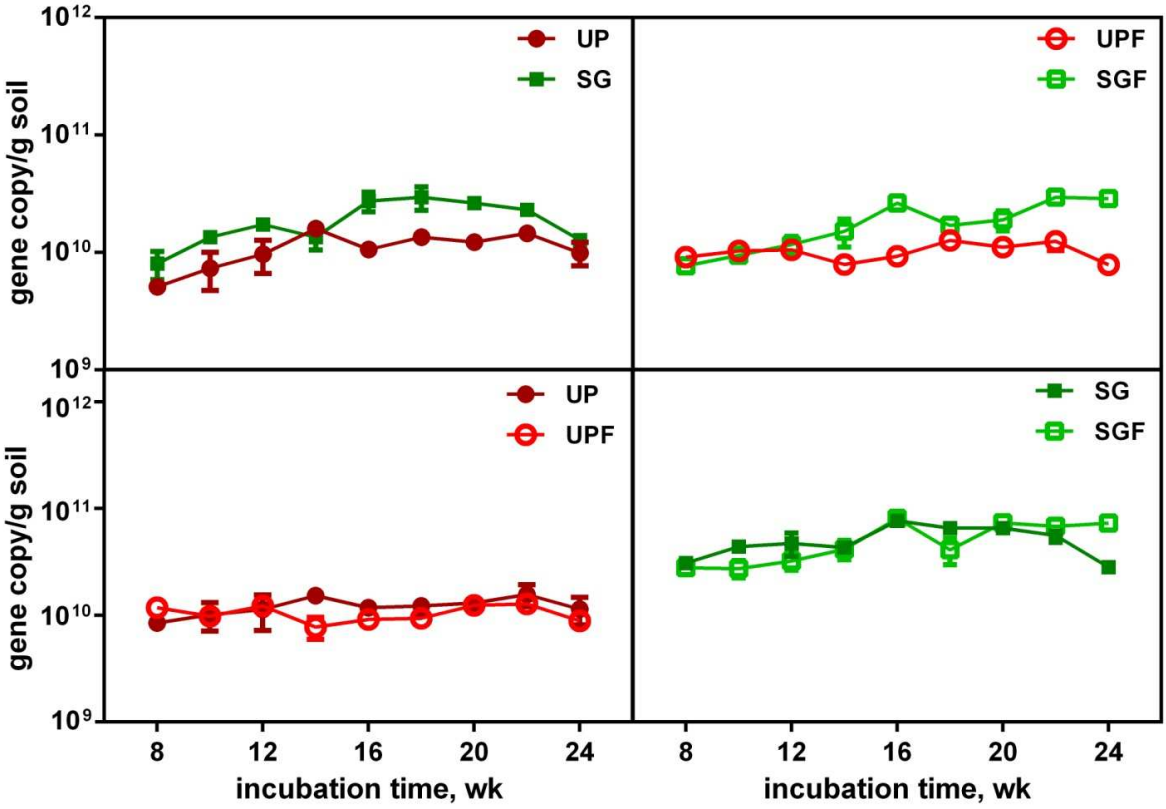


Figure 6.9. qPCR analysis of bacterial 16S rRNA gene in unplanted soil (UP), switchgrass treated soil (SG), unplanted soil with redox cycling (UPF), switchgrass treated soil with redox cycling (SG F). Error bars indicate the range of two soil subsamples from the same reactor.

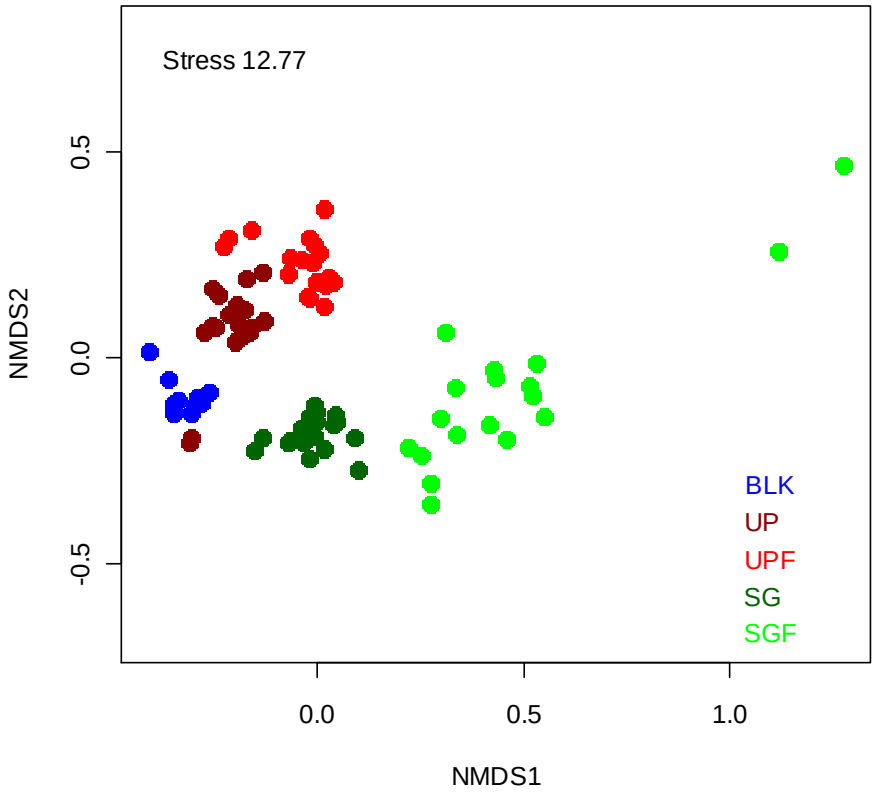


Figure 6.10. NMDS ordination of T-RFLP profiles from blank soil without PCB spiking (BLK), unplanted soil (UP), switchgrass treated soil (SG), unplanted soil with redox cycling (UPF), switchgrass treated soil with redox cycling (SG F).

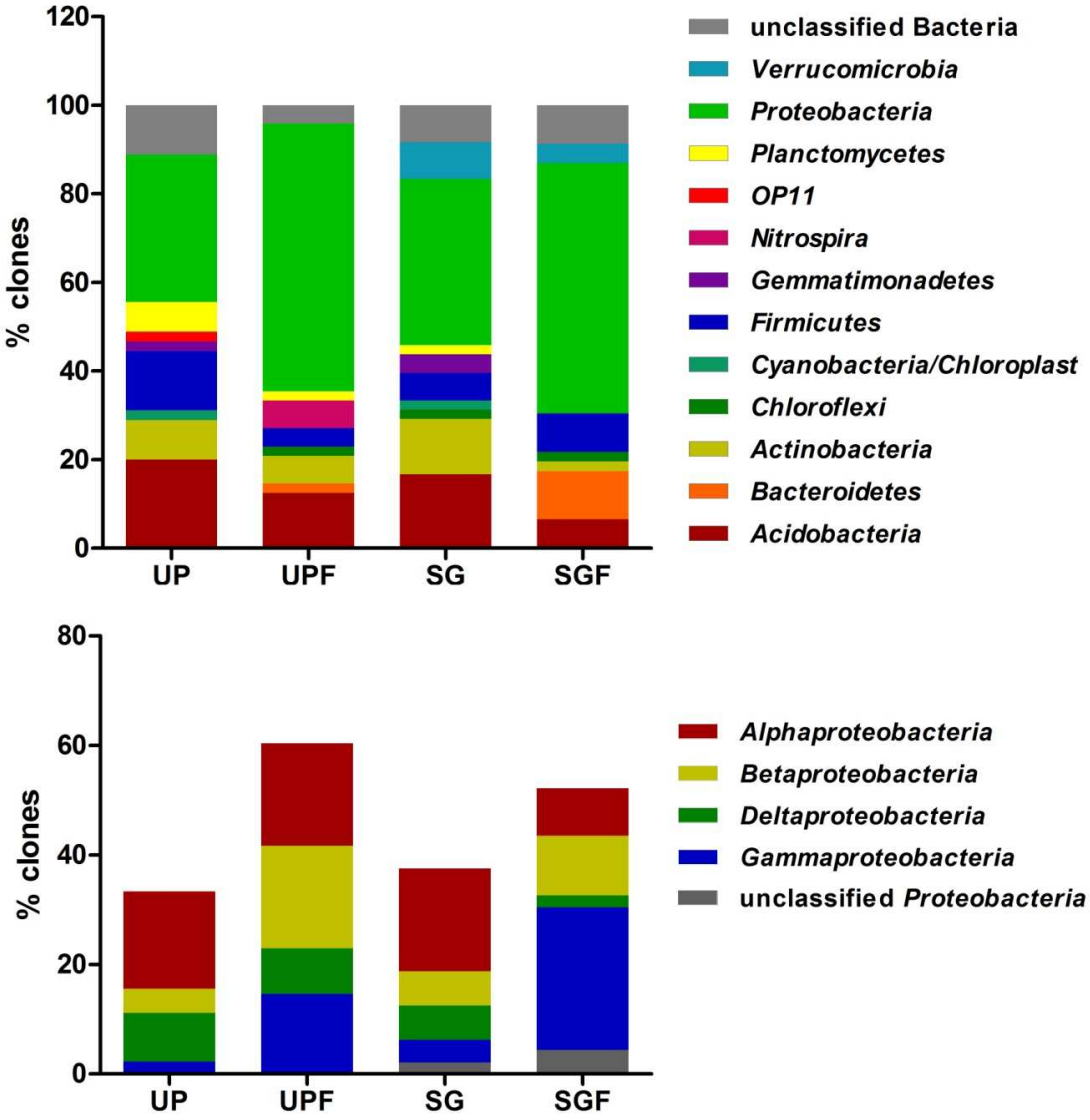


Figure 6.11. Clone library analysis of bacterial 16S rRNA gene in unplanted soil (UP), switchgrass treated soil (SG), unplanted soil with redox cycling (UPF), switchgrass treated soil with redox cycling (SG F). Error bars indicate the range of two soil subsamples from the same reactor.

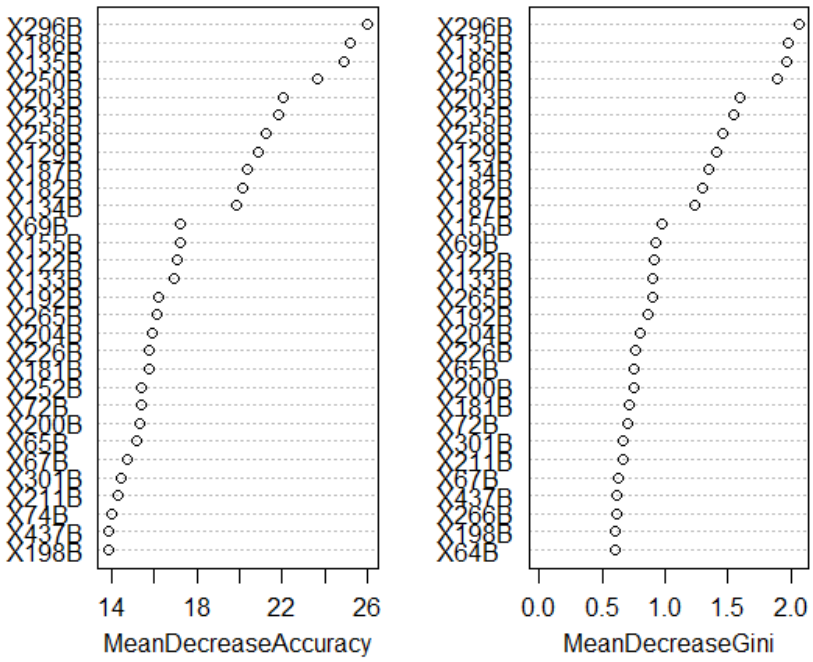


Figure 6.12. T-RF importance for classification as determined by random forest algorithm.

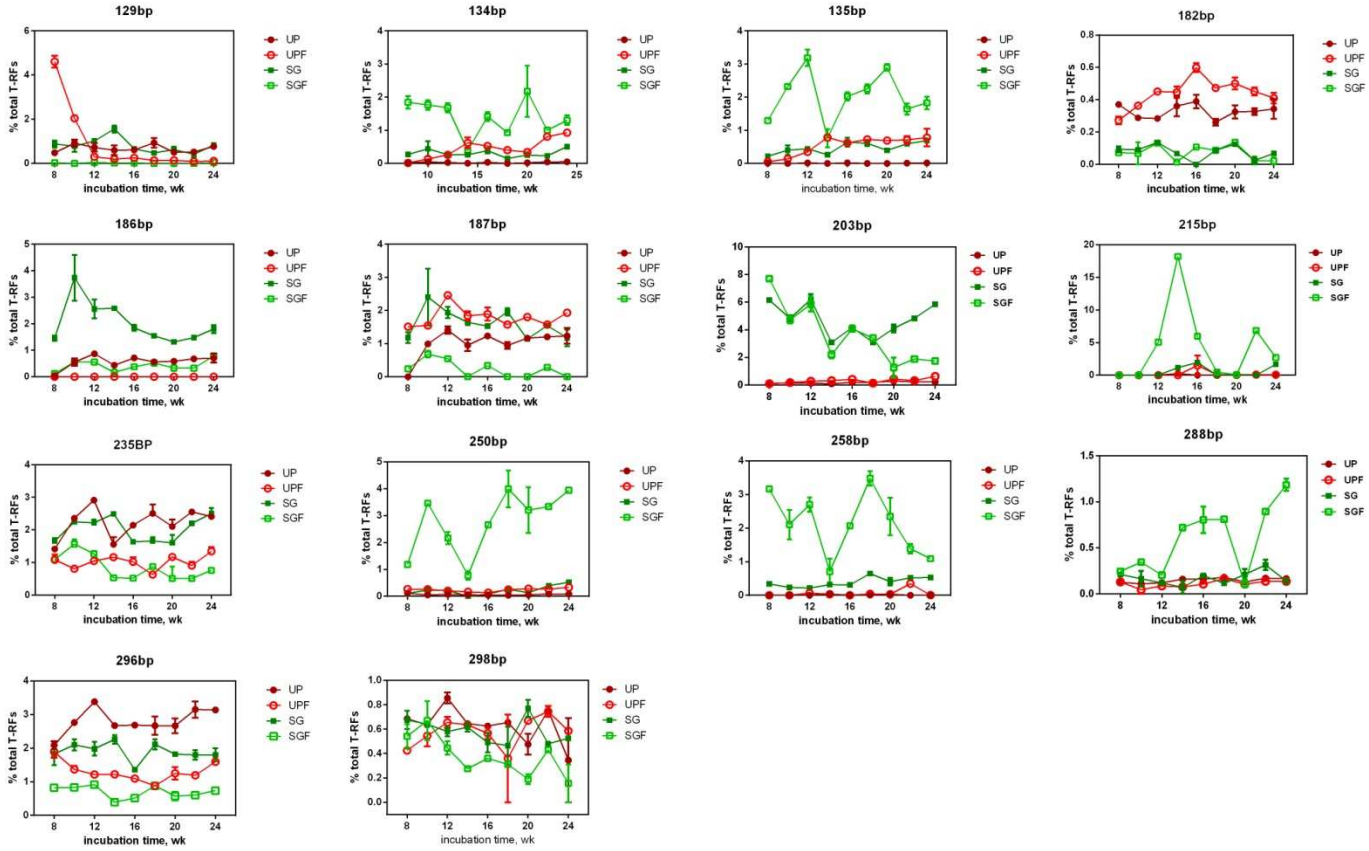


Figure 6.13. The abundance of T-RFs that were identified by random forest algorithm or corresponded to dehalorespiring bacteria in unplanted soil (UP), switchgrass treated soil (SG), unplanted soil with redox cycling (UPF), switchgrass treated soil with redox cycling (SG F).

# CHAPTER VII. ENGINEERING AND SCIENTIFIC SIGNIFICANCE

This work contributes to the understanding of microbial PCB degradation as a remediation strategy. The research objectives included evaluating the *in situ* microbial PCB degradation potential at a PCB contaminated site, improving PCB removal by manipulating switchgrass rhizosphere, and investigating the microbial community variation during the remediation processes.

The selection and optimization of bioremediation strategies for PCB contaminated site clean-up requires a comprehensive understanding about the *in situ* PCB degradation potential. In Chapter III and IV, the aerobic and anaerobic microbial PCB degradation potential was evaluated for two PCB contaminated sediment cores from IHSC (core 1 and core 2) by the combination use of PCB congener analysis and molecular analysis of microbial communities along sediment depth. The correlation between PCB congener profiles and microbial communities was explored, in an attempt to identify the key members that might be involved in the PCB degradation in the deep sediments.

Core 2 exhibited a higher PCB dechlorination potential than core 1, as suggested by congener analysis. The increasing MDPR along core 2 sediment depth suggested a higher degree of dechlorination in deep sediments. The microbial PCB degradation potential was estimated by quantifying biomarker genes along sediment depth, including *bph*A, the gene indicative of aerobic PCB degradation, and the putative dechlorinating *Chloroflexi* 16S rRNA genes. Both *bph*A and putative dechlorinating *Chloroflexi* 16S rRNA genes were more abundant in upper 1.83 m sediments, suggesting the occurrence of both aerobic and anaerobic PCB degradation in the upper sediments. The low abundance of *bph*A in deep sediments may be the result of the stricter anaerobic environment. The discrepancy between PCB congener data and putative dechlorinating *Chloroflexi* 16S rRNA gene data revealed that targeting solely the *Chloroflexi* may be not enough for the evaluation of anaerobic PCB degradation potential. Other dehalorespiring

microorganisms may also be involved in the PCB dechlorination process at IHSC. To identify possible dechlorinating bacteria, microbial communities were characterized by T-RFLP and clone library. *Proteobacteria*, which include many known aerobic PCB degraders, were found dominant. Especially, *Acidovorax* and *Acinetobacter* were abundant. And the abundance of *Acidovorax* was correlated with sediment and pore water MDPR values.

The results from the studies described in Chapter III and IV confirmed the potential of PCB degradation at the contaminated sediments at IHSC, suggesting that the indigenous microbial communities possessed the capability to degrade PCBs in sediments, and that monitored natural attenuation could be a feasible remediation strategy. Another question is how to enhance microbial PCB degradation during the bioremediation process. In Chapters V and VI, phytoremediation with switchgrass was performed on soil microcosms with PCB spiking (PCB 52, 77 and 153). To further enhance the aerobic PCB degradation, bioaugmentation with *Burkholderia xenovorans* LB400 was performed, and to improve the anaerobic PCB degradation, soil microcosms were subjected to redox cycling (two weeks of flooding and two weeks of non-flooding).

Chapter V described the results of phytoremediation and bioaugmentation. The presence of switchgrass significantly enhanced the PCB removal in the soil after 24 weeks. The improved PCB removal was found to be the result of phytoextraction and enhanced microbial activity. The microbial PCB degradation was not improved as much as expected, which was also indicated by RT-qPCR of *bph*A. The reason may be the availability of other carbon sources in the rhizosphere. It is also noted that microbial activity had different effect on different PCB congeners. PCB 52 is more susceptible to aerobic degradation by indigenous microorganisms, despite of its *ortho* chlorine substituents. After bioaugmentation, PCB 52 removal was improved the most, while the removal of PCB 77 and 153 was not significantly improved. The active aerobic microbial PCB degradation was also confirmed by the successful detection of *bph*A transcripts. The

presence of switchgrass was found to benefit LB400 survival and its ability to degrade PCBs. Thus the combination use of phytoremediaiton and bioaugmentation was recommended.

While Chapter V shows aerobic PCB degradation can be enhanced by bioaugmentation with LB400, in Chapter VI, an alternating anaerobic-aerobic condition was created by redox cycling, in an attempt to promote the anaerobic PCB degradation. The removal of PCB 153 was significantly improved in redox cycled and unplanted soils, as compared with unplanted soil, suggesting the enhanced anaerobic PCB degradation. The first two weeks of flooding of the redox cycling treatment successfully promoted the putative dechlorinating *Chloroflexi* population, which may be associated with the improved PCB 153 removal. However, the transformation products were detected in all soils. Especially, *ortho* dechlorinaiton product from PCB 153 was observed. Then the microbial community analysis was performed to search for the presence of taxa that contain a dechlorination potential in the rhizosphere. *Proteobacteria* was again found to be the most dominant, followed by *Acidobacteria*. *Geobacter*, known as iron reducers, and *Clostridium*, which was able to dechlorinate other chlorinated compounds, were detected, indicating their possible role in the PCB dechlorination.

One innovation of this work is assessing the *in situ* microbial PCB degradation by congener analysis in conjunction with culture-independent microbial analysis for core sediment samples. The depth pattern of congener data can provide evidence that anaerobic dechlorination has occurred, while the microbial analysis targeting biomarker genes shows the potential for both aerobic and anaerobic PCB degradation by indigenous microbial communities. The obtained information is valuable to decide whether microbial PCB degradation (natural attenuation) is feasible for the specific contaminated site.

Another innovation of this work is the detection of dehalorespiring bacteria in the sediments and soils undergoing PCB dechlorination. Currently most microbial PCB dechlorination research focuses on *Chloroflexi*, which include the only confirmed PCB

dechlorinators (*Dehaloccoccoides* spp. and o-17/DF1). Although the capability of dechlorinating PCBs is unknown for many other dehalorespiring bacteria, the lack of analysis of these bacteria can lead to incomplete understanding of the PCB degradation potential in contaminated sediments and soils, and a biased selection of remediation strategies. In this research, the presence of *Acinetobacter*, *Geobacter*, *Clostridium* and *Dehalogenimonas* in the contaminated sediments and soils may suggest their potential in PCB dechlorination. *Dehalogenimonas* was found present in PCB contaminated sediments from IHSC and in the soil microcosms under laboratory condition, making them a promising candidate for PCB dechlorination. Especially, flooding treatment was found to be able to increase *Dehalogenimonas* population, suggesting that flooding was effective in promoting the dechlorinating group. Effort was also made to identify the taxa that might be important to PCB biodegradation by linking microbial data and congener data. For example, the abundance of *Acidovorax* was found to be correlated with an indicator of PCB dechlorination. These observations provide the starting point for future studies about microbial PCB dechlorination. It is hoped that the use of high-throughput sequencing technologies could provide more insight into the microbial communities involved in the dechlorination process.

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