AEROBIC BACTERIAL DEGRADATION OF HYDROXYLATED PCBs: POTENTIAL IMPLICATIONS FOR NATURAL ATTENUATION OF PCBs

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ABSTRACT

Polychlorinated biphenyls (PCBs) are toxic and persistent chemicals that have been largely dispersed into the environment. The biological and abiotic transformations of PCBs often generate hydroxylated derivatives, which have been detected in a variety of environmental samples, including animal tissues and feces, water, and sediments. Because of their toxicity and widespread dispersion in the environment, hydroxylated PCBs (OH-PCBs) are today increasingly considered as a new class of environmental contaminants.

Although PCBs are known to be susceptible to microbial degradation under both aerobic and anaerobic conditions, bacterial degradation of OH-PCBs has received little attention. The overall objective of this study is therefore to evaluate the transformation of mono-hydroxylated PCBs by the well characterized aerobic PCB-degrading bacterium, *Burkholderia xenovorans* LB400. In order to achieve our overall objective, a series of model mono-hydroxylated PCBs have been selected and they are used to determine the toxicity of hydroxylated congeners toward the bacterium *B. xenovorans* LB400. The biodegradation kinetics and metabolic pathways of the selected OH-PCBs by *B. xenovorans* LB400 are then characterized using GC/MS. To understand further the molecular basis of the metabolism of OH-PCBs by *B. xenovorans* LB400, gene expression analyses are conducted using reverse-transcription real-time (quantitative) polymerase chain reaction (RT-qPCR) and microarray technology.

More formally, the specific aims of the proposed research are stated as follows:

(1) To evaluate the toxicity of selected mono-hydroxylated derivatives of lesser-chlorinated PCBs toward the bacterium *B. xenovorans* LB400.

(2) To assess the degradation of the selected OH-PCBs by *B. xenovorans* LB400.

(3) To gain further understanding of the molecular bases of the metabolism of the selected OH-PCBs by *B. xenovorans* LB400.

Three hydroxylated derivatives of 4-chlorobiphenyl and 2,5-dichlorobiphenyl, including 2'-hydroxy-, 3'-hydroxy-, and 4'-hydroxy- congeners, were significantly transformed by *Burkholderia xenovorans* LB400 when the bacterium was growing on biphenyl (biphenyl pathway-inducing conditions). On the contrary, only 2'-OH-4-chlorobiphenyl and 2'-OH-2,5-dichlorobiphenyl were transformed by the bacterium growing on succinate (conditions non-inductive of the biphenyl pathway). Gene expression analyses showed that only exposure to 2'-OH-4-chlorobiphenyl and 2'-OH-2,5-dichlorobiphneyl resulted in induction of key genes of the biphenyl pathway, when cells grown on succinate. These observations suggest that 2'OH-PCBs were capable of inducing the genes of biphenyl pathway. These results provide the first evidence that bacteria are able to cometabolize PCB derivatives hydroxylated on the non-chlorinated ring. Genome-wide transcriptional analyses using microarrays showed that 134 genes were differentially expressed in cells exposed to biphenyl, 2,5-dichlorobiphenyl, and 2'-OH-2,5-dichlorobiphneyl as compared to non-exposed cells. A significant proportion of differentially expressed genes were simultaneously expressed or down regulated by exposure to the three target compounds i.e., biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB, which suggests that these structurally similar compounds induce similar transcriptional response of *B.xenovorans* LB400.

Results of this study may have important implications for the natural attenuation of PCBs and fate of OH-PCBs in the environment. The recalcitrance to biodegradation and the high toxicity of some OH-PCBs may provide a partial explanation for the persistence of PCBs in the environment.

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ABBREVIATION LIST

B. xenovorans: Burkholderia xenovorans
BSTFA: *N,O*-Bis(trimethylsilyl)trifluoroacetamide
CBA: Chlorobenzoic Acid
Ct: Threshold cycle
DCB: Di-Chlorobiphenyl
DCBA: Di-Chlorobenzoic Acid
EC50: Effective Concentration 50% (half-maximum effective concentration)
GC-MS: Gas Chromatography Mass Spectrometry
IC50: Inhibitory Concentration 50% (half-maximum inhibitory concentration)
LB: Luri-Bertani
mRNA: Messenger RNA
NRTEE: (Canadian) National Round Table on the Environment and the Economy
OH-PCB: Hydroxylated Polychlorinated Biphenyl
OD 600 nm: Optical density (measured at 600 nm)
PCB: Polychlorinated Biphenyl
PCR: Polymerase Chain Reactions
PTFE: Polytetrafluoroethylene
RPM: Revolution per Minute
RT-qPCR: Reverse Transcription-quantitative PCR
TCB: Tri-Chlorobiphenyl
TCBA: Tri-Chlorobenzoic Acid
TeCB: Tetra-Chlorobiphenyl
TMCS: Trimethylchlorosilane
USEPA: U.S. Environmental Protection Agency

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CHAPTER 1

INTRODUCTION

Polychlorinated biphenyls (PCBs) represent a class of 209 different congeners made of a biphenyl core to which 1 to 10 chlorine atoms are attached. From the 1920s to 1970s, PCBs were widely used for a variety of industrial applications, including lubricants, dielectric fluids, and plasticizers (Borja et al. 2005). Due to their toxicity and persistence in the environment, the production and usage of PCBs were banned in most countries by the late 1970's. In the meanwhile, it is estimated that over 1.5 million ton of PCBs were produced worldwide. PCBs have been largely dispersed and they are today detected in virtually every compartment of the ecosystem, including air, water, soil, sediments, and living organisms (Field and Sierra-Alvarez 2008).

Hydroxylated polychlorinated biphenyls (OH-PCBs) are the major metabolites generated by higher organisms exposed to PCBs (Rezek et al. 2007, Zhai et al. 2010). OH-PCBs can be therefore released into the environment and they are today increasingly considered as a new class of environmental contaminants (Kawano et al. 2005). OH-PCBs have been detected in a variety of environmental samples, including animal tissues and feces, water, and sediments (Jansson et al. 1975; Flanagan and May 1993; Ueno et al. 2007). OH-PCBs have raised serious environmental concerns because they are more toxic than the parent PCBs and they exert various endocrine disruption effects (Arulmozhiraja et al. 2005).

1.1 Problem Statement

Since natural attenuation of PCBs occurs primarily as a result of microbial activity, aerobic and anaerobic degradation of PCBs by bacteria has been extensively studied (Pieper and Seeger, 2008). On the contrary, the bacterial biodegradation of OH-PCBs, even though they constitute the major toxic metabolites of PCBs, has received little attention.

In the current literature, only three studies have focused specifically on the bacterial degradation of OH-PCBs (Francova et al. 2004; Sondossi et al. 2004; Wiegel et al. 1999). Two of these reports have investigated the aerobic degradation of lesser-chlorinated congeners bearing hydroxyl and chlorine substituents both located on the same aromatic ring, i.e., having one unsubstituted ring (Francova et al. 2004; Sondossi et al. 2004). However, a review of the current literature on the topic reveals that hydroxylation of lower-chlorinated PCBs by higher organisms occurs preferentially on the non- (or lesser-) chlorinated ring, therefore generating primarily congeners hydroxylated on the *non-chlorinated ring* (Safe et al. 1975; Kucerova et al. 2000;Sietman et al. 2006; Zhai et al 2010). The existing literature therefore critically lacks information regarding the bacterial degradation of OH-PCB congeners bearing hydroxyl and chlorine substituents located on the different rings, which are the most susceptible to be generated by metabolic transformation of parent PCBs.

1.2 Overall Objective

The overall objective of this research was to demonstrate and, if applicable, to characterize the transformation of a series of derivatives of model lesser-chlorinated PCBs that are hydroxylated on the *non-chlorinated ring* by the well-characterized PCB-degrading bacterium, *B. xenovorans* LB400. The central hypothesis underlying this research is that OH-PCBs, due to their similarity of structure with biphenyl and PCBs, can be degraded by (aerobic) PCB-degrading bacteria and that this degradation occur through mechanisms similar as those involved in biphenyl and PCB metabolic transformations, i.e., through the biphenyl upper and lower pathways (Furukawa and Fujihara 2008).

1.3 Specific Objectives

In order to achieve the overall objective of the proposed research, I pursued the following the specific aims:

(1) To evaluate the toxicity of mono-hydroxylated derivatives of lesser-chlorinated PCBs toward the *B. xenovorans* LB400.

(2) To determine and characterize the degradation of selected mono-hydroxylated of PCBs by *B. xenovorans* LB400.

(3) To gain further understanding of the molecular basis of the metabolism of mono-hydroxylated PCBs by *B. xenovorans* LB400.

1.4 Selection of Mono-Hydroxylated PCBs

Three lesser chlorinated PCBs and their hydroxylated derivatives were chosen for this study: 4-chlorobiphenyl (4-CB), 2,5-dichlorobiphenyl (2,5-DCB), and 2,4,6-trichlrobiphenyl (2,4,6-TCB), and their three isomers hydroxylated on the non-chlorinated ring (*ortho*, *meta*, and *para*). Lesser chlorinated PCBs and OH-PCBs were chosen because it has been demonstrated that the efficiency and extent of aerobic degradation of PCBs by bacteria is reduced when the degree of chlorination increased (Pieper and Seeger 2009). We also selected hydroxylated derivatives with the phenolic group located on the non-chlorinated ring because the hydroxylation of PCBs by most living organisms have been shown to occur primarily on the non-chlorinated ring (Safe et al. 1975; Kucerova et al. 2000;Sietman et al. 2006; Zhai et al 2010). The only two reports published on the degradation of OH-PCBs focused on congeners in which chlorine and hydroxyl substituent were attached to the same ring of the biphenyl core. The proposed research is intends to be the first study of focusing specifically on the degradation of three different mono-hydroxylated PCB derivatives, 2'-OH, 3'OH, and 4'OH- of mono-, di-, and tri-cholorbiphenyls, in which hydroxyl groups and chlorine atoms are attached to different rings.

Mono-hydroxylated isomers of 4-chlorobiphenyl (4-CB) were selected because 4-CB, as one of the least-chlorinated congener is a model to study PCB biodegradation. Also 4-CB has been found in commercial PCB mixtures, in Aroclor 1242 and 1016, albeit at low levels (molar fractions of 0.22 and 1.0, respectively) (Albro et al. 1979). Hydroxylated derivatives of 4-CB have also been shown to be produced by the metabolism of living organisms, including plant, mammals, and fungi (Francova et al. 2004; Rezek et al. 2007;

Safe et al. 1975). Studying the transformation of PCBs in plants, Zhai et al. (2010) reported that hydroponic poplar plants metabolized 4-CB into a range of mono-hydroxylated derivatives, including mainly 2'-OH-, 3'-OH-, and 4'-OH-4-CB. Mono-hydroxylated isomers of 2,5-dichlorobiphenyl (2,5-DCB) were chosen because 2,5-DCB has been detected in Aroclor 1242 and 1016 with molar fraction of 0.31 and 0.34, respectively (Albro et al 1979). As was the case for 4-CB, hydroxylated derivatives of 2,5-DCB have also been shown to be produced by the metabolism of living organisms. For instance, studying the phytotransformation of PCBs, Rezek et al. (2007) showed the formation of 3'-OH-2,5-dichlorobiphenyl and 4'-OH-2,5-dichlorobiphenyl in *Nicotiana tabacum* (tobacco) cell cultures exposed to 2,5-DCB. Mono-hydroxylated isomers of 2,4,6-trichlorobiphenyl (2,4,6-TCB) were selected because 2,4,6-TCB is the only trichlorobiphenyl for which all the three hydroxylated isomers with the OH- group on the non-chlorinated ring were commercially available.

1.5 Selection of the Bacterial Strain, *Burkholderia xenovorans* LB400

Burkholderia xenovorans strain LB400 is one of the most characterized aerobic PCB degraders. The strain is capable of degrading more than 20 individual congeners including hexa-chlorinated compounds. *B. xenovorans* LB400 was originally isolated from a PCB-contaminated landfill in New York State. *B. xenovorans* is a member of the *Burkholderia graminis clade*, which are common inhabitants in the rhizosphere of plants (Chain et al. 2006). *Burkholderia* genus is highly widespread and members of the genus are found in various ecological niches, including soil, aquatic environments, and in association with plants, fungi, animals, and humans, from saprophytes to endosymbionts,

from biocontrol agents to pathogens. Members of the genus are plant and animal pathogens, including *B. mallei, B. pseudomallei,* and *B. cepacia*. *Burkholderia* species possess one of the largest prokaryotic genomes that involves the presence of many insertion sequences resulting in a high diversity of metabolic pathways. The genome of *B. xenovorans* LB400 has been recently sequenced. The *B. xenovorans* LB400 genome is 9.73 Mbp-wide, and involves about 9,000 – 10,000 coding sequences that are carried by three large circular chromosomes. *B. xenovorans* has high metabolic versatility, including an ability to degrade aromatic compounds that originate from root exudates and/or root turnover in rhizosphere. The strain is enriched in aromatic catabolic pathways (Chain et al. 2006).*B. xenovorans* is gram-negative, motile, non-sporulating, and is *catalase*- and *oxidase*-positive (Goris et al. 2004).

CHAPTER 2

LITERATURE REVIEW: HYDROXYLATED POLYCHLORINATED BIPHENYLS IN THE ENVIRONMENT

For the most part, the sections presented in this chapter will be published in Tehrani R and Van Aken B (2013) Hydroxylated polychlorinated biphenyls in the environment: source, fate, and toxicities. Journal of Environmental Science and Pollution Research DOI 10.1007/s11356-013-1742-6

2.1 Abstract

Hydroxylated polychlorinated biphenyls (OH-PCBs) are produced in the environment by the oxidation of PCBs through a variety of mechanisms, including metabolic transformation in living organisms and abiotic reactions with hydroxyl radicals. Consequently, OH-PCBs have been detected in a wide range of environmental samples, including animal tissues, water, and sediments. OH-PCBs have recently raised serious environmental concerns because they exert a variety of toxic effects at lower doses than the parent PCBs and they are disruptors of the endocrine system. Although evidence has accumulated about the widespread dispersion of OH-PCBs in various compartments of the ecosystem, little is currently known about their biodegradation and behavior in the environment. OH-PCBs are today increasingly considered as a new class of environmental contaminants that possess specific chemical, physical, and biological

properties not shared with the parent PCBs. This article reviews recent findings regarding the sources, fate, and toxicities of OH-PCBs in the environment.

2.2 Introduction

Polychlorinated biphenyls (PCBs) are toxic and persistent pollutants that have been widely dispersed in the environment before they were banned by most countries in the 1970s. PCBs exert various detrimental effects on wildlife and humans, including immunotoxicity, neurotoxicity, developmental toxicity, and reproductive toxicity, and there are classified by several agencies as suspected carcinogens (Field and Sierra-Alvarez 2008; Takeuchi et al. 2011).

PCBs may undergo hydroxylation through both natural and anthropogenic mechanisms, including metabolism by living organisms, reactions with atmospheric reactive oxygen species, and transformation in wastewater treatment plants (Totten et al. 2002; Ueno et al. 2007). The first step of the PCB metabolism by higher organisms frequently involves oxidation by the cytochrome P-450 system, resulting in the formation of hydroxylated derivatives (OH-PCBs) (Safe et al. 1975; Kaminski et al. 1981; Letcher et al. 2000; Rezek et al. 2007). Although transformation of PCBs by aerobic bacteria is also well documented, it results primarily in the formation of unstable di-hydroxylated metabolites, which may not constitute a significant source of OH-PCBs in the environment (Borja et al. 2005; Furukawa and Fujihara 2008; Pieper and Seeger 2008). On the other hand, increasing evidence has been provided that PCBs in gas phase react with hydroxyl radicals, which strongly suggests the formation of OH-PCBs in the atmosphere (Totten et al. 2002; Mandalakis et al. 2003). Although they are intermediates of a detoxification

sequence potentially leading to their conjugation and excretion, OH-PCBs exert a range of toxic effects at concentrations lower that their parent PCBs, including inhibition of mitochondrial respiration, generation of reactive oxygen species, oxidative damage to DNA, and endocrine disrupting effects (Narasimhan et al. 1991; Schultz et al. 1998; Purkey et al. 2004; Kitamura et al. 2005; Takueshi et al. 2011). PCBs are ubiquitous contaminants of the environment and their OH-derivatives have been detected in most compartments of the ecosystem (Jansson et al. 1975; Flanagan and May 1993; Ueno et al. 2007).

Although environmental concerns associated with PCBs have been the topic of an abundant literature, the formation of OH-PCBs and their detection in the environment have received comparatively little attention. Very few review articles focusing specifically on OH-PCBs have been published several years ago (Letcher et al. 2000; Kawano et al. 2005). Since then, additional research has been conducted in response to emerging environmental issues raised by OH-PCBs. OH-PCBs have been detected in more environmental samples, including animal tissues, water, snow, and sediments (Sakiyama et al. 2007; Ueno et al. 2007). In addition, new evidence has been provided suggesting that OH-PCBs are produced in significant amounts by abiotic processes (Persoon et al. 2010; Samuel et al. 2012). Following the observation that PCBs were taken up by plants and metabolized in plant tissues into OH-derivatives, several studies have investigated the biodegradation of OH-PCBs by bacteria (Sondossi et al. 2004; Francova et al. 2004; Tehrani et al. 2012). Because of their high toxicity and widespread detection in the environment, OH-PCBs are today increasingly considered as a new class of environmental contaminants (Schultz et al. 1998; Cámara et al. 2004; Purkey et al.

2004; Kawano et al. 2005; Kitamura et al. 2005). The objective of the present review is to provide updated information focusing on the sources, environmental fate, and toxicity of OH-PCBs.

2.3 PCBs as Ubiquitous Environmental Contaminants

2.3.1 Sources of PCBs

Polychlorinated biphenyls (PCBs) are toxic pollutants that are exclusively generated from human sources. They consist in a suite of 209 congeners made of a biphenyl core to which one to ten chlorine atoms are attached. PCBs are extremely stable and recalcitrant to biodegradation and they are therefore classified as persistent organic pollutants (POPs). The high chemical and physical stability and high dielectric constant of PCBs made them useful for a range of industrial applications, including lubricants, dielectric fluids, and plasticizers. Most PCBs were produced as mixtures commercialized under various brand names, such as Aroclor (USA), Kaneclor (Japan), Pyralene (France) or Clophen (Germany). Commercial production of PCBs started in 1929, until the recognition of their toxicity and persistence in the environment led to their interdiction in most countries in the late 1970s (Stockholm Convention) (Kawano et al. 2005). In the meanwhile, an estimated 1.5 million tons of PCBs were produced worldwide, of which a significant fraction has been released into the environment. As a consequence, PCBs are today detected in every compartment of the ecosystem, including air, water, soil, sediments, and living organisms. Although the production of PCBs has been generally banned in most countries, small amounts of non-Aroclor PCBs are still found in a series of products currently manufactured, including pigments, paints, and resins. For instance,

3,3'-dichlorobiphenyl (DCB) or PCB11, which is only present as traces in Aroclor mixtures, has been recently detected in environmental samples, including Chicago air (Illinois, USA) and water of Delaware River (New Jersey, USA) and Hudson River (New York, USA) (Hu et al. 2009; Rodenburg et al. 2010). Further analysis of the PCB profiles in the Delaware River and Hudson River provided evidence that 3,3'-DCB did not result from anaerobic dechlorination of Aroclor PCBs (Rodenburg et al. 2010).

2.3.2 Environmental Fate of PCBs

PCBs enter the environment through normal manufacture, usage, and disposal operations. Because of their high stability and relative volatility, PCBs are subject to environmental cycling, which typically involves volatilization from contaminated sites, atmospheric transport, and deposition in different areas. Although they are notoriously persistent in the environment, PCBs are metabolized by most living organisms, including mammals, plants, fungi, and bacteria. The formation of highly toxic PCB metabolites (including OH-PCBs) is suspected to play an important role in the PCB recalcitrance to biodegradation (Flanagan and May 1993; Cámara et al. 2004). Recent studies have also suggested that atmospheric reactions could constitute a significant sink for environmental PCBs (see section "Oxidation of PCBs in the atmosphere").

2.3.3 Toxicity and Environmental Concerns of PCBs

Although the occupational toxicity of PCBs has been known since the 1930s, their environmental impact was not reported until 1964, when the Swedish researcher, Soren Jensen, detected them in human blood. PCBs exhibit low-to-moderate toxicity, with chronic lethal dose 50% (LD50) in test animals ranging from 0.5 to 11.3 g kg^{-1} of body

weight (Borja et al. 2005). PCBs enter the body through ingestion, inhalation, and dermal contact, and, because of their hydrophobicity, they tend to accumulate in the liver and fatty tissues. In humans, PCBs are commonly detected in breast milk and blood, with concentrations increasing with the age. Reported effects on humans include fatigue, chloracne, liver damage, weight loss, and various effects on the immune, reproductive, and nervous systems (ATSDR 2000). Studies conducted on workers exposed to PCBs showed an increase in liver cancers and malignant melanomas. PCBs are classified as suspected human carcinogens by the U.S. Environmental Protection Agency (EPA) and International Agency for Research on Cancer (IARC). PCBs are also suspected to be mild endocrine disruptors that can be responsible for the decreased fertility observed in aquatic species. PCBs have been shown to negatively impact phytoplankton populations, with potential long-term effects on the oceanic food chain, oxygen production, and carbon dioxide mitigation (Borja et al. 2005). PCBs are included in the U.S. EPA List of Priority Pollutants (http://oaspub.epa.gov/), the 2007 CERCLA (Comprehensive Environmental Response, Compensation, and Liability Act) Priority List of Hazardous Substances (http://www.atsdr.cdc.gov/), and the Stockholm Convention on Persistent Organic Pollutants (http://chm.pops.int/default.aspx).

2.4 Sources of OH-PCBs in the Environment

In essence, OH-PCBs are secondary contaminants that are produced by oxidative transformation of PCBs through both biological and abiotic processes. OH-PCBs formed by metabolic activity in living tissues can be released into the environment and enter the food chain by the ways of excretion, predation, and natural vegetation cycling.

2.4.1 Metabolism of PCBs in Mammals

The metabolism of PCBs in mammals follows a three-phase sequence that applies to the transformation of most xenobiotic contaminants (reviewed in Letcher et al. 2000). This sequence involves initial activation (phase I), conjugation with an endogenous molecule (phase II), and excretion from the body or sequestration in tissues (phase III). Phase I of the mammalian metabolism of PCBs typically occurs in the liver and involves hydroxylation mediated by a cytochrome P-450-dependent monooxygenase (CYP). Initial hydroxylation of PCBs proceeds either by direct insertion of a hydroxyl group (-OH) or by formation of an arene oxide intermediate that spontaneously rearranges into an OH-product (Figure 2.2) (Connor et al. 1997; Letcher et al. 2000). Although the resulting OH-PCBs may undergo further reactions (phases II and III), some congeners seem to accumulate preferentially in the blood, prior to their excretion from the body. Further metabolism of OH-PCBs includes glucuronidation and sulfonation reactions (phase II), producing conjugates that are more soluble than the parent PCBs are susceptible to fast excretion from the body (phase III) (Letcher et al. 2000). Phase I of the metabolism of PCBs in mammals may therefore constitute a significant source of OH-PCBs in the environment.

2.4.2 Metabolism of PCBs in Plants

The metabolism of PCBs in plants has been abundantly documented (reviewed in Van Aken et al. 2010). Plants transform PCBs following a sequence of reactions that shares similarities with the PCB mammalian metabolism and is referred to as the green liver model (Sanderman et al. 1994). As in other eukaryotic cells, the initial biotransformation

of PCBs in plants is catalyzed the cytochrome P-450 system or other oxidative enzymes, such as peroxidases (phase I), resulting in the generation of a variety of mono- and di-hydroxylated metabolites (Chroma et al. 2003; Harms et al. 2003). These hydroxylated compounds may undergo transferase-mediated conjugation with sulfonic acid, glucuronic acid or glutathione (phase II), which is followed by sequestration in the cell vacuole or incorporation into plant structures (phase III) (Sanderman et al. 1994). Several publications have reported the formation of OH-derivatives from mono- to tetrachlorinated congeners by different plant species, including *Atropa belladonna*, *Lactuca sativa*, *Populus nigra* × *deltoides*, *Solanum nigrum*, and *Rosa sp*. (Lee and Fletcher 1992; Wilken et al. 1995; Mackova et al. 1997; Rezek et al. 1997; Harms et al. 2003; Zhai et al. 2010). Hydroxylation of PCBs in plants occurs preferentially with lesser-chlorinated congeners and seems to be limited to compounds with less than five chlorine atoms. In the absence of further reactions, OH-PCBs formed in plant tissues are likely to be released into the environment through natural vegetation turnover.

2.4.3 Metabolism of PCBs in Bacteria, Fungi and Yeast

Microbial degradation of PCBs is well documented (reviewed by Borja et al. 2005, Furukawa and Fujihara 2008, and Pieper and Seeger 2008). Higher-chlorinated congeners (with four or more chlorine atoms) are susceptible to reductive dechlorination mediated by anaerobic bacteria (Bedard et al. 2005), while lesser-chlorinated congeners (with three or less chlorine atoms) undergo preferentially aerobic oxidative degradation mediated by Pseudomonas, *Burkholderia*, *Comamonas*, *Rhodococcus*, and *Bacillus* species (Pieper and Seeger 2008). Aerobic oxidation of PCBs is catalyzed by dioxygenases and results primarily in the formation of catechol-like di-hydroxylated metabolites, which typically

undergo breakdown of the biphenyl core by opening one of the aromatic rings (Furukawa and Fujihara 2008). For this reason, the bacterial metabolism of PCBs may not constitute a significant source of OH-PCBs in the environment. Although the metabolism of PCBs by fungi has received little attention, a yeast (*Trichosporon mucoides*) and a filamentous fungus (*Paecilomyces lilacinus*) have been shown to oxidize 4-chlorobiphenyl (4-CB) into hydroxylated congeners (phase I), which led to the formation of muconic acid and lactone conjugates (phase II), suggesting that these organisms are also capable of metabolizing PCBs through a three-step sequence (Sietmann et al. 2006).

2.4.4 Oxidation of PCBs in the Atmosphere

Several studies have demonstrated that PCBs in gas phase react with atmospheric hydroxyl radials (•OH), which may contribute to the formation of large amounts of OH-PCBs (Figure 2.2). PCBs are semi-volatile compounds that are present in significant concentrations in the atmosphere, which may have profound implications on their environmental cycling and reactivity (Anderson and Hites 1996; Persoons et al. 2010). Based on measured concentrations of PCBs in air and water, Hornbuckle et al. (1996) calculated seasonal PCB fluxes in Lake Superior and established that PCB volatilization and deposition were primarily governed by the ambient temperature and gas-phase PCB concentration. On the other hand, Totten et al. (2002) reported the depletion of tropospheric gas-phase PCBs during daytime in Chicago (Illinois, USA), Baltimore (Maryland, USA), and Jersey City (New Jersey, USA). Based on the observation that depletion rates followed diurnal variations, the authors concluded that PCB removal occurred through reaction with sunlight-generated hydroxyl radicals. This conclusion was supported further by the observation that PCB reduction decreased with the degree of

chlorination, which determines the reactivity of PCBs with hydroxyl radicals (i.e., 10 - 20% less reactivity for each additional chlorine substituent). Similarly Mandalakis et al. (2003) provided direct evidence of the reaction of gas-phase PCBs with atmospheric hydroxyl radicals in subtropical regions (Crete, Greece). As it was previously reported, the authors observed diurnal variations of PCB depletion that correlated the level of atmospheric hydroxyl radicals. The reactivity of PCBs with hydroxyl radicals was also shown to be higher for lesser-chlorinated congeners. The observed diurnal variations of PCBs could not be explained by temperature-controlled volatilization, which would result in higher PCB levels during the day, as it was described by Hornbuckle et al. (1996). It has also been suggested that the reduction of PCBs during daytime could be related to the extension of the boundary layer of the troposphere, leading to larger dilution of PCBs. However, this explanation does not account for the higher reduction rates observed with lesser-chlorinated congeners (Mandalakis et al. 2003). Based on the measured reaction constants, the authors calculated half-lives of approx. 10 days for 2,4'-dichlorobiphenyl (DCB) and 20 days for 2,3,3',4',6-pentachlorobiphenyl (PCB) in tropical and subtropical regions (versus 60 and 120 days in polar regions, respectively). Hydroxyl radicals are instable, short-life species that are among the strongest known oxidants, and they are susceptible to react with most organic molecules, including PCBs. Hydroxyl radicals are formed by photochemical reactions and they are present at low concentrations in the atmosphere and surface water. Hydroxyl radicals have been reported to be present in the troposphere at average levels of approx. 6.5 105 molecules cm^{-3} (Sinkkonen and Paasivirta 2002). Based on calculated reaction constants, Anderson and Hites (1996) estimated that approx. 8,300 tons of PCBs are removed annually from the atmosphere by

reaction with hydroxyl radicals (as comparison values, 240 and 2 tons of PCBs are estimated to be removed annually by sediments burial in marine and fresh water, respectively). However, although several publications have presented evidence of reaction between PCBs and hydroxyl radicals in the atmosphere, no study has demonstrated the actual formation OH-PCBs through this mechanism. It is noteworthy that reaction between PCBs and hydroxyl radicals in the atmosphere likely results in the formation of a range of oxidation products in addition to OH-PCBs. For instance studying the gas-phase reaction of PCBs with hydroxyl radicals under laboratory conditions, Brubaker and Hites (1998) reported the detection of several oxidation products, including mainly chlorobenzoic acids and only traces of OH-PCBs. The reaction between hydroxyl radicals and aromatic compounds proceeds through complex, multistep mechanisms resulting in transient loss of aromaticity and generating unstable structures with multiple resonance (mesomeirc) forms. The complex nature of such radical reaction was reflected in Figure 2.2 (pathway b) by the structure under brackets representing the resonance forms of a hypothetical radical intermediate.

2.4.5 Oxidation of PCBs in Water and Sediments

Although hydroxyl radicals have been suggested to be responsible for the formation of OH-PCBs in water and sediments, their concentrations in these matrices are likely to be very low due both to their slow formation rate (low irradiation energy) and their high reactivity with organic matter (Sinkkonen and Paasivirta 2002).

2.4.6 Oxidation of PCBs by Advanced Oxidation Processes

Analyzing OH-PCBs in surface water in several sites in Canada, Ueno et al. (2007) reported relatively higher concentrations of total OH-PCBs in the vicinity of sewage treatment plants in urban areas (130 pg L^{-1} and 35 pg L^{-1}) than offshore Lake Ontario (1.6 pg L^{-1}), suggesting the formation of these compounds during wastewater treatment processes. As suggested by the authors, OH-PCBs could be formed in sewage treatment plants either through microbial oxidation of PCBs or by reactions with hydroxyl radicals generated by advanced oxidation processes (e.g., ozonation) (Dasary et al. 2012). Indeed, PCBs have been reported to be present in significant amounts in the influents of wastewater treatment plants: by the analysis of large data sets on PCB congener concentrations in the influents and effluents of wastewater treatment plants in the Delaware River Basin (Delaware, New Jersey, and Pennsylvania, USA) and New York City metropolitan area (New York, USA), Rodenburg et al. (2010, 2012) provided evidence that significant dechlorination occurred in the sewage collection system. The resulting lesser-chlorinated congeners would therefore be more susceptible to microbial and abiotic oxidation in wastewater treatment facilities. On the other hand, the documented reactivity of PCBs with hydroxyl radicals under laboratory conditions has led to the idea of developing potential in situ remediation strategies that could generate OH-PCBs, including treatment by Fenton reagent and photocatalytic degradation by titanium dioxide (TiO2) (Huang and Hong 2000; Manzano et al 2003).

2.5 OH-PCBs as a New Class of Environmental Contaminants

The first publication referring to the presence of OH-PCBs in the environment is due to Jansson et al. (1975), who detected a suite of OH-PCBs in the feces of Baltic guillemot (*Uria algae*) and grey seals (*Halichoerus grypus*). Since then, numerous OH-PCB congeners have been found in living tissues, water, and sediments (Letcher et al. 2000; Darling et al. 2004; Sakiyama et al. 2007; Kawano et al. 2005; Ueno et al. 2007).

2.5.1 OH-PCBs in Wildlife

OH-PCBs can accumulate in higher trophic organisms by three ways: hydroxylation of ingested PCBs, ingestion of contaminated organisms, and ingestion of OH-PCBs produced by microorganisms in water and sediments (Letcher et al. 2000). Besides humans, animal models, plants, and fungi (see section "Sources of OH-PCBs in the environment"), OH-PCBs have been detected in the tissues and blood of a range of animals, including fishes, marine mammals, polar bears, and birds (Letcher et al. 2000; Kawano et al., 2005). Because of their higher polarity and their stereochemistry allowing specific binding to plasma proteins, OH-PCBs were previously believed to partition preferentially in the blood of higher organisms, prior to their rapidly excretion or transformation into glucuronide and sulfonate conjugates (Bergman et al. 1994; Letcher et al. 2000; Robertson et al. 2001). However, recent studies showed that OH-PCBs, even though more water soluble than their corresponding parent PCBs, retain a high hydrophobicity leading to their potential accumulation in the liver and adipose tissues or their persistence in blood by association with plasma proteins (Tampal et al. 2002). Moreover, some OH-PCBs seem to exhibit structural resistance to conjugation (e.g.,

glucuronation) and they have been shown to persist in the body for long periods of time (Tampal et al. 2002). The concentration and congener profiles of OH-PCBs detected in human and animal tissues are highly variable among species, but they involve mostly higher chlorinated congeners (with five or more chlorine atoms) (Letcher et al. 2000; Kawano et al. 2005). Reported ratios total OH-PCBs-to-total PCBs are also species-specific: relatively low ratios have been reported in fishes and marine mammals (< 0.01% in Great Lakes fishes), although higher ratios have been reported in birds and terrestrial mammals (Kawano et al. 2005). Different ratios total OH-PCBs-to-total PCBs among species may reflect different metabolic capabilities and kinetics. Major congeners frequently detected in animal and human tissues include 4-OH-2,3,3',4',5-pentachlorobiphenyl (PeCB) or PCB107, 4'-OH-2,3,3',4,5'- PeCB or PCB108, 4-OH-2,2',3,4',5,5'-hexachlorobiphenyl (HCB) or PCB146, 3-OH-2,2',4,4',5,5'-HCB or PCB153, 4'-OH-2,3,3',4,5,5'-HCB or PCB159, 4-OH-2,2',3,4',5,5',6-heptachlorobiphenyl (HeCB) or PCB187, and 4'-OH- 2,2',3,3',4,5,5'-HeCB or PCB172, whose suspected parent PCBs are major constituents of Araclor 1254 and Aroclor 1260 (from 0.82 to 13.59%) (Letcher et al. 2000; Kawano et al. 2005). Interestingly, these congeners all bear two chlorine atoms adjacent to the hydroxyl group and are mostly dissociated at near neutral pH (pKa ranging from 5.57 to 4.48), which may explain their recalcitrance to conjugation and persistence in tissues and body fluids (see section "Chemical and physical properties of OH-PCBs").

To the best of our knowledge, no study has reported the detection of OH-PCBs in the tissues of natural plants collected from PCB-contaminated sites (Van Aken et al. 2010). However, detectable levels of PCBs have been measured in plant species cultivated in the

greenhouse using soil collected from actual Aroclor-contaminated sites (Zeeb et al. 2006; Aslund et al. 2008). Together with the detection of OH-PCBs in plants artificially-exposed to PCBs in the greenhouse and the laboratory (see section "Metabolism of PCBs in plants"), this observation strongly suggests that natural plants growing on PCB-contaminated environments would contain traces of OH-PCBs. Due to their high hydrophobicity, PCBs are poorly taken up inside plant tissues, which also suggests that OH-PCB concentrations in natural plants would be very low.

2.5.2 OH-PCBs in Water and Sediments

Increasing concerns regarding the toxicity of OH-PCBs have led to attempts to detect them in various environmental samples (Table 2.1). To date, a number of lesser-chlorinated OH-PCBs have been identified in water and sediments samples (Darling et al. 2004; Sakiyama et al. 2004; Ueno et al. 2007; Kuch et al. 2010). Darling et al. (2004) measured low concentrations of OH-PCBs in various samples, including snow, surface water, and fish tissues, collected in the Great Lakes region (Canada). Several mono- and dichlorinated OH-PCBs were detected in surface water near wastewater treatment plants, which was attributed to microbial oxidation of lesser-chlorinated PCB congeners in the activated sludge and/or hydroxylation of PCBs by advanced oxidation processes. Only a few of the 14 lesser-chlorinated OH-PCBs detected in surface water were structurally identified. A wider variety of congeners, ranging from mono- to heptachlorinated (including in majority 2'-OH-2,3',4,6-TeCB, 3'-OH-2,3,5,6-TeCB, and 2'-OH-2,3,3',5,6-PCB), were detected from snow samples, reaching a total concentration of 1 to 7 pg L^{-1} (as compared with 100 to 4,000 pg L^{-1} for the total PCBs). Plasma samples from fish (brown bullhead) contained mainly hexa- to nonachlorinated congeners, but no mono- or

dichlorinated ones. Based on these results, the authors suggested that OH-PCBs were formed by three major processes: microbial oxidation of PCBs in wastewater treatment plant, metabolic transformation of PCBs in fish tissues, and abiotic oxidation in the atmosphere. The authors also mentioned that mono- and dichlorinated OH-PCBs could exist as impurities in other industrial compounds. Ueno et al. (2007) reported the detection of OH-PCBs, ranging from mono- to nonachlorinated, in rain, snow, and surface water samples from different sites in Ontario (Canada). Total OH-PCB concentrations ranged from 0.87 to 130 pg L^{-1} and 230 to 990 pg g^{-1} in surface water and particulate organic matter, respectively. Total OH-PCB fluxes ranged from <1 to 100 pg m^{-2} and from <1 to 44 pg m^{-2} day^{-1} in snow and rainwater, respectively. Higher OH-PCB fluxes were found near urban areas and the highest concentrations in surface water were detected in vicinity of two sewage treatment plants in eastern Toronto (130 pg m^{-2}) and Hamilton harbor (35 pg m^{-2}), suggesting higher source of PCBs and OH-PCBs from urban areas. A recent study by Sakiyama et al. (2007) showed the presence of a large number of OH-PCB congeners in sediments samples collected from rivers and estuaries of Osaka Bay (Japan). Total OH-PCBs were detected at the average concentration of 24 ng g^{-1} (0.90 to 150 ng g^{-1}) of sediments (dry weight). The ratio total OH-PCBs-to-total PCBs ranged from 1.4 to 13%. The ratios OH-PCB-to-PCB decreased significantly with the degree of chlorination: from 3.8 to 82% for monochlorinated congeners to below 0.2 % for octachlorinated congeners, which may reflect the lower reactivity of higher-chlorinated PCBs or the enrichment of sediments by atmospheric deposition of the more volatile lesser-chlorinated OH-PCBs (Sakiyama et al. 2007). Investigating estrogenic compounds in groundwater downstream of several abandoned landfills, Kuch et al.

(2010) recently reported the detection of OH-PCBs, together with other potential estrogens, such as bisphenol A, phthalic acid esters, and hydroxylated polycyclic aromatic hydrocarbons (OH-PAHs). The estrogenic activity exceeded the provisional benchmark of 0.5 ng 17β-estradiol (E2) L^{-1} at three out of seven sites tested, which was attributed in part to the presence of OH-PCBs.

2.6 Chemical and Physical Properties of OH-PCBs

As for PCBs, the environmental behavior of OH-PCBs is largely determined by the degree and pattern of chlorine substitution of the biphenyl core, resulting in important variations in solubility, toxicity, and biodegradation rate (Rayne and Forest 2010). For instance, it has been reported that a higher degree of chlorination results in lower reactivity of PCBs and OH-PCBs by oxidation reactions, likely because of the electron-withdrawing effect of chlorine atoms decreasing the electron density of the aromatic rings (Anderson and Hites 1996; Ueno et al. 2007). As a basis for discussing the effects of hydroxylation on the environmental fate and bioavailability of PCBs, relevant physical and chemical constants, including the octanol-water partition coefficient (log K_{ow}), Henry's constant, dissociation constant (Ka), and water solubility, were estimated using the on-line SPARC calculator (version 4.6; http://sparc.chem.uga.edu/sparc/) for a suite of PCBs and OH-PCBs (Hilal et al. 2004). PCBs and OH-PCBs were selected based on their relative proportion in Aroclor mixtures and their detection in animal and human tissues (Table 2.2). Table 2.2shows that increasing the degree of chlorination affects OH-PCBs in the same way as PCBs: higher log K_{ow}, lower Henry's constant and lower water solubility (Tampal et al. 2002; Anderson and Hites 1996; Kawano et al. 2005). On the

other hand, hydroxylation of PCBs results in a slightly lower log K_{ow}, which may reduce the adsorption on organic matter and increase the bioavailability of the molecules. Nevertheless, OH-PCBs remains largely hydrophobic (with log K_{ow} ranging from 4.5 to 9.0) because of the presence of one or two chlorinated phenyl rings (Letcher et al. 2000). OH-PCBs also exhibit significantly higher water solubility as compared to the parent PCBs, because of both the increase of polarity and the susceptibility to form hydrogen bonds. Hydroxylation then reduces the volatility of PCBs (lower Henry's constant), making them less susceptible to atmospheric transport. Unlike PCBs, OH-PCBs are weak acids because of the presence of the ionizable phenolic group. Table 2.1 shows that the pKa of OH-PCBs decreases with the degree of chlorination of the entire molecule and, more significantly, of the phenolic ring. OH-PCBs with two chlorine atoms adjacent to the hydroxyl group are mostly dissociated at neutral pH (pKa < 7.0) (Letcher et al. 2000; Tampal et al. 2002; Ueno et al. 2007). Ionization of OH-PCBs largely determines their fate and partitioning in the environment by reducing adsorption on organic matter, reducing volatility, and increasing water solubility (Schwarzenbach et al. 2003).

2.7 Bacterial Degradation of OH-PCBs

Although higher organisms have been shown to be capable of metabolizing PCBs, there is little information about their contribution to the natural attenuation of PCBs in the environment. Unlike higher organisms, whose PCB metabolism constitutes a detoxification process, bacteria transform PCBs as a side effect of their energy metabolism. Biodegradation by competent bacteria is therefore one of the major sinks of PCBs in the environment. Although bacterial degradation of PCBs is well documented

(see section "Metabolism of PCBs in bacteria"), little is known about the bacterial metabolism of OH-PCBs. A few publications have shown that lesser-chlorinated OH-PCBs were transformed by aerobic PCB-degrading bacteria through the biphenyl pathway: Sondossi et al. (2004) reported the transformation of variety of OH-biphenyls (2-, 3-, and 4-OH-biphenyl) and OH-PCBs (4-OH-2-CB, 4-OH-3-CB, and 4-OH-5-CB) by the bacterium *Comamonas testosteroni* B-356 and by a recombinant Pseudomonas putida strain harboring the biphenyl pathway system. Francova et al. (2004) reported the transformation of a series of *ortho*-substituted OH-PCBs (2-OH-3-CB, 2-OH-5-CB, and 2-OH-3,5-DCB) by biphenyl dioxygenases of *Burkholderia xenovorans* LB400 and *C. testosteroni* B-356. Unlike these reports that focused on congeners bearing the hydroxyl and chlorine substituents on the same ring, our group has recently showed that *B. xenovorans* LB400 was capable of transforming 4-CB and three of its derivatives hydroxylated on the non-chlorinated ring (i.e., 2'-OH-, 3'-OH-, and 4'-OH-4-CB) when cultivated under conditions inductive of the biphenyl pathway (Tehrani et al., 2012). Besides oxidative transformation of OH-PCBs, Wiegel et al. (1999) have reported reductive dehalogenation of higher-chlorinated OH-PCBs by the anaerobic PCB-degrader, *Desulfitobacterium dehalogenans*.

2.8 Biphenyl Pathway and Transformation of PCBs

Enzymes that are part of the upper biphenyl (bph) pathway catalyze the aerobic transformation of biphenyl and PCBs. Different studies on strains isolated from PCB contaminated sites have shown that PCBs with lower degree of chlorination and with chlorine substituents located on one ring are more easily degraded that those with a

higher degree of chlorination and/or chlorines on both rings. Some strains, such as *Burkholderia xenovorans* LB400 and *Rhodococcus sp*. RHA1 can transform a wide range of PCBs congeners (Pieper and Seeger 2008; Bopp 1986; Haddock et al. 1995; and Seeger et al. 1995). The following figure (Figure 2.1) illustrates the upper biphenyl pathway for degradation of 3-chlorobiphenyl.

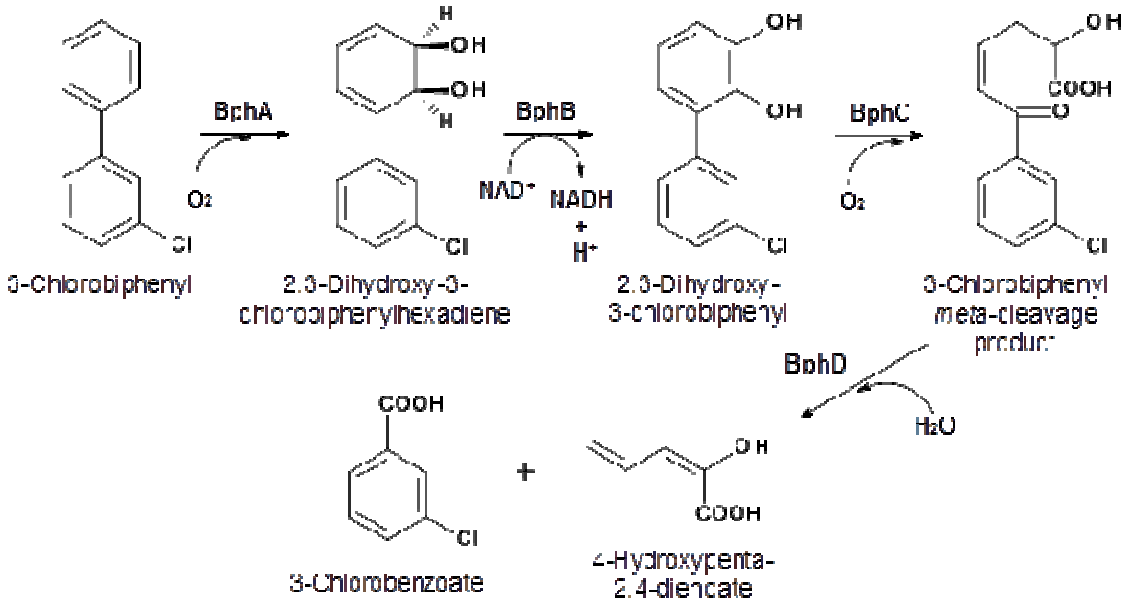

Figure 2.1. Bacterial aerobic degradation of lower-chlorinated PCBs is catalyzed by biphenyl dioxygenase (bph) gene cluster (upper pathway). (Figure from Furukawa and Fujihara 2008)

The upper pathway, which is similar for all described aerobic PCB degraders, involves seven genes grouped into one operon (biphenyl dioxygenase, bph) (Figure 2.1). A multicomponent dioxygenase *(bphA)* initiates hydroxylation of two adjacent biphenyl carbons to form an arene cis-diol. In the second step, a cis-2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase *(bphB)* further oxidizes the biphenyl ring to produce a dihydroxychlorobiphenyl. In the third step, a second dioxygenase, 2,3-dihydroxybiphenyl 1,2-dioxygenase *(bphC)*, opens the ring in *ortho*-or *meta*-position. The four step of the

upper pathway involves a hydrolase *(bphD)* that cleaves the resultingmolecule into chlorobenzoate and 2-hydroxypenta-2,4-dienoate (Borja et al. 2005; Van Aken et al. 2010).

Although biphenyl is a natural compound, it is not commonly found in environment. There are possibilities that other naturally occurring compounds with similar structure induce genes encoding biphenyl-degrading enzymes (Furukawa and Fujihara 2008; Master and Mohn 2001). Coal tar, crude oil, and natural gas are considered to be natural sources of biphenyl in the environment. Anthropogenic sources can be oil and coal process plants, wood preserving facilities, and municipal waste disposal sites (WHO 1999).

2.9 Toxicities and Environmental Concerns of OH-PCBs

2.9.1 Toxicity toward Higher Organisms

Increasing evidence suggests that the toxicity of PCBs originates, at least partly, from their OH-metabolites. OH-PCBs may be more toxic than their parent compounds and they have been found to exert a range of toxic effects that are not shown by the parent PCBs, including the inhibition of mitochondrial respiration, generation of reactive oxygen species (ROS), damage to DNA, thyroid hormone-disrupting activity, and estrogenic activity.

Several OH-PCBs have been shown to inhibit the mitochondrial respiration in mouse liver (Narasimhan et al., 1991). Different congeners were reported to act through different mechanisms, such as uncoupling oxidative phosphorylation or inhibiting

ATPase activity. Srinivasan et al. (2001) showed that several di-hydroxylated PCBs and PCB quinones produced ROS, including hydroxyl radicals, superoxide ions, and singlet oxygen, in vitro and in HL-60 human leukemia cells. The authors also reported that the di-hydroxylated PCBs and PCB quinones tested induced oxidative DNA damage in vitro. van den Hurk et al. (2002) demonstrated the inhibitory effect of OH-PCBs on the conjugating enzymes, sulfotransferase and glucuronosyltransferase, involved in the metabolism of environmental pollutants, such as 3-hydroxybenzo[a]pyrene, by catfish intestinal cells, which may have important implications for the detoxification capability of wildlife exposed to OH-PCBs.

In addition to other kinds of toxicity, OH-PCBs have been recognized endocrine disrupting compounds. OH-PCBs are potent inhibitors of the iodothyronine sulfotransferase activity toward the hormone triiodothyronine (T3) in vitro assays (Schuur et al., 1998). T3 is an important hormone regulating somatic and brain development, which may explain the reported developmental neurotoxicity of OH-PCBs. Sinjari and Darnerud (1998) have shown that exposure to some OH-PCBs (including 4-OH-3,5,3',4'-TeCB, which is a major fetal metabolite of the coplanar congener, 3,4,3',4'-TeCB) induced a reduction of tetraiodothyronine (T4) levels in maternal and fetal mice. The reduction of T4 was suspected to be caused by the disruption of T4 plasma transport by inhibition of transthyretin (TTR) binding. Certain OH-PCB congeners bearing an OH-group in *para* position with adjacent chlorine atoms resemble structurally the thyroid hormones T3 and T4 and they have been shown to compete for binding TTR, which is a major thyroid hormone transport protein in mammals (Letcher et al. 2000). It has been suggested that some thyroidogenic OH-PCBs might be one of the causes of thyroid

dysfunctions (i.e., large goiters and thyroid hyperplasia) that has been reported in salmonids of the Great Lakes for more than 30 years (Ueno et al. 2007).

In addition, OH-PCBs have been shown to exert a range of estrogenic and anti-estrogenic activities. Korach et al. (1988) reported the binding activity of a series of OH-PCBs to a soluble uterine estrogen receptor protein, with the highest activity recorded in congeners bearing a *para*-hydroxyl group. Using the yeast estrogen screen (YES) assay, Schultz et al. (1998) demonstrated the estrogenic activity of several OH-PCBs. The authors observed that hydroxylation was necessary to induce the estrogenic response and that the presence of chlorine atoms on the phenolic ring reduced the estrogenicity. OH-PCBs have also been recognized as inhibitors of human estrogen sulfotranferase (hEST) (Kester et al., 2000). hEST is a key enzyme in the metabolism of 17β-estradiol (E2). Disruption of the hEST pathway increases the cellular level of E2, which may be responsible for the estrogenic activity of OH-PCBs. Several other studies based on different bioassays have reported the estrogenic or anti-estrogenic activity of OH-PCBs. Structure-activity relationships (STARs) have recently been conducted on large suites of OH-PCBs, allowing identify key molecular features contributing to the estrogenic activity, such a *para*-hydroxyl group and *ortho*-chlorine substituents on the non-phenolic ring (Arulmozhiraja et al. 2005; Takeuchi et al. 2011). It is noteworthy that besides specific toxic mechanisms, hydroxylation is also known to increase the solubility and bioavailability of the molecules, which is susceptible to explain the higher toxicity of some OH-congeners (Cámara et al. 2004).

2.9.2 Toxicity toward Bacteria

OH-PCBs have been shown to be toxic for bacteria, which may have important implications for the biodegradation of PCBs by these organisms. Sondossi et al. (2004) measured a significant decrease of the oxygen uptake in the PCB-degrader, C. testosteroni B-356, exposed to OH-PCBs. Similarly, Cámara et al. (2004) reported decreased cell viability in a recombinant *E. coli* expressing genes involved in the PCB metabolism (biphenyl pathway) following exposure to PCBs, which was attributed to the high toxicity of di-hydroxylated derivatives originating from the oxidation of PCBs. Exposure of the PCB-degrader, *B. xenovorans* LB400, to the commercial mixture Aroclor 1242 resulted in induction of several detoxification genes, again suggesting the generation of toxic metabolites (Parnell et al. 2006). Similarly, we have recently showed that exposure to OH-metabolites of 4-CB resulted in severe inhibition of *B. xenovorans* LB400 under conditions inductive of the biphenyl pathway (Tehrani et al. 2012). OH-PCBs have been shown or suggested to affect bacterial cells by different mechanisms, including changes in membrane structure, uncoupling protonophoric shuttle mechanism, oxidative damage to DNA, inactivation of cytochrome c oxidase, and reduction of cell DNA content (Cámara et al. 2004). The increased toxicity of PCBs following their bacterial transformation resembles the metabolic activation of xenobiotics that is commonly observed in higher organisms.

2.10 Remediation of PCB Contaminated Sites

There are about 350 superfund sites in United States (USEPA 2011), total of 294,000 reported sites contaminated with PCBs (USEPA 20004), and in Canada, 148 sites are

listed on federal contaminated sites inventory (TBS-SCT 2011), total of 30,000 PCB contaminated sites were reported by NRTEE in 2004 (Gomes et al.2013; Saldana et al. 2010). Generally, remediation strategies fall under two main categories, *in situ* and *ex situ* approaches. *In situ* approach usually involves biological treatments (including bioremediation and phytoremediation), natural attenuation, physical, thermal and chemical treatments. In *ex situ* category of treatments, contaminated substance is moved to another site for chemical, thermal, and/or biological treatment (Gomes et al. 2013).

Removal of contaminated soil and sediments for incineration or landfill disposal is a common practice in the United States; however, such approaches are expensive and unsustainable. For examples excavation, dredging and landfilling of contaminated soil and sediments of Shiawassee River in Michigan has cost nearly 13 million USD (during 1981 to 1982, for 1341 m^3 of contaminated soil and 1216 m^3 of contaminated sediments). The most costly superfund site is Hudson River in New York where the cost of dredging and landfilling has reached 500 million USD (during 2009 to 2012, for 2 million m^3 of contaminated sediments) (Gomes et al. 2013).

Most of current methods for remediation or clean up of PCB contaminated sites are expensive and ecologically challenging (Schnoor et al. 1995), as they require excavation and manipulation of the sites. Efficient, cost effective, environmentally friendly and more sustainable alternative techniques are needed. Bioremediation techniques such as, anaerobic and aerobic biodegradation, and phytoremediation are less costly and more environmentally friendly (Gomes et al. 2013).

Phytoremediation is an environmentally friendly approach by utilizing plants to clean up and to detoxify organic pollutants such as PCBs, but unfortunately this process is usually slow and leads to accumulation of more toxic metabolites, and more hydrophilic compounds that eventually they can be released to the environment (Sylverter et al. 2009; Tehrani et al. 2012, Zhai et al. 2010a, Zhai et al. 2010b, Van Aken et al. 2010). Few studies have shown that transgenic plants may act better in uptake and metabolism of xenobiotics but in general the metabolisms of contaminants by plants are incomplete and may cause more environmental complications (Van Aken 2008). Genetically modified plants combined with applicable bacteria can be used as a new eco-friendly approach for treatment of soil, sediment, and water bodies contaminated with PCBs (Van Aken et al. 2010)

Rhizoremediation of PCBs is another method that can be used for remediation of PCBs. Many reports have proven that vegetated soils can play a better role in natural attenuation of PCBs compared to none-vegetated soil (Van Aken et al. 2010), and eventually through anaerobic bacterial degradation of PCBs, *meta* and/or *para* chlorines atoms can be removed, and *ortho* substituted PCBs can be formed (Wiegel and Wu 2000). Some PCBs congeners with lower degree of chlorination can be transformed through aerobic bacterial degradation and be degraded to chlorobenzoic acids (Borja et al. 2005).

Many of these remediation methods are investigate in a bench scale and their outcomes are very case dependent and may not globally apply to different sites with different profile of PCB congeners contamination with dissimilar environmental conditions. Besides, many of published reports have not thoroughly investigated the environmental factors affecting the efficiency or application of the methods to different sites. Therefore,

there is no comprehensive, global and sustainable clean-up method for removal of PCBs that is also technically and economically viable.

2.11 Conclusions

Although OH-PCBs have raised moderate concerns as environmental contaminants, emerging evidence suggests that they may constitute more dangerous species that the corresponding parent PCBs. Because of their diversity of structures, OH-PCBs can interfere with many biological systems (e.g., endocrine system), exhibiting activities that are not shown by the parent PCBs. Because of their higher solubility and lower volatility, OH-PCBs also exhibit environmental behaviors that are expected to be different than with PCBs.

As it is the case with many other environmental contaminants, limited information is available regarding the toxicology, metabolism, and environmental fate of PCB metabolites. This lack of environmental and toxicological information regarding OH-PCBs may be explained by the limited availability of synthetic OH-congeners, the large variety of structures of OH-PCBs (more than 800 for the mono-hydroxylated group only), each of them exhibiting different physical and chemical properties and biological activities, and the specificity of analytical methods that are different for OH-PCBs and parent PCBs.

Regulations regarding environmental contaminants are primarily based on the parent molecules and usually disregard their potential metabolites. Recently, the European Medicines Agency has set specific guidelines for risk assessment of pharmaceuticals entering the environment, as well as any metabolite formed at a concentration greater

than 10% of the parent compound (Celiz et al. 2009). However, to the best of our knowledge, such regulations do not exist regarding other classes of environmental pollutants. The U.S. Safe Drinking Water and Food Quality Protection Act require monitoring estrogenic substances in drinking water, but they do not formally list OH-PCBs (Wiegel et al. 1999).

PCBs exemplify a larger group of environmental pollutants that exert estrogenic activity after metabolic activation by the cytochrome P-450 system and referred to as proestrogens. Besides PCBs, a variety of xenobiotic pollutants are proestrogens, including trans-stilbene, biphenol A, benzophenone, and methoxychlor. As it is the case with PCBs, proestrogens typically become estrogenic (or more estrogenic) after addition of a hydroxyl group on an aryl core in *para* of a bulky hydrophobic group (Kitamura et al. 2008).

Ironically, many remediation processes potentially or actually used for the treatment of PCB-contaminated soil and water are susceptible to generate OH-PCBs, including bioremediation by bacteria or plants (phytoremediation) and advanced oxidation processes (e.g., Fenton oxidation, ozonation) used for in situ soil remediation or wastewater treatment.

Table 2.1. Detection of hydroxylated polychlorinated biphenyls (OH-PCBs) in various environmental samples, excluding living organisms, which were reviewed in Letcher et al. (2000) and Kawano et al. (2005).

Environmental Matrix	Collection site	Detected congeners [a]	OH-PCBs	PCBs	Ratio OH-PCBs/PCBs	Reference
River sediments	Upper Hudson River, USA	2-OH-2'-CB, 3-OH-2'-CB, 2,3-diOH-2'-CB	N.D.	N.D.	N.D.	Flanagan and May (1993)
Snow	Great Lakes basin, Canada	2'-OH-2,3',4,6-TeCB, 4'-OH-2,3',4,6-TeCB, 3'-OH-2,3,5,6-TeCB, 2-OH-2,3,3',5,6 PCB	4.0 (1.0-7.0) pg L^{-1}	2.05 (0.1-4.0) ng L^{-1}	N.D.	Darling et al. (2004)
Surface water	Toronto harbor, Canada	OH-CBs, OH-DCBs	N.D.	N.D.		Darling et al. (2004)
Sediments	Osaka Bay, Japan	OH-CB, OH-DCB, OH-TCB, OH-TeCB, OH-PeCB, OH-HCB, OH-HeCB, OH-OCB	2.4 (0.017-0.71) 1.6 (0.044-6.9) 7.2 (0.36-41) 8.1 (0.19-53) 5.2 (0.21-36) 1.5 (0.016-14) 0.41(0.024-3.5) 0.091 ng g^{-1}		38% (3.8-82) 9.9% (1.1-38) 6.2%(1.8-16) 5.3%(0.66-15) 6.5% (1.3-22) 3.4%(0.37-9.6) 2.3%(0.62-7.0) 0.19%	Sakiyama et al. (2007)
Snow	Southern Ontario, Canada	OH-PCBs OH-DCBs	43 (0-100) pg m^{-2}	23000 (10000-54000) pg m^{-2}	0.2 %	Ueno et al. (2007)
Rain		OH-DCBs, OH-TCBs, OH-TeCBs	28 (0-44) pg m^{-2} d^{-1}	3000 (1500-7300) pg $m^{-2}d^{-1}$	1.1%	
Surface water		OH-DCBs OH-CBs	22 (0.87-130) pg L^{-1}	590 (190-980) pg L^{-1}	2.1%	
Particulate organic matter		OH-TeCBs, OH-TCBs, OH-HCB	610 (230-990) pg g^{-1}	19000 (9500-29000) pg g^{-1}	0.3 %	
Groundwater	Unspecified abandoned landfill, USA	4-OH-BP, Unspecified OH-PCBs	N.D.	N.D.	N.D.	Kuch et al. (2010)

N.D.: Not determined.

[a] BP: biphenyl, CB: chlorobiphenyl, DCB: dichlorobiphenyls, TCB: trichlorobiphenyl, TeCB: tetrachlorobiphenyl, PeCB: pentachlorobiphenyl, HCB: hexachlorobiphenyl, HeCB: heptachlorobiphenyl, and OCB: octachlorobiphenyl.

Table 2.2. Environmentally-relevant chemical and physical constants of selected PCBs and OH-PCBs estimated using the SPARC Calculator (Hilal et al. 2004).

Compound[b]	Henry's Constant (log)	$logK_{ow}$	pK_a	Water Solubility[b] ($mg\ L^{-1}$)
2-CB	-1.7	4.65	N/A[c]	3.27 - 1.07
4-OH-2-CB	-5.43	4.62	8.77	144.2 - 40.87
4'-OH-2-CB	-5.42	4.54	9.55	153.6 - 43.57
2,4'-DCB	-2.14	5.11	N/A	0.91 - 0.29
4-OH-2,4'-DCB	-5.88	5.11	8.73	62.85 - 17.62
2,4-DCB	-1.94	5.22	N/A	0.79 - 0.25
4'-OH-2,4-DCB	-5.68	5.13	9.51	36.67 - 10.11
3,5-DCB	-2.02	5.18	N/A	0.87 - 0.28
4-OH-3,5-DCB[d]	-3.02	5	6.36	1.02 - 0.31
2,2',5-TCB	-2.05	5.74	N/A	0.25 - N.D.[e]
4-OH- 2,2',5-TCB	-5.43	5.54	7.12	13.10 - 3.69
4'-OH-2,2',5-TCB	-5.67	5.75	8.64	20.12 - 5.59
4-OH-2,3,5-TCB[d]	-3.3	5.65	5.66	0.18 - N.D.
2,2',3,5'-TeCB	-2.43	6.37	N/A	N.D.
4'-OH-2,2',3,5'-TeCB	-5.81	6.17	7.07	2.47 - 0.67
4-OH-2,2',3,5-TeCB[d]	-3.31	6.22	5.58	N.D.
2,2',3,4',6-PeCB	-2.41	7.04	N/A	N.D.
4-OH-2,2',3,4',6-PeCB	-5.94	6.78	6.27	0.66 - 0.17
4-OH-2,3,3',4',5-PeCB[d]	-4.06	6.75	5.57	N.D.
2,2',3,4',5',6-HCB	-2.83	7.67	N/A	N.D.
4-OH-2,2',3,4',5',6-HCB	-6.3	7.42	6.22	0.11 - N.D.
4-OH-2,2',3,4',5,5'-HCB[d]	-3.77	7.47	5.49	N.D.
2,2',3,3',4,5,6'-HeCB	-2.99	8.39	N/A	N.D.
4'-OH-2,2',3,3',4,5,6'-HeCB	-6.42	8.12	6.17	N.D.
4-OH-2,2',3,4',5,5',6-HeCB[d]	-4.07	8.13	4.68	N.D.
2,2',3,3',4,5,5',6'-OCB	-3.02	9.04	N/A	N.D.
4'-OH-2,2',3,3',4,5,5',6'-OCB[d]	-4.18	8.83	4.62	N.D.

[a] CB:chlorobiphenyl, DCB: dichlorobiphenyl, TCB: trichlorobiphenyl, TeCB: tetrachlorobiphenyl, PeCB: pentachlorobiphenyl, HCB: hexachlorobiphenyl, HeCB: heptachlorobiphenyl.

[b] Estimation of the water solubility requires knowledge of the melting point which is unknown for most OH-PCBs. The values presented were computed using the range of reported PCB melting points (24 to 149 ºC).

[c] N/A: Not applicable.

[d] These OH-PCB congeners were selected based on the presence of two chlorine atoms adjacent to the hydroxyl group, leading to low pK_a. The ones with five or more chlorine atoms were detected at high levels in animal and mammal tissues.

[e] N.D.: Not determined.

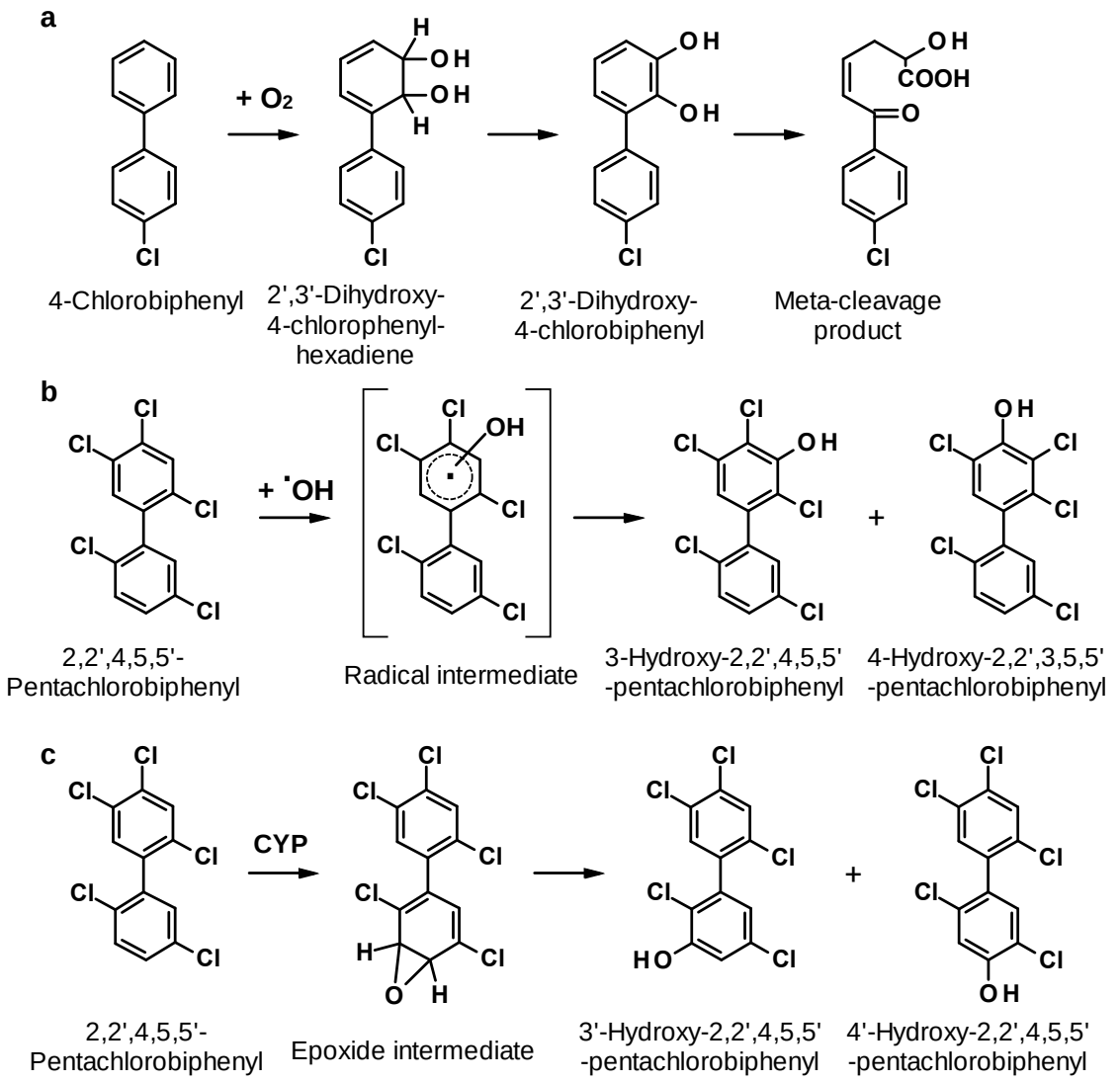

Figure 2.2 Potential mechanisms of formation of hydroxylated polychlorinated biphenyls (OH-PCBs). Pathway a: aerobic bacterial transformation of 4-chlorobiphenyl (CB) through the upper biphenyl pathway (adapted from Furukawa and Fujihara 2008), pathway b: oxidation of 2,2',4,5,5'-pentachlorobiphenyl (PeCB) by abiotic reaction with a hydroxyl radical(adapted from Letcher et al. 2000), pathway c: cytochrome P-450-mediated oxidation of 2,2',4,5,5'-PeCB through the formation of an arene oxide (adapted from Letcher et al. 2000). The structure under brackets shown in pathway b represents the resonance forms of a hypothetical radical intermediate.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Biphenyl (99% purity) was obtained from Acros Chemicals (Geel, Belgium). 2,5-Dichlorobiphenyl (2,5-DCB), 2,4,6-trichlorobipehnyl (2,4,6-TCB), 2'-hydroxy-2,4,6-trichlorobiphenyl (2'-OH-2,4,6-TCB), 3'-OH-2,4,6-TCB, 4'-OH-2,4,6-TCB, 4-CBA, 2,5-DCBA, and 2,4,6-TCBA (99% purity) were purchased from Accustandard (New Haven, CT). 4-Chlorobiphenyl (4-CB), 2'-hydroxy-4-chlorobiphenyl(2'-OH-4-CB), 3'-OH-4-CB, 4'-OH-CB, 2'-hydroxy-2,5-dichlorobiphenyl (2'-OH-2,5-DCB), 3'-OH-2,5-DCB, and 4'-OH-2,5-DCB were custom-synthesized by the Superfund Research Program Synthesis Core at the University of Iowa (Iowa City, IA). Hydroxylated PCBs (OH-PCBs) were synthesized by coupling a chlorinated boronic acid with a chlorinated bromoanisole followed by demethylation with boron tribromide (BBr3) as described by Lehmler and Robertson (2001). Synthesized compounds had purity greater than 99% as determined by gas chromatography-mass spectrometry (GC-MS). Except when otherwise stated, other chemicals were of analytical grade, solvents were of HPLC grade, and they were obtained from Acros Chemicals, Fischer Scientific (Pittsburgh, PA), or Sigma-Aldrich (St. Louis, MO). Moelcular biology reagents and kits, including DNA and RNA extraction and purification kits, reverse transcription kits, DNA polymerase, real-time PCR reagents, DNA and RNA laters and controls, and microarray reagent for attachment of polyA tail and labeling were from Qiagen (Germmantown, MD), Invitrogen (Grand Island, NY), Ambion (Carlsbad, CA), Applied Biosystems (Grand Island, NY), Agilent

(Santa Clara,CA), etc. Primers used for RT-qPCR were custom-synthesized by Integrated DNA Technology (Coralville, IA).

3.2 Bacterial Strain and Culture Conditions

Burkholderia. xenovorans LB400 was obtained from the U.S. Department of Agriculture (Agricultural Research Service Culture Collection (NRRL), Peoria, IL). The bacterium was routinely maintained and propagated on Luria-Bertani (LB) Lennox agar or broth. Before toxicity and biodegradation tests, the bacterium was acclimated on K1 mineral medium supplemented with 10 mM (1,180 mg L^{-1}) sodium succinate (i.e., conditions non-inductive of the biphenyl pathway) or 5 mM (770 mg L^{-1}) biphenyl (i.e., biphenyl pathway-inducing conditions) as the carbon source as described by Denef et al. (2004). Cell suspensions were cultivated on an orbital shaker at 185 rpm, 30°C, aliquots were collected in late exponential phase (approx. 24 hours on succinate and 48 hours on biphenyl), and used to subculture the bacterium under the same conditions. The cells were sub-cultured twice on succinate or biphenyl before to be exposed to 4-CB, 2,5-DCB, 2,4,6-TCB, and their mono-hydroxylated derivatives.

3.3 Growth Inhibition Testing

The inhibitory effect of 4-CB, 2,5-DCB, 2,4,6-TCB, and their mono-hydroxylated derivatives was determined by cultivating the bacterium in 12-mL (16 × 100 mm) culture tubes (Kimble,Vineland, NJ) closed with polytetrafluoroethylene (PTFE)-lined stopper. The tubes contained 4-mL of K1 medium supplemented with succinate (10 mM) or biphenyl (5 mM) and dosed with increasing concentrations (from 0.0 to 50 mg L^{-1}) of 4-

CB, 2'-OH-, 3'-OH-, and 4'-OH-4CB, 2,5-DCB, 2'-OH-, 3'-OH-, and 4'-OH-2,5-DCB, 2,4,6-TCB, 2'-OH-, 3'-OH-, and 4'-OH-2,4,6-TCB, and 4-chlorobenzoic acid(4-CBA), 2,5-dichlorobenzoic acid (2,5-DCBA). The tubes were inoculated with 0.5% v/v acclimated cells in late exponential phase and incubated on a rotary shaker at 185 rpm, 30°C for 96 hours. Experiments were conducted in triplicate. Bacterial growth was monitored by the optical density at 600 nm (OD600).

The concentrations of target compounds used in this study were relatively high considering concentrations of PCBs and OH-PCBs expected in the most environment. However, I decided to work with high concentrations for a practical reason. First, high concentrations greatly facilitated the detection of the target compounds and their metabolites. Second, higher concentrations ensure to observe a clear cell response to the target compounds. Toxicity screening was conducted by acute exposure: cells were exposed to relatively high concentrations of target compounds (up to 50 mg L^{-1}) over a short period (24 h to 96 h), even though typical exposure to contaminants at low dosage in the environment is better described by chronic exposure. This approach is further justified by the fact that typical PCB contaminated sites can show vey high concentrations (Nogales et al. 1999; Lunsdorf et al. 2000). As an example, a former Soviet Union missile-testing site was reported with an average total PCBs concentration of 28 g kg^{-1} soil (10 to 150 g kg^{-1})(Nogales et al. 1999).

3.4 Exposure Experiments for Biodegradation and Gene Expression

For biodegradation experiments, cells were grown in 12-mL tubes closed with PTFE-lined stopper and containing 4 mL of K1 medium supplemented with succinate or

biphenyl as described previously. The tubess were dosed individually with 10 mg L^{-1} of 4-CB and its hydroxylated derivatives (growing on succinate), 2 mg L^{-1} of 4-CB and its hydroxylated derivatives (growing on biphenyl), and 5 mg L^{-1} of 2,5-DCB, 2,4,6-TCB, and their hydroxylated derivatives (growing on biphenyl and succinate, two different groups), inoculated with 0.5% v/v acclimated cells, and incubated on an orbital shaker at 185 rpm, 30°C for 96 hours. All experiments were conducted in triplicate.

To determine the induction of genes of the biphenyl pathway by biphenyl, cells were cultivated in 125-mL Erlenmeyer flasks as described above using either succinate (1,180 mg L^{-1}) or biphenyl (770 mg L^{-1}) as the carbon source. To determine the induction of biphenyl pathway genes by 4-CB and its hydroxylated derivatives, 2,5-DCB and its hydroxylated derivatives, cells were grown on succinate (770 mg L^{-1}) and exposed individually to 4-CB, 2'-OH-, 3'-OH-, and 4'-OH-4-CB, 2,5-DCB, 2'-OH-, 3'-OH-, and 4'-OH-2,5-DCB dosed at 5 mg L^{-1}. Aliquots of cell suspensions were collected after 18 hours of growth/exposure, immediately mixed with two volumes of RNA Protect® (Qiagen, Carlsbad, CA), and frozen at -80°C prior to total RNA (mRNA). Gene expression experiments were conducted in triplicate.

3.5 Extraction and Analysis of PCBs, OH-PCBs, and Their Metabolites

PCBs, OH-PCBs, and their metabolites were extracted and analyzed using protocols adapted from Masse et al. (1989) and Maltseva et al. (1999). Briefly, parent PCBs (4-CB, 2,5-DCB and 2,4,6-TCB) were extracted using a mixture of Triton X100 and n-hexane (Masse et al. 1989). OH-PCBs (OH-4-CBs, OH-2,5-DCBs and OH-2,4,6-TCBs) and their metabolites (4-chlorobenozic acid, 2,5-dichlorobenzoic acid and 2,4,6-trichlorobenzoic

acid) were extracted with ethyl acetate and derivatized using a mixture of BSTFA/1-% TMCS. Analyses were conducted using a 7890A GC equipped with a 5975C MSD (Agilent, Santa Clara, CA) and a HP-5MS high-efficiency capillary column (30 m × 0.25 mm, 0.25 µm; Agilent). 4-CB, 2,5-DCB, 2,4,6-TCB, and their hydroxylated derivatives were extracted and analyzed using protocols adapted from Masse et al. (1989) and Maltseva et al. (1999). For PCBs analysis, the tubes were spiked with 10 mg L^{-1} of 3,3',4,4'-tetrachlorobiphenyl (3,3',4,4'-TeCB) as a surrogate and extracted with 40 µL of Triton X100 and 4 mL of n-hexane by horizontal shaking at 300 rpm for 30 min. The supernatant was collected and extraction was repeated three times with 4 mL of n-hexane. The extracts were combined and analyzed by GC-MS. For OH-PCBs analysis, the tubes were spiked with 10 mg L^{-1} of 3,3',4,4'-TeCB and extracted with one drop of 6 M hydrochloric acid and 4 mL of ethyl acetate by horizontal shaking at 300 rpm for 30 min. Extraction was repeated three times with 4 mL of ethyl acetate and the combined extracts were dried on a rotary vacuum drier at 35°C. Dried OH-PCBs were redissolved in 400µL of n-hexane and 400 µL of a mixture of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)and 1% trimethylchlorosilane (TMCS) (Thermo Scientific, Bellefonte, PA), vortexed, and incubated 20 minutes in a water bath at 60°C for derivatization. The reaction solution was then mixed with 3,400 µL of n-hexane, vortexed, and analyzed by GC-MS. PCBs and hydroxylated derivatives were analyzed on an Agilent 7890A GC coupled with a 5975C MSD and load with a high-efficiency capillary column (30 m × 0.25 mm, 0.25 µm). The GC was used in splitless injection mode with a purge flow of 50 mL min^{-1} at 0.8 min and injection volume of 1 µL. The temperature of the injection port was 280ºC and the one of the transfer line was 250 ºC. The initial temperature of the oven

was set at 70ºC for 2 min, then ra4-mped sequentially to 150ºC at a rate of 25 °C min^{-1},to 200ºC at a rate of 3°C min^{-1}, and to 280ºC at a rate of 8°C min^{-1}, and was then kept at 280ºC for 10 min. The carrier gas was high purity helium with a flow rate of 1.935 mL min^{-1}. The ion source was operated in the electron impact (EI) ionization mode and full-scan mass spectra (m/z 50 to 550) were recorded (Agilent 2000). Agilent Chemstation software (version E.02.00493) with 2008 NIST/EPA/NIH Mass Spectral Library (Agilent) was used for analyte identification. Quantification of PCBs and hydroxylated derivatives was done based on standard curves obtained with authentic standards.

3.6 RNA Extraction and Gene Expression Analyses

The expression of key genes of the biphenyl pathway, bphA, bphB, bphC, and bphD, was characterized using reverse-transcription real-time PCR (RT-qPCR). Total RNA was extracted from exposed cell suspensions (premixed with RNA Protect® reagent) using RNeasy® Tissue Mini Kit (Qiagen) according to the modified protocol for bacterial cells. Residual DNA was digested in the silica gel column using RNase-free DNase (Qiagen). RNA extracts were quantified by recording the OD260 using a NanoDrop™ ND-1000 (NanoDrop, Wilmington, DE). The quality of RNA was assessed by the ratio OD260/OD280. RNA was then reverse-transcribed using SuperScriptTM Reverse Transcriptase III (Invitrogen, Carlsbad, CA) and nonamer random primers (Integrated DNA Technologies, Coralville, IA). Negative controls were run without reverse-transcriptase. Quantitative PCRs were performed on an ABI StepOne™ System using SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Cycling conditions were as prescribed by the manufacturer and included an initial

activation/denaturation step at 95ºC for 10 min followed by 40 cycles of denaturation at 95ºC for 15 sec and annealing-extension at 60ºC for 30 sec. Post-cycling melting curves were generated from 60 to 95 ºC. Control reactions contained nucleic acid-free water or negative-control complementary DNA (cDNA) as template. DNA sequences of bphA, bphB, bphC, and bphD of *B. xenovorans* LB400 used to design specific primers were retrieved from the National Center for Biotechnology Information (NCBI) database. Primers were designed using Primer Express (version 3.0) with factory settings (Applied Biosystems) and synthesized by Integrated DNA Technologies. The amplification levels of target genes were normalized by the amplification levels of 16S ribosomal DNA (rDNA) used as an internal standard. Normalized amplification levels of exposed cells suspensions were expressed by reference to non-exposed controls using the Comparative CT(cycle threshold) method (or ΔΔCT method) (Applied Biosystems 2004) (StepOne™ Software, version 2.1, Applied Biosystems).The amplification efficiency was determined for all primer sets using 5-log dilutions of cDNA from treated and non-treated samples according to standard protocols (Applied Biosystems 2004) (Table 3.1).

3.7 Microarray Design and Analysis

A DNA (or cDNA) microarray is a solid surface with many immobilized polynucleotides (probes) attached in defined arrangements. The probes are usually printed by a robotic arm. Microarray is used as an assay to measure the types and quantity of mRNA transcripts extracted from cells after they are labeled with fluorescent dye. The DNA microarrays used for this study were custom-designed and printed by Agilent (e-Array platform, https://earray.chem.agilent.com/earray/). A large number of 45-nucleotide-long

probes representing 8,651 transcripts (covering 99.4% of the *B. xenovorans* genome) were retrieved from the OligoArray database (http://berry.engin.umich.edu/oligoarraydb/index.html). Of these probes, 91.4% were specific to only the corresponding transcript.

In this work, I extracted mRNA from cells exposed to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB and growing on succinate as primary carbon source. mRNA of cells growing on succinate (unexposed to target compounds) was also extracted to be used as control(untreated) samples.

The general workflow of microarray was as follows (Simon et al. 2003): Total RNA was reverse-transcribed and labeled to cDNA. The labeled cDNA was hybridized to the complementary probes immobilized on the array surface. After hybridization the array was washed to remove unbind cDNA. The microarray was then scanned with a laser light tuned to frequency of the fluorescent dye, and the fluorescence intensity was measured for each probe. Through image analysis, the pixel-level data were processed and converted to signal intensities, For technical reasons signal intensities must be normalized prior to data analysis. The goal of normalization is to adjust the signal intensities so that genes that are not in fact differentially expressed have similar intensity values across the array. The signal intensities of each probe is proportional to the transcript abundance in the RNA sample.

Total RNA was extracted from exposed cell suspensions (premixed with RNA Protect® reagent) using RNeasy® Tissue Mini Kit (Qiagen) according to the modified protocol for bacterial cells. Residual DNA was digested in the silica gel column using RNase-free

DNase (Qiagen). RNA was eluted in nuclease free water. The concentration of RNA samples were measured using a NanoDrop™ ND-1000 spectrophotometer (NanoDrop) and the quality of RNA was assessed by the ratios OD260/OD280 and OD260/OD230. Further assessment of the RNA quality was performed using an Agilent 2100 Bioanalyzer (Agilent Cat. No. G2938C, Santa Clara, CA) and computing the RNA integrity number - RIN (only RNA samples with a RIN equal or higher than 8 were utilized for microarray analysis). The RNA concentrations were between 541 to 612 ng μl^{-1}. Minimum volume required for microarray (8 X 15K format) experiment is 5 µl. Attachment of a polyA tail to RNA fragments was performed using the Poly(A)Polymerase tailing kit (Epicentre, Madison, WI) . PolyA-tailed RNA was then reverse-transcribed into cDNA labeled with Cy3 (Cyanine dyes fluoresce yellow-green) using the Low Input QuickAmp labeling kit (Agilent, Cat. No. 5190-2305).

Hybridization of cDNA, microarray scanning, and preliminary data analysis were performed at the Gene Expression Core Facility at the Fox Chase Cancer Center (Fox Chase, PA). These procedures are briefly described in the section below. 0.825 µg of labeled cDNA was then hybridized to the microarray probed at 65 ºC for 16 hours using the manufacturer protocols (Agilent one-color microarray-based gene expression analysis low input quick amp labeling, Version 6.6, Agilent Manual No.G4140-90040). Array slides were then washed following the manufacturer recommendations; they were scanned immediately using an Agilent microarray scanner (Agilent SureScan microarray scanner, Cat. No.G4900DA, Santa Clara, CA). Fluorescent signals were normalized and filtered using the Agilent Feature Extraction software (Agilent, version 10.7.1 , Santa Clara, CA). The Biometric Research Branch (BRB) Array Tools (developed by National

Cancer Institute) was used for data analysis. A F-test was performed (p-values 0.005) and the genes with less than 20 % of expression were filtered out. 134 genes were shown to significantly expressed at the nominal 0.005 level of the univariate test.

3.8 Data Processing and Statistical Analyses

In chapter4, the growth curve data were fit using a classical 3-parameter sigmoidal. The model parameters, standard deviations, and coefficients of determination of the regression (R^2) were estimated using SigmaPlot v8.4.0 (San Jose, Ca). In chapter 6, inhibition of *B. xenovorans* LB400 was modeled using a first-order inactivation model with shoulder using the non-linear regression procedure in Prism 5.0 (La Jolla, CA). The efficiency of real-time PCR amplifications was calculated by plotting CT values against log cDNA concentrations according to standard procedures. The statistical significance of the results (for biodegradation experiments and gene expression analyses) was assessed by performing unpaired t-tests at 95% confidence level.

Table 3.1. Primers used in this study for reverse-transcription real-time PCR analyses. Gene and primer names, primer sequences (in a 5' to 3' direction), and sequence accession numbers (NCBI) are presented

Gene	Primer	Sequence	**Accession**
Biphenyl dioxygenase (*bphA*)	bphA-92f	AAAAAGGGCTGCTTGATCCA	AAB63425
	bphA-211r	CGGTTTCAGGCACATGACTCT	
cis-2,3-Dihydrobiphenyl-2,3-diol	bphB-258f	TGCAGGCATCTGGGATTACTC	ABE37054
dehydrogenase (*bphB*)	bphB-377r	CAGGCCTTCACTGCATGGAT	
2,3-Dihydroxybiphenyl 1,2	bphC-556f	TACTTCCTGCACTGCAACGAA	ABE37053
dioxygenase (*bphC*)	bphC-675r	AAATGCAAAGCCGACGTCAT	
2-Hydroxy-6-oxo-6-phenylhexa-,4-	bphD-667f	GGAGAAATCAAGGCCAAGACAT	ABE37048
dienoate hydrolase (*bphD*)	bphD-786r	CTTGGAGAAAACGTGCAAACG	
16S ribosomal DNA (16S rDNA)	EB-916f	GAATTGACGGGGGCCCGCACAAG	
	EB-1115r	AGGGTTGCGCTCGTTG	

CHAPTER 4

TOXICITY OF PLANT METABOLITES OF POLYCHLORINATED BIPHENYLS TOWARD THE BACTERIUM *BURKHOLDERIA XENOVORANS* LB400

For the most part, the sections presented in this chapter have been published in Tehrani R, Van Aken B, Lehmler H and Schnoor J (2012) Toxicity of Plant Metabolites of Polychlorinated Biphenyls toward the Bacterium Burkholderia xenovorans LB400. Proceeding of Battelle 8th International Conference on Remediation of Chlorinated and Recalcitrant Compounds. Monterey California, May 19-24 2012

4.1 Abstract

Hydroxylated polychlorinated biphenyls (PCBs) are major metabolites generated by plants exposed to PCBs. The objective of this study was to determine the toxicity of mono-hydroxylated derivatives of 4-chlorobiphenyl (4-CB) and 2,5-dichlorobiphenyl (2,5-DCB) toward the PCB-degrading bacterium, *Burkholderia xenovorans* LB400. These hydroxylated metabolites have been previously detected in black nightshade and poplar plants exposed to 4-CB and 2,5-DCB. Cell suspensions of *B. xenovorans* LB400 were exposed to increasing concentrations (0 to 25 mg L^{-1}) of 4-CB, 2,5-DCB, and six of their mono-hydroxylated derivatives. Only mild reduction of the growth rate was observed when cells were exposed to the parent PCBs, even at the highest concentration applied. On the other hand, exposure to mono-hydroxylated derivatives resulted in a significant inhibition of the cell growth, depending on the congener and the carbon source

(i.e., biphenyl or succinate). When the bacterium was growing on biphenyl (i.e., under biphenyl pathway-inducing conditions), the degree of growth inhibition was consistently higher than when cells were growing on succinate (i.e., conditions non-inductive of the biphenyl pathway), suggesting that transformation of 4-CB, 2,5-DCB, and their derivatives through the biphenyl pathway resulted in the generation of toxic metabolites. Inhibition of the microbial growth was generally observed at lower concentrations with hydroxylated congeners from 4-CB than with congeners from 2,5-DCB, which was explained as a result of the lower solubility and bioavailability of higher chlorinated congeners. These results have environmental significance as the toxicity of hydroxylated metabolites of PCB toward PCB-degrading bacteria may be partly responsible for the recalcitrance of PCBs to biodegradation.

4.2 Introduction

The inhibitory effect of 4-CB, 2,5-DCB, and their hydroxylated derivatives was determined by cultivating the bacterium in 10-mL (16 × 100 mm) culture tubes closed with PTFE-lined stoppers. The tubes were filled with 4 mL of K1 medium supplemented with succinate (10 mM) or biphenyl (5 mM) and dosed with the target analytes (formulated as 5 mg mL^{-1} solution in acetone) at concentrations ranging from 2.0 to 25 mg L^{-1}. The tubes were agitated horizontally overnight before being inoculated with 0.5% v/v acclimated bacteria in late exponential phase. Cell suspensions were incubated on a rotary shaker at 185 rpm, 30 °C for 96 hours. Experiments were conducted in triplicate. Positive controls were dosed with an equivalent volume of acetone. The bacterial growth

was monitored by the optical density at 600 nm (OD600) using an Agilent 8453 UV-visible spectrophotometer (Palo Alto, CA).

Dose – response curves were drawn by plotting the growth inhibition against the concentration of toxicant. The experimental data were fit using a classical 3-parameter sigmoidal model:

$$Y = \frac{A}{1 + e^{-\left(\frac{X - X_0}{b}\right)}}$$

where Y is the percentage of growth inhibition as compared to non-exposed controls recorded in the early stationary phase (24 and 48 hours in the presence of succinate and biphenyl, respectively), X is the concentration of PCBs or hydroxylated derivatives (mg L^{-1}), and A, b, and X0 are model parameters. X0 represents the effective concentration 50 % (EC50), which is defined as the concentration of toxicant (mg L^{-1}) that results in a 50% growth inhibition as compared to non-exposed controls. A represents the maximum growth inhibition (%). The model parameters, standard deviations, and coefficients of determination of the regression (R^2) were estimated using SigmaPlot (v8.4.0).

4.3 Results and Discussion

The first step of the PCB metabolism by higher organisms, including plants, results primarily in the formation of toxic mono-hydroxylated derivatives (Kawano et al., 2005). Because hydroxylated PCBs have been detected in the environment, it is important to investigate whether they could inhibit the growth of bacteria responsible for PCB biodegradation. The PCB-degrading bacterium, *B. xenovorans* LB400, growing on biphenyl or succinate as sole carbon source, was exposed to increasing concentrations

(from 0 to 25 mg L^{-1}) of 4-CB and 2,5-DCB, and six of their mono-hydroxylated derivatives. These hydroxylated congeners were previously described as the major metabolites detected in black nightshade (*Solanum nigrum*) and poplar plants (*Populus deltoides* × *nigra*) exposed to 4-CB and 2,5-DCB (Resek et al., 2007; Zhai et al., 2010). We chose to expose the bacterium to high concentrations of PCB and metabolites (up to 25 mg L^{-1}) because PCBs were sometimes detected at high levels in soil and sediments, e.g., a maximum of 28,000 mg kg^{-1} was observed in the sediments a former missile testing base of the Soviet Army (Nogales et al., 1999).

The bacterial growth was monitored by the optical density at 600 nm (OD600) over 4 days. In the absence of inhibition, the microbial growth reached a maximum after 24 hours in the presence of succinate and 48 hours in the presence of biphenyl (i.e., early stationary phase). The presence of inhibitory concentration of hydroxylated PCB resulted in a longer lag phase, as compared to non-exposed controls. In order to compare the inhibitory effect of 4-CB, 2,5-DCB, and their hydroxylated metabolites on *B. xenovorans*, the plots of growth inhibition (based on OD600) against the concentration of toxicant were fit using a 3-parameter sigmoidal model, which was used to calculate the EC50 (i.e., concentration that resulted in 50% of growth inhibition, as recorded in the early stationary phase). As an example, Figure 4.1 shows the inhibition profiles of cells exposed to the hydroxylated isomers of 4-CB in the presence of biphenyl.

The dose – response data fitted generally well the sigmoidal model, as shown by the calculated coefficients of determination, R^2 (Table 4.1). Exceptions were observed when cells were exposed to 4-CB and 2,5-DCB in the presence of succinate, which resulted in the absence of significant inhibition, making the sigmoidal model unfit ($R^2 << 1$).

When cells were exposed to the parent PCBs in the presence of succinate as a carbon source (i.e., conditions non-inductive of the biphenyl pathway), no significant inhibition of the growth rate was recorded, even at the highest concentration tested (25 mg L^{-1}) (Table 4.1). On the other hand, a significant inhibition of growth was recorded when cells were exposed to the parent PCBs in the presence of biphenyl (i.e., under biphenyl pathway-inducing conditions), with the maximum inhibition (A) equal to 57.4% and 63.2% for 4-CB and 2,5-DCB, respectively. Exposure to mono-hydroxylated derivatives resulted consistently in higher inhibition of the microbial growth as compared to the parent PCBs, with the maximum inhibition (A) close to 100% and EC50 ranging from 3.0 to 9.6 mg L^{-1} for 4-CB and from 5.8 to 20.2 mg L^{-1} for 2,5-DCB, respectively. As observed with the parent PCBs, the degree of inhibition was generally higher when the bacterium was growing on biphenyl as compared to succinate, suggesting that induction of the biphenyl pathway resulted in generation of toxic metabolites.

Generally speaking, the inhibition of microbial growth was lower with hydroxylated congeners from 2,5-CB than with congeners from 4-CB, which can be explained as a result of the lower solubility and bioavailability of higher chlorinated congeners. Except with hydroxylated isomers from 2,5-DCB in the presence of succinate, the strongest inhibitory effect was observed with 3'-(*meta*-)hydroxylated congeners, with EC50 ranging from 3.0 to 5.8 mg L^{-1}.

Consistent with our results, a few publications have reported that hydroxylation of PCBs results in a higher toxicity for bacteria. Based on the oxygen uptake, Sondossi et al. (1991) reported a high toxicity of three hydroxylated PCBs (4-OH-2-CB, 4-OH-3-CB, and 4-OH-5-CB) at the concentration of 0.5 and 3.0 mM (~100 and ~600 mg L^{-1}) toward

the PCB degrader, *Comamonas testosteroni* B-356. The hydroxylated PCBs were reported significantly more toxic than the hydroxybiphenyls and chlorobiphenyls tested. Camara et al. (2004) studied the toxicity of PCBs using recombinant E. coli expressing subsets of biphenyl dioxygenase genes (bph). Based on the cell viability, the authors observed a high toxicity of di-hydroxylated derivatives originating from the metabolism of parents PCBs. Parnell et al. (2006) exposed the PCB degrader, *B. xenovorans* LB400, to the commercial mixture Aroclor 1242 (500 mg L^{-1}) and observed that induction of the biphenyl pathway resulted in overexpression of several detoxification genes, suggesting that the toxicity associated with PCBs was due to the formation of PCB metabolites. Hydroxylation of other aromatic compounds has been reported to have a similar effect (e.g., catechols are two orders of magnitude more toxic than benzene), likely because of the higher solubility of hydroxylated derivatives as compared with parent compounds (Camara et al., 2004).

The toxicity of hydroxylated PCBs for bacteria has environmental significance because bacteria are known as the major actors of PCB biodegradation under aerobic and anaerobic conditions (Borja et al., 2005; Pieper and Seeger, 2008). Despite their potential biodegradability, field analyses showed that PCBs are extremely persistent contaminants that can remain in the environment for years or decades. The toxicity of hydroxylated metabolites could inhibit the bacterial activity, potentially explaining the recalcitrance of PCBs to biodegradation.

Table 4.1. Growth inhibition of *B. xenovorans* LB400 exposed to 4-CB, 2,5-DCB, and their mono-hydroxylated metabolites, as measured in the early stationary phase (24 and 48 hours with succinate and biphenyl as carbon source, respectively). The effective concentrations 50% (EC50) ± standard deviations, maximum inhibition levels (A) ± standard deviations, and coefficients of determination (R^2) of the sigmoidal fitting are presented.

	Succinate				Biphenyl			
Compounds	**4-CB**	**2'-OH-4CB**	**3'-OH-4CB**	**4'-OH-4CB**	**4-CB**	**2'-OH-4CB**	**3'-OH-4CB**	**4'-OH-4CB**
EC50 (mg L^{-1})	ND	9.0±1.7	4.6±0.1	9.6±0.2	11.6±0.4	8.1±0.8	3.0±0.0	9.0±0.1
***A* (%)**	ND	99.7±0.1	99.1±0.3	98.6±0.1	57.4±46.8	98.0±2.6	99.7±0.3	103.0±4.2
R^2	0.15	0.99	1.00	0.99	0.67	1.00	1.00	0.96

	Succinate				Biphenyl			
Compounds	**2,5-DCB**	**2'-OH-2,5-DCB**	**3'-OH-2,5-DCB**	**4'-OH-2,5-DCB**	**2,5-DCB**	**2'-OH-2,5-DCB**	**3'-OH-2,5-DCB**	**4'-OH-2,5-DCB**
EC50 (mg L^{-1})	ND	8.7±1.3	13.9±4.3	20.2±3.1	13.8±2.7	7.4±1.4	5.8±0.7	9.5±1.7
***A* (%)**	ND	98.9±1.6	47.2±25.3	107.3±2.0	63.2±24.2	96.9±5.3	99.7±1.6	102.2±3.1
R^2	0.1	0.99	0.96	0.98	0.65	0.94	0.97	0.98

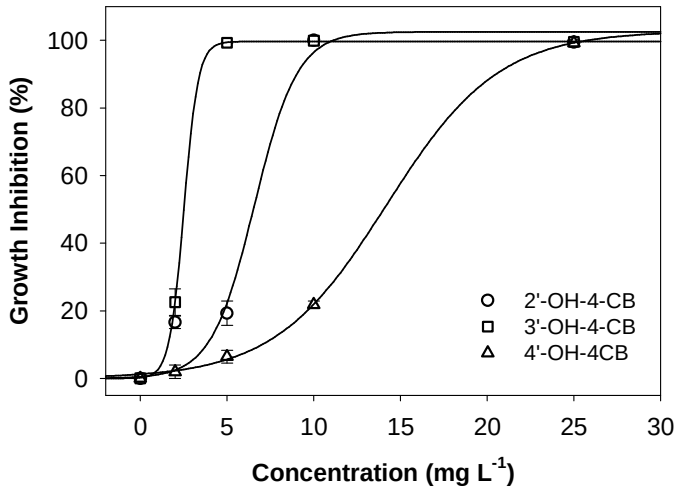

Figure 4.1. Growth inhibition (%) as a function of the concentration of 2'-OH-4-CB, 3'-OH-4-CB, and 4'-OH-4-CB (mg L^{-1}) when cell were growing in the presence of biphenyl as a carbon source. Error bars show the standard deviations between experimental replicates. Solid lines show the fitting to a 3-parameter sigmoid model.

CHAPTER 5

BIODEGRADATION OF MONO-HYDROXYLATED PCBS BY *BURKHOLDERIA XENOVORANS* LB400

For the most part, the sections presented in this chapter have been published in Tehrani R, Lyv M, Kaveh R, Schnoor J, and Van Aken B (2012) Biodegradation of Mono-Hydroxylated PCBs by Burkholderia xenovorans LB400. Biotechnology Letters, 34:12:2247-2252

5.1 Abstract

Three hydroxylated derivatives of PCBs, including 2'-hydroxy-4-chlorobiphenyl (2'-OH-4-CB), 3'-hydroxy-4-chlorobiphenyl (3'-OH-4-CB), and 4'-hydroxy-4-chlorobiphenyl (4'-OH-4-CB), were transformed by the PCB degrader, *Burkholderia xenovorans* LB400. When the bacterium was growing on biphenyl (biphenyl pathway-inducing conditions), all three hydroxylated isomers were significantly transformed. On the contrary, only 2'-OH-4-CB was transformed by the bacterium growing on succinate (conditions non-inductive of the biphenyl pathway). Gene expression analyses showed a strong induction of key genes of the biphenyl pathway (bph) when cells were grown on biphenyl, which is consistent with the transformation of the three isomers by biphenyl-grown cells. When cells were grown on succinate, only exposure to 2'-OH-4-CB resulted in overexpression of biphenyl pathway genes, which suggests that this isomer was capable of inducing the biphenyl pathway. These results provide the first evidence that bacteria are able to metabolize PCB derivatives hydroxylated on the non-chlorinated ring.

5.2 Introduction

Polychlorinated biphenyls (PCBs) are toxic environmental contaminants that are rather recalcitrant to biodegradation. Due to their high chemical and physical stability and high dielectric constant, PCBs have been used widely for a variety of industrial applications, including lubricants, dielectric fluids, and plasticizers. Because of their toxicity and recalcitrance to biodegradation, the production and usage of PCBs were banned in most countries by the late seventies (Field et al. 2008; Pieper and Seeger 2008). In the meanwhile, PCBs have been largely dispersed in the environment and they are today detected in every compartment of the ecosystem.

The first step of the PCB metabolism by higher organisms results in the formation of hydroxylated derivatives, which are increasingly suspected to be responsible for the toxicity of PCBs. Hydroxylated PCBs (OH-PCBs) are known to induce various deleterious effects, including disruption of transmembrane proton-gradient, generation of reactive oxygen species (ROS), oxidative damage to DNA, inhibition of proteins (e.g., transthyretin), and endocrine disruption (Camara et al., 2004; Kitamura et al. 2005). OH-PCBs have been detected in a variety of environmental samples, including animal tissues and feces, water, and sediments, and they are today increasingly considered as a new class of environmental contaminants (Kawano et al. 2005; Ueno et al 2007).

Although PCBs are known to be susceptible to microbial degradation under both aerobic and anaerobic conditions, little information is available about the bacterial metabolism of OH-PCBs (Field et al. 2008; Pieper and Seeger 2008). Two publications reported the bacterial transformation of OH-PCBs bearing both hydroxyl and chlorine substituents on

the same ring (Francova et al. 2004; Sondossi et al. 2004). On the other hand, bacterial degradation of PCB metabolites hydroxylated on the non-chlorinated ring has received little attention, even though hydroxylation of lower-chlorinated PCBs by higher organisms have been shown to occur on both chlorinated and non-chlorinated rings (Safe et al. 1975; Rezek et al. 2007).

The objective of this study was to characterize the biodegradation of three mono-hydroxylated isomers of 4-chlorobiphenyl (4-CB), with the hydroxyl group located on the non-chlorinated ring, by the PCB degrader, *B. xenovorans* LB400.

5.3 Results

The potential inhibitory effect of 4-CB and its three hydroxylated derivatives, 2'-OH-, 3'-OH-, and 4'-OH-4-CB, on *B. xenovorans* LB400 was determined by growing cells in the presence of increasing concentrations of the target compounds and monitoring the OD600. Although exposure to 4-CB did not result in significant growth inhibition at the highest concentration tested (50 mg L^{-1}), the three OH-4-CBs exerted inhibitory effects above 10 mg L^{-1} when the cells were growing on succinate (i.e., conditions non-inductive of the biphenyl pathway) and above 2 mg L^{-1} when cells were growing on biphenyl (i.e., biphenyl pathway-inducing conditions).

Transformation of OH-PCBs by *B. xenovorans* LB400 was studied by growing the bacterium on K1 mineral medium containing sub-inhibitory concentrations of 4-CB, 2'-OH-, 3'-OH-, and 4'-OH-4-CB (i.e., 10 mg L^{-1} in the presence of succinate and 2 mg L^{-1} in the presence of biphenyl). Concentrations of 4-CB, OH-4-CBs, and the metabolite, 4-chlorobenzoic acid (4-CBA), were determined after 96 hours of incubation (Table 5.1).

In the presence of biphenyl, all congeners tested were significantly transformed after 4 days: ~100% of 4-CB and 2'-OH-4-CB, 81% of 3'-OH-4-CB, and 54% of 4'-OH-4-CB. In the presence of succinate, ~100% of 4-CB and 2'-OH-4-CB were transformed, but not significant transformation of 3'-OH-4-CB (0%) and 4'-OH-4-CB (3%) were recorded over the time of the experiment. The metabolite, 4-CBA, was only detected in cultures growing on succinate and exposed to 4-CB and 2'-OH-4-CB. No other metabolites were detected. Relevant GC-MS chromatograms are presented in Figure 5.2, Figure 5.3 and Figure 5.4. Abiotic losses recorded in the non-inoculated controls were negligible, suggesting that disappearance of 4-CB and OH-4-CBs observed in the presence of cells accounted for biotransformation.

The expression of key genes of the biphenyl pathway, bphA, bphB, bphC, and bphD, in response to 4-CB and OH-4-CBs was characterized using Q-RT-PCR (Table 5.2.). We first compared the expression level of biphenyl pathway genes (bph) in cells growing in the presence of biphenyl (5 mM) and succinate (10 mM): biphenyl-grown cells showed expression of bph genes as compared to succinate-grown cells, with relative expression levels ranging from 6.5 to 8.8. On the other hand, when cells were grown on succinate as the carbon source, expression of bph genes was observed in the presence of biphenyl, 4-CB, and 2'-OH-4-CB (5 mg L^{-1}) as compared to non-exposed controls, with relative expression levels ranging from 2.5 to 3.8, 2.0 to 2.8, and 1.6 to 2.4, respectively. On the contrary, down regulation of bph genes was observed in the presence of 3'-OH-4-CB and 4'-OH-4-CB.

Based on t-tests, expression of bph genes upon exposure to biphneyl, 4-CB, and 2'-OH-4-CB relative to non-exposed controls was statistically significant for all genes tested ($p <$

0.05), with the exception of bphC exposed to 2'-OH-4-CB ($p = 0.09$). The amplification efficiencies for all sets of primers ranged from 97 to 105% (regression line slopes between -3.4 and -3.2, with R^2 higher than 0.98).

5.4 Discussion

We showed that exposure of *B. xenovorans* LB400 to hydroxylated derivatives of 4-CB resulted in a stronger inhibition when the cells were growing in the presence of biphenyl as compared to succinate, suggesting that induction of the biphenyl pathway produced toxic metabolites from OH-4-CBs. Although an increase of toxicity associated with the oxidative metabolism of OH-PCBs has not been reported, a few publications have showed that hydroxylation of PCBs by bacteria resulted in a higher toxicity (Sondossi et al. 2004; Camara et al. 2004; Parnell et al. 2006). Besides potential specific toxic mechanisms, hydroxylation results in an increase of solubility and bioavailability of the molecules susceptible to explain a higher toxic effect on cells (Camara et al. 2004).

We have also shown that under biphenyl pathway-inducing conditions (in the presence of biphenyl), *B. xenovorans* LB400 was able to transform 4-CB and all hydroxylated congeners tested, while only 4-CB and 2'-OH-4-CB were significantly transformed without biphenyl induction (in the presence of succinate). The enhanced transformation rates observed in the presence of biphenyl as compared to succinate, as well as the detection of the metabolite, 4-CBA, strongly suggest that the metabolism of OH-4-CBs occurred through the biphenyl pathway. The quantitative conversion of 2'-OH-4-CB into 4-CBA in the presence of succinate also suggests that only the hydroxylated ring was attacked by dioxygenases. The absence of 4-CBA detection in the presence of biphenyl

seems to indicate that 4-CBA was further transformed under biphenyl pathway-inducing conditions. Although *B. xenovorans* LB400 is commonly believed to be unable to transform 4-CBA, a recent publication from Gilmartin et al. (2003) suggests that strain LB400 metabolizes 4-CBA by the action of a glutathione S-transferase (GST) encoded by a bphK gene located in the bph operon. The important question of the degradation of 4-CBA by PCB-degrading bacteria needs to be further investigated. Previous publications have shown that hydroxylated lesser-chlorinated PCBs were transformed by PCB-degrading bacteria. Sondossi et al. (2004) reported the metabolism of 2-, 3-, and 4-hydroxybiphenyl, 2-, 3-, and 4-CB, and 4-hydroxyl-2-chlorobiphenyl (OH-2-CB), 4-hydroxyl-3-chlorobiphenyl (4-OH-3-CB), and 2-hydroxyl-5-chlorobiphenyl (2-OH-5-CB) by Comamonas testosteroni B-356 and a recombinant Pseudomonas putida strain harboring the biphenyl pathway system. As it was observed with most PCBs, the metabolism of these compounds involved dihydroxylation of the unsubstituted ring in positions 2,3 followed by *meta*-cleavage in position 1,2. Francova et al. (2004) reported the transformation of a series of *ortho*-substituted hydroxylated PCBs, including 2-hydroxyl-3-chlorobiphenyl (2-OH-3-CB), 2-OH-5-CB, and 2-hydroxyl-3,5-dichlorobiphenyl (2-OH-3,5-DCB), by biphenyl dioxygenase enzymes of *B. xenovorans* LB400 and C. testosteroni B-356. However, these two studies have focused on PCB derivatives that were hydroxylated on the chlorinated ring. In contrast, our investigation showed that bacteria are able to metabolize PCBs hydroxylated on the non-chlorinated ring, which may have important implications for natural attenuation of PCBs. Indeed, published evidence shows that the metabolism of lesser-chlorinated PCBs by plants, fungi, and mammals yields derivatives hydroxylated on both chlorinated and non-

chlorinated rings. For instance, the metabolism of PCBs by plants has been reported to generate derivatives hydroxylated on both chlorinated and non-chlorinated rings (Francova et al. 2004; Rezek et al. 2007). On the other hand, 4'-OH-4-CB and 3',4'-dihydroxy-4-chlorobiphenyl were the major urinary metabolites identified in rabbits inoculated with 4-CB (Safe et al. 1975).

The upper biphenyl pathway that is responsible for the bacterial transformation of PCBs into chlorobenzoates and chlorinated aliphatic acids typically involves seven genes (bphA to bphG) grouped into one operon (bph). Our gene expression results showed overexpression of four key genes of the biphenyl pathway (bphA, bphB, bphC, and bphD) when cells were growing on biphenyl as the primary carbon source. Biphenyl, as the natural substrate of the biphenyl pathway, is a strong inducer of bph genes, resulting in significant transformation of 4-CB, 2'-OH-, 3'-OH-, and 4'-OH-4-CB (Denef et al. 2004; Parnell et al. 2006). On the other hand, when cells were growing on succinate as the carbon source, a slight overexpression of *bph* genes was observed in the presence of 4-CB and 2'-OH-4-CB, which were the only compounds significantly degraded in the absence of biphenyl. The relatively low induction levels of *bph* genes in the presence of 4-CB and 2'-OH-4-CB (as compared with biphenyl) may account for the absence of significant transformation of their metabolite, 4-CBA, when cells were growing on succinate. The toxicity and recalcitrance of selected hydroxylated PCBs to biodegradation may provide a partial explanation for the persistence of PCBs in the environment.

Table 5.1. Molar fraction (%) of 4-CB, 2'-OH-, 3'-OH-, 4'-OH-4-CB, and the metabolite, 4-CBA, recorded in cell suspensions of *B. xenovorans* LB400 growing on biphenyl (5 mM) and succinate (10 mM). Results are presented as mean and standard deviation of three replicates

	Succinate as Carbon Source			Biphenyl as Carbon Source		
Compounds	**Initial (0 h)**	**Final (96 h)**	**4-CBA (96 h)**	**Initial (0 h)**	**Final (96 h)**	**4-CBA (96 h)**
4-CB	100.0±10.3	0.0±0.0	25.6±5.9	100.0±10.1	0.7±0.4	0.0±0.0
2'-OH-4-CB	100.0±7.7	0.0±0.0	99.4±4.7	100.0±2.2	0.0±0.0	0.0±0.0
3'-OH-4-CB	100.0±11.9	103.3±6.9	0.0±0.0	100.0±1.1	19.0±1.4	0.0±0.0
4'-OH-4-CB	100.0±0.6	96.9±1.8	0.0±0.0	100.0±8.4	45.7±5.7	0.0±0.0

[a] 100% corresponded to 10 mg L^{-1} of 4-CB, 2'-OH-, 3'-OH-, and 4'-OH-4-CB.

[b] 100% corresponded to 2 mg L^{-1} of 4-CB, 2'-OH-, 3'-OH-, and 4'-OH-4-CB.

Table 5.2. Expression levels of genes of the biphenyl pathway, bphA, bphB, bphC, and bphD, in *B. xenovorans* LB400 after 18 hours of cultivation. Left Panel: cells growing on biphenyl (BP) (770 mg L^{-1}, 5 mM) relative to cells growing on succinate (1,180 mg L^{-1}, 10 mM). Right Panel: cells growing on succinate (1,180 mg L^{-1}, 10 mM) exposed to 5 mg L^{-1} of BP, 4-chlorobiphenyl (CB), 2'-hydroxy- (OH-), 3'-OH-, and 4'-OH-4-CB relative to non-exposed cells

	A	**B**				
Genes	**BP (770 mg L^{-1})**	**BP (5 mg L^{-1})**	**4-CB (5 mg L^{-1})**	**2'-OH-4-CB (5 mg L^{-1})**	**3'-OH-4-CB (5 mg L^{-1})**	**4'-OH-4-CB (5 mg L^{-1})**
bphA	6.5 (5.9-7.1)	2.5 (2.4-2.6)	2.0 (1.9-2.2)	1.6 (1.4-1.7)	0.25 (0.23-0.28)	0.29 (0.28-0.30)
bphB	6.7 (5.1-8.7)	3.1 (2.9-3.2)	2.2 (1.9-2.6)	2.2 (1.9-2.5)	0.66 (0.51-0.85)	0.46 (0.37-0.58)
bphC	7.6 (6.9-8.3)	3.8 (3.6-3.9)	2.8 (2.4.-3.2)	2.4 (1.8-3.2)	0.46 (0.42-0.49)	0.41 (0.33-0.50)
bphD	8.8 (8.0-9.6)	2.9 (1.8-4.7)	2.3 (1.6-3.2)	2.2 (1.8-2.5)	0.21 (0.19-0.24)	0.16 (0.14-0.17)

Results are presented as mean of three replicates, the ranges of amplification levels were calculated based on the standard deviations of ΔΔCT values.

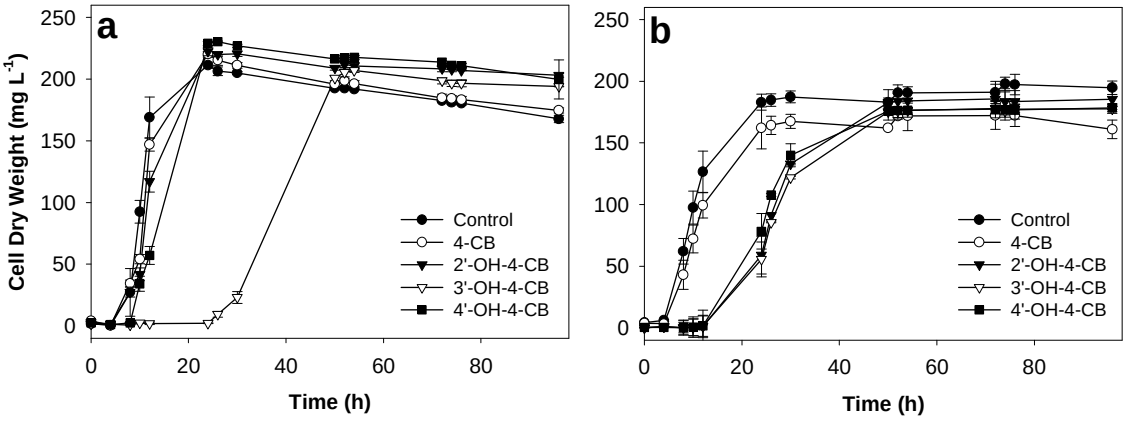

Figure 5.1. Growth curves of *B. xenovorans* LB400 exposed to 4-chlorobiphenyl (CB), 2'-hydroxy- (OH-), 3'-OH-, and 4'-OH-4-CB. Bacterial growth was expressed as cell dry weight (mg L^{-1}). Panel a: Cells were growing on succinate (1,180 mg L^{-1}, 10 mM) as the carbon source and were exposed to 4-CB, 2'-OH-, 3'-OH-, and 4'-OH-4-CB (10 mg L^{-1}). Panel b: Cells were growing on biphenyl (770 mg L^{-1}, 5 mM) as the carbon source and were exposed to 4-CB, 2'-OH-, 3'-OH-, and 4'-OH-4-CB (2 mg L^{-1}).

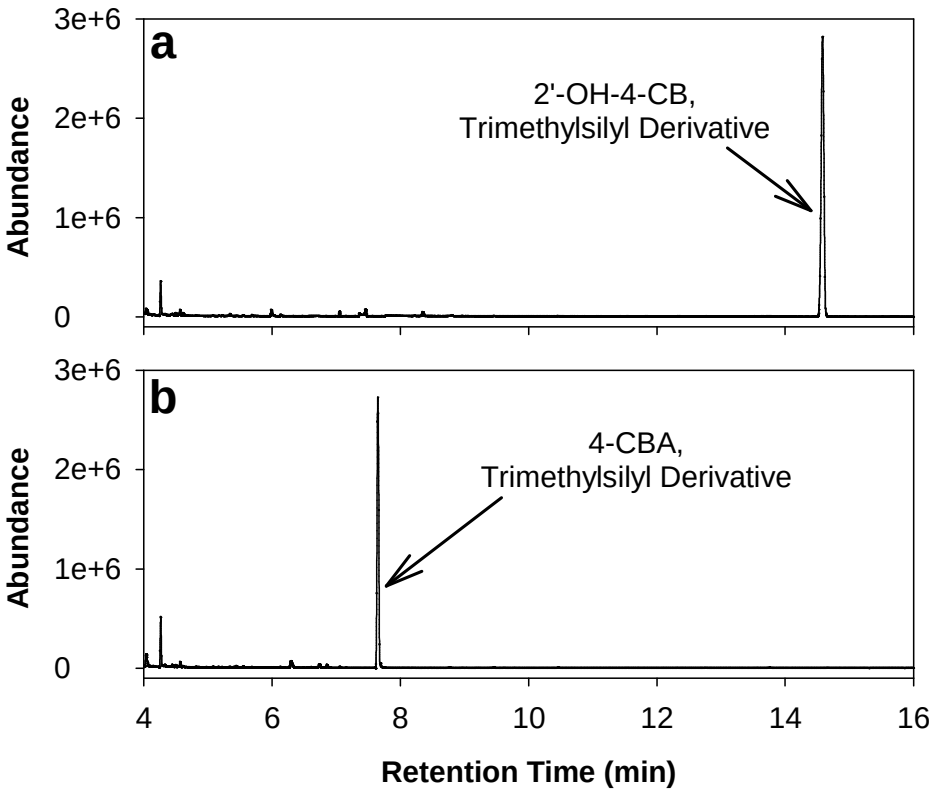

Figure 5.2. Full scan GC-MS chromatograms of extracts of *B. xenovorans* LB400 cultures using succinate (1,180 mg L^{-1}) as the carbon source and exposed to 2'-hydroxy-4-chlorobiphenyl (2'-OH-4-CB) (10 mg L^{-1}). The chromatograms were recorded at time 0 (Panel a) and after 96 hours of incubation (Panel b). 2'-OH-4-CB was detected by its trimethylsilyl derivative showing characteristic spectral features (Masse et al., 1989): M+ at m/z = 276, M-15 at m/z = 261. 4-chlorobenzoate (4-CBA) was detected by its trimethylsilyl derivative showing characteristic spectral features (Masse et al., 1989): M+ at m/z = 228, M-15 at m/z = 213

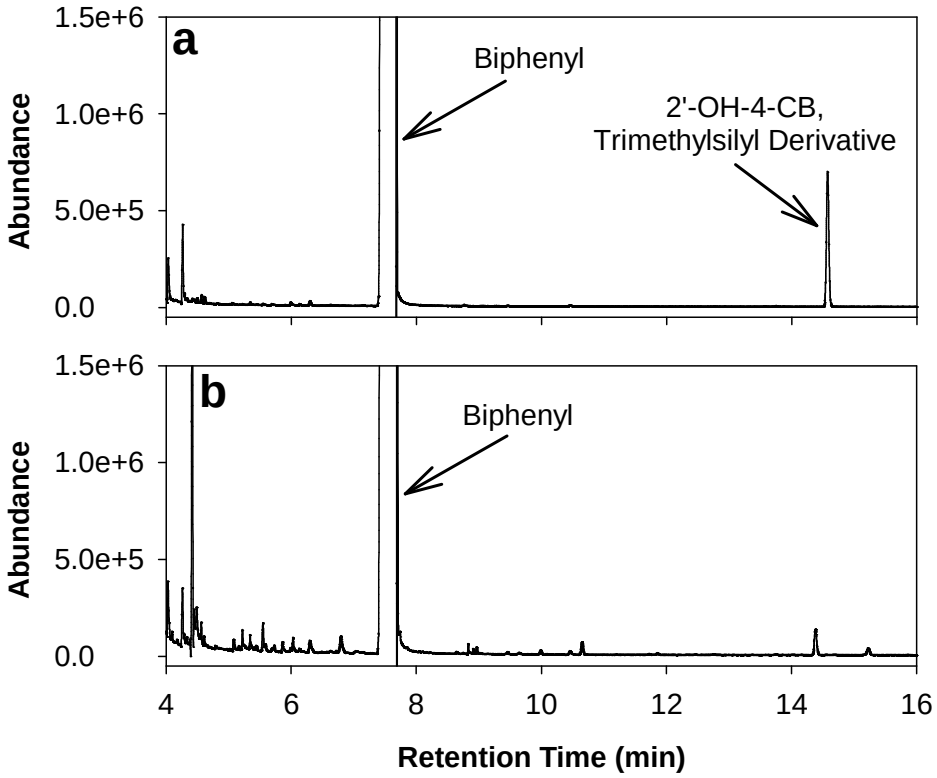

Figure 5.3. Full scan GC-MS chromatograms of extracts of *B. xenovorans* LB400 cultures using biphenyl (770 mg L^{-1}) as the carbon source and exposed to 2'-hydroxy-4-chlorobiphenyl (2'-OH-4-CB) (2 mg L^{-1}). The chromatograms were recorded at time 0 (Panel a) and after 96 hours of incubation (Panel b). 2'-OH-4-CB was detected by its trimethylsilyl derivative showing characteristic spectral features (Masse et al., 1989): M+ at m/z = 276, M-15 at m/z = 261

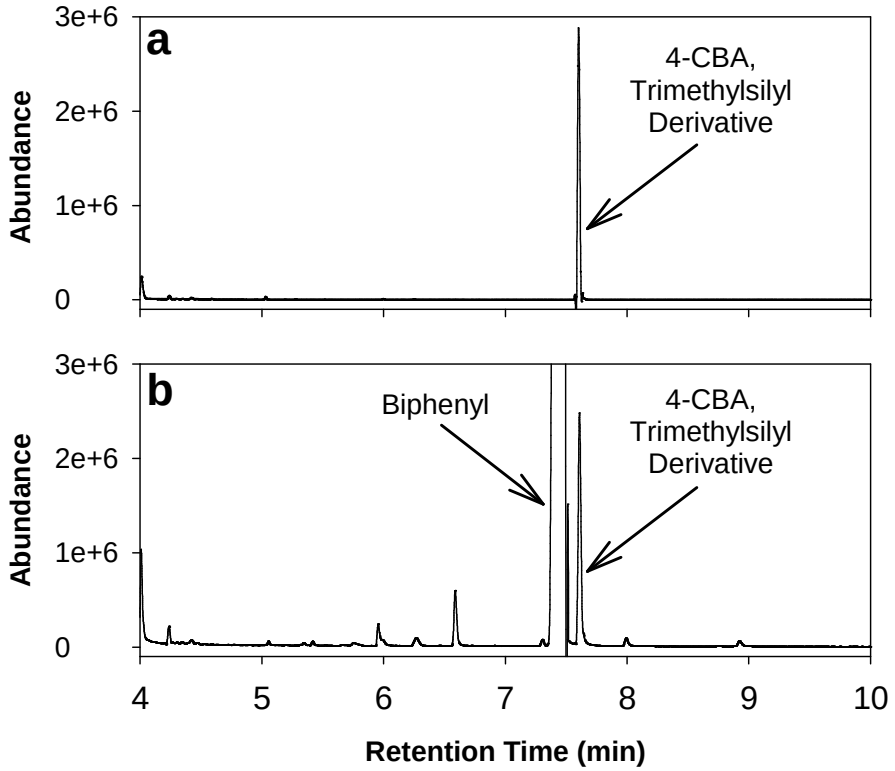

Figure 5.4. Full scan GC-MS chromatograms of 4-chlorobenzoate (4-CBA) standards. Panel a: The standard contained 10 mg 4-CBA L^{-1} and 1,180 mg L^{-1} succinate. Panel b: The standard contained 10 mg 4-CBA L^{-1} and 770 mg L^{-1} biphenyl. 4-CBA was detected by its trimethylsilyl derivative showing characteristic spectral features (Masse et al., 1989): M+ at m/z = 228, M-15 at m/z = 213

CHAPTER 6

TRANSFORMATION OF HYDROXYLATED DERIVATIVES OF 2,5-DICHLOROBIPHENYL AND 2,4,6-TRICHLOROBIPHENYL BY *BURKHOLDERIA XENOVORANS* LB400

For the most part, the sections presented in this chapter will be published in Tehrani R, Lyv M, and Van Aken (2013) Transformation of hydroxylated derivatives of 2,5-dichlorobiphenyl and 2,4,6-trichlorobiphenyl by Burkholderia xenovorans LB400, journal of Environmental Science and Pollution Research DOI 10.1007/s11356-013-1629-6

6.1 Abstract

The PCB-degrading bacterium, *Burkholderia xenovorans* LB400, was capable of transforming three hydroxylated derivatives of 2,5-dichlorobiphenyl (2,5-DCB) (2'-hydroxy- (2'-OH-), 3'-OH-, and 4'-OH-2,5-DCB) when biphenyl was used as the carbon source (i.e., biphenyl pathway-inducing condition), although only 2'-OH-2,5-DCB was transformed when the bacterium was growing on succinate (i.e., condition non-inductive of the biphenyl pathway). On the contrary, hydroyxlated derivatives of 2,4,6-trichlorobiphenyl (2,4,6-TCB) (2'-OH-, 3'-OH-, and 4'-OH-2,4,6-TCB) were not significantly transformed by *B. xenovorans* LB400, regardless of the carbon source used. Gene expression analyses showed a clear correlation between the transformation of OH-

2,5-DCBs and expression of genes of the biphenyl pathway *(bph)*. The PCB metabolite, 2,5-dichlorobenzoic acid (2,5-DCBA), was produced following the transformation of OH-2,5-DCBs. 2,5-DCBA was not further transformed by *B. xenovorans* LB400. The present study is significant because it provides evidence that PCB-degrading bacteria are capable of transforming hydroxylated derivatives of PCBs, which are increasingly considered as a new class of environmental contaminants.

6.2 Results

The half-maximum inhibitory concentration (IC50) of the target compounds, 2,5-DCB, 2,4,6-TCB, and their hydroxylated derivatives (2'-OH-, 3'-OH-, and 4'-OH-), toward *B. xenovorans* LB400 was determined by performing growth inhibition tests in which the cells were individually exposed to increasing concentrations of the compounds. The bacterial growth was monitored by the OD600.Overall, exposure to PCBs and OH-PCBs resulted in higher inhibition of cell growth when biphenyl was used as the carbon source as compared to succinate (Figure 6.1 and Table 6.1).

The capability of *B. xenovorans* LB400 to metabolize 2,5-DCB, 2,4,6-TCB, and their hydroxylated derivatives (2'-OH-, 3'-OH-, and 4'-OH-) was tested by growing the bacterium on K1 mineral medium containing succinate or biphenyl and dosed with 5 mg L^{-1} of target compounds. The residual concentrations of the compounds were determined after 96 hours of incubation (Table 6.2). In the presence of biphenyl, 2,5-DCB and the three OH-2,5-DCBs tested were significantly transformed after 4 days: 100% of 2,5-DCB and 2'-OH-2,5-DCB, 69.0 ± 3.3% of 3'-OH-2,5-DCB, and 60.7 ± 0.9 % of 4'-OH-2,5-DCB. In the presence of succinate, 100% of 2,5-DCB, 46.6 ± 0.6% of 2'-OH-2,5-DCB,

and 19.6 ± 5.3% of 4'-OH-2,5- DCB were transformed over the time of the experiment, while no significant transformation of 3'-OH-2,5-DCB was recorded. The expected metabolite, 2,5-DCBA, was detected in all cultures where a significant degradation of 2,5-DCB and OH-2,5-DCBs occurred (Table 6.2, upper section). 2,5-DCBA was measured at concentrations suggesting almost quantitative conversion of 2,5-DCB and OH-2,5-DCBs into 2,5-DCBA, except for 2'-OH-2,5-DCB in the presence of succinate, whose recovery accounted for 82.7 ± 1.6% of the parent compound. The estimated extraction efficiencies (expressed as mean ± standard deviation of three replicates) were 99.7 ± 3.8% for 2,5-DCB, 95.2 ± 1.8% for 2,4,6-TCB, 105.8 ± 6.5 for 2,5-DCBA, 100.1 ± 0.4% for 2,4,6-TCBA, 89.4 ± 1.3% to 95.6 ± 2.8% for OH-2,5-DCBs, and 94.5 ± 4.0% to 95.2 ± 1.8% for OH-2,4,6-TCBs. On the other hand, neither 2,4,6-TCB nor any of the OH-2,4,6-TCBs tested, except one, were significantly transformed by *B. xenovorans* LB400 over the time of the experiment, regardless of the carbon source used. Only 3'-OH-2,4,6-TCB was slightly transformed by *B. xenovorans* LB400 in the presence of succinate (12.3 ± 2.7%). The predicted biphenyl pathway metabolite, 2,4,6-TCBA, was not detected (Table 6.2, bottom section).

Because 2,5-DCBA is the only metabolite detected from the transformation of 2,5-DCB and OH-2,5-DCBs, the inhibitory effect and biodegradation of 2,5-DCBA by *B. xenovorans* LB400 were tested in the presence of succinate and biphenyl as carbon sources. The corresponding dose-response curves are presented in Figure 6.2. Biodegradation experiments did not show significant transformation of 2,5-DCBA regardless of the carbon source used (Table 6.3).

In order to understand further the metabolism of 2,5-DCB and its hydroxylated derivatives, the expression of key genes of the biphenyl pathway, bphA, bphB, bphC, and bphD, was determined using RT-qPCR (Table 6.4). The induction of bph genes by biphenyl was assessed by comparing their expression levels in cells growing in the presence of succinate (1,180 mg L^{-1}) and biphenyl (770 mg L^{-1}). Biphenyl was shown to result in significant overexpression of bph genes as compared to succinate, with relative expression levels ranging from 6.5 ± 0.5 to 8.8 ± 1.2.

On the other hand, when cells were grown on succinate (1,180 mg L^{-1}) as the carbon source, an overexpression of bph genes was observed in the presence of 5 mg L^{-1} of 2,5-DCB, and 2'-OH-2,5-DCB as compared to non-exposed controls, with relative expression levels ranging from 1.8±0.0 to 2.5±0.5 and 1.8±0.3 to 2.0±0.4, respectively (Table 6.4). (Exposure to 5 mg L^{-1} of biphenyl resulted in expression levels of 2.7±0.9 to 5.7±2.3.) On the contrary, a downregulation of bph genes was observed in the presence of 3'-OH-2,5-DCB and 4'-OH-2,5-DCB (5 mg L^{-1}). The expression levels of bph genes upon exposure to biphenyl, 2,5-DCB, and OH-2,5-DCBs relative to non-exposed controls were statistically significant for all genes tested ($p < 0.05$) except bphD. The amplification efficiencies for all sets of primers ranged from 91 to 101% (regression lines slopes between -3.5 and -3.3, with R2 higher than 0.99).

6.3 Discussion

In this study, we chose to study hydroxylated derivatives of a dichloro- and a trichlorobiphenyl: 2,5-DCB was selected because it is present in the commercial mixtures, Aroclor 1016 and 1242, and because hydroxylated isomers could be

synthesized; 2,4,6-TCB is not detected in commercial mixtures but it was chosen based on the commercial availability of its hydroxylated isomers. We showed that exposure of *B. xenovorans* LB400 to hydroxylated derivatives of 2,5-DCB and 2,4,6-TCB resulted in a stronger inhibition when the cells were growing in the presence of biphenyl as compared to succinate, suggesting either that induction of the biphenyl pathway produced toxic metabolites or that hydroxylated derivatives of 2,5-DCB and 2,4,6-TCB inhibited the biphenyl pathway. A few publications showed that hydroxylation of PCBs resulted in increasing toxicity toward bacteria. Sondossi et al. (2004) measured a significant decrease of the oxygen uptake rate in *C. testosteroni* B-356 exposed to hydroxylated PCBs as compared to hydroxybiphenyls and PCBs. Similarly, Cámara et al. (2004) reported decreasing cell viability in recombinant E. coli expressing subsets of bph genes following exposure to PCBs, which was attributed to the high toxicity of di-hydroxylated derivatives originating from the PCB metabolism. Exposure of *B. xenovorans* LB400 to the commercial mixture Aroclor 1242 was shown to result in the induction of detoxification genes under biphenyl pathway-inducing condition, again suggesting the generation of toxic metabolites (Parnell et al. 2006). We have recently showed that exposure to hydroxylated metabolites of 4-CB resulted in higher inhibition of *B. xenovorans* LB400 under condition inductive of the biphenyl pathway. Besides specific toxic mechanisms, hydroxylation is known to result in increasing solubility and bioavailability of the molecules susceptible to explain higher toxicity (Cámara et al. 2004).

Our results also showed that the extent of transformation of 2,5-DCB and its hydroxylated derivatives was consistent with the pattern of expression of genes of the

upper biphenyl pathway (bph). When cells were growing on biphenyl, a strong induction of bph genes was recorded in the presence of all target compounds, which resulted in significant transformation of 2,5-DCB and the three OH-2,5-DCBs. On the other hand, when cells were growing on succinate, only 2,5-DCB and 2'-OH-2,5-DCB slightly induced expression of bph genes, leading to transformation of these two compounds, while 3'-OH- and 4'-OH-2,5-DCB were left unchanged. These results strongly suggest that the metabolism of OH-2,5-DCBs occurred through the biphenyl pathway. Biphenyl, as the natural substrate of the biphenyl pathway, is known as a strong inducer of bph genes (Denef et al. 2004; Denef et al. 2005; Parnell et al. 2010). Previous publications have shown that lesser-chlorinated OH-PCBs were transformed through the biphenyl pathway. A variety of hydroxylated biphenyls (2-, 3-, and 4-OH-biphenyl) and PCBs (4-OH-2-CB, 4-OH-3-CB, and 4-OH-5-CB) was shown to be transformed by C. testosteroni B-356 and by a recombinant Pseudomonas putida strain harboring the biphenyl pathway system (Sondossi et al. 2004). As it was observed with most PCBs, the metabolism of these compounds involved dihydroxylation in positions 2,3 of the unsubstituted ring followed by meta-cleavage in position 1,2. Francova et al. (2004) reported the transformation of a series of *ortho*-substituted hydroxylated PCBs (2-OH-3-CB, 2-OH-5-CB, and 2-OH-3,5-DCB) by biphenyl dioxygenases of *B. xenovorans* LB400 and C. testosteroni B-356. However, these reports focused on congeners bearing hydroxyl and chlorine substituents located on the same ring, even though hydroxylation of PCBs has been reported on both chlorinated and non-chlorinated rings (Kaminski et al. 1981; Kucerova et al. 2000; Sietmann et al. 2006; Rezek et al. 2007; Rezek et al. 2008; Zhai et al. 2010). We have recently showed that *B. xenovorans* LB400 was capable of

transforming 4-CB and three of its derivatives hydroxylated on the non-chlorinated ring (2'-OH-, 3'-OH-, and 4'-OH-4-CB) (Tehrani et al., 2012). The specific contribution of the present study is to provide evidence of the metabolism of higher-chlorinated biphenyl derivatives that are hydroxylated on the non-chlorinated ring (i.e., 2'-OH-, 3'-OH-, and 4'-OH-2,5-DCB).

Our results showed that transformation of 2,5-DCB and OH-2,5-DCBs was associated with the accumulation of the PCB metabolite, 2,5-dichlorobenzoate (2,5-DCBA), which accounted for a large fraction of the parent compounds, suggesting that the initial attack by dioxygenases occurred primarily on the non-chlorinated ring. Our results showed that transformation of 2,5-DCB and OH-2,5-DCBs was associated with the accumulation of the PCB metabolite, 2,5-dichlorobenzoate (2,5-DCBA), which accounted for a large fraction of the parent compounds, suggesting that the initial attack by dioxygenases occurred primarily on the non-chlorinated ring.

Indeed, the biphenyl 2,3-dioxygenase (BphA) of *B. xenovorans* LB400, which catalyzes the initial hydroxylation of the biphenyl core, was shown to oxidize preferentially the non-chlorinated ring. Moreover, oxidation of 2,5-dichlorinated rings by BphA of *B. xenovorans* LB400 (3,4-dioxygenation) is known to produce dead-end metabolites, which were not detected in our experiments (Peeper and Seeger, 2008). Our results also suggest that 2,5-DCBA was not efficiently transformed by *B. xenovorans* LB400. Some chlorobenzoic acids are known to be dead-end products of the bacterial metabolism of PCBs and their toxicity has been suggested to be responsible for the limited degradation of PCBs observed in the environment (Billingsley et al. 1999; Flanagan and May 1993; Cámara et al. 2004; Martínez et al. 2007). In order to test this hypothesis, we measured

the toxicity and biodegradability of 2,5-DCBA by *B. xenovorans* LB400 and observed that the compound was not significantly transformed by the bacterium, even under biphenyl pathway-inducing condition, nor did it result in significant inhibition of cell growth at concentration up to 20 mg L^{-1}. As for PCBs, biodegradation of chlorobenzoic acids by bacteria depends largely on the degree and pattern of chlorine substitution of the aromatic ring. Billingsley et al. (1999) have reported that *B. xenovorans* LB400 growing on biphenyl could transform some chlorobenzoic acids (i.e., 2-CB, 2,3-DCBA), while others were not metabolized (i.e., 4-CBA, 2,4-DCBA). On the other hand, a more recent study reported that the same strain was not capable of metabolizing 2- and 4-CBA when using glucose as the carbon source (Martinez et al. 2007). In agreement with our results, Cámara et al. (2004) reported a mild toxicity of 2,3-DCBA for Escherichia coli cells.

Our results also showed that *B. xenovorans* LB400 was not capable of transforming 2,4,6-TCB or any of its hydroxylated derivatives over the time of the experiment, which could be explained by the inability of these compounds to induce the biphenyl pathway. Alternatively, the recalcitrance of 2,4,6-trichlorinated congeners to degradation by *B. xenovorans* may be related to the specificity of the biphenyl 2,3-dioxygenase (BphA) known to be highly dependent on the substitution pattern of the substrate (Peeper and Seeger, 2008). Our findings are also consistent with the general observation that higher-chlorinated PCBs are less susceptible to oxidative transformation, which is explained by the electron-withdrawing effect of chlorine atoms, reducing the electron density of the aromatic rings (Borja et al., 2005).

Our results may have important implications for the natural attenuation of PCBs because hydroxylated PCBs are the most common PCB metabolites generated by higher

organisms and they have been detected in the environment (Flanagan and May 1993; Jansson et al. 1975; Kawano et al. 2005; Ueno et al. 2007). In addition, the recalcitrance to biodegradation and toxicity of some hydroxylated PCBs may provide an explanation for the persistence of PCBs in the environment (Cámara et al. 2004).

Table 6.1 Half-maximum inhibitory concentrations (IC50) of *B. xenovorans* LB400 exposed to 2,5-dichlorobiphenyl (2,5-DCB), 2,4,6-trichlorobiophenyl (2,4,6-TCB), and their respective 2'-hydroxy- (2'-OH-), 3'-hydroxy- (3'-OH-), and 4'-hydroxy- (4'-OH-) derivatives

	Succinate[a]				**Biphenyl[b]**			
Compounds	**2,5-PCB[c]**	**2'-OH-**	**3'-OH-**	**4'-OH-**	**2,5-DCB[c]**	**2'-OH-**	**3'-OH-**	**4'-OH-**
IC50[e] (mg L^{-1})[g]	N.D.[f]	6.9	16.0	14.9	N.D.[f]	5.7	5.2	11.4
CI 95% (mg L^{-1})[h]	-	-	-	-	-	-	-	-
R^2		1.00	1.00	1.00	1.00	1.00	1.00	1.00
	2,4,6-TCB[d]	**2'-OH-**	**3'-OH-**	**4'-OH[c]**	**2,4,6-TCB[d]**	**2'-OH-**	**3'-OH-**	**4'-OH-**
IC50[e] (mg L^{-1})[g]	N.D.[f]	19.3±10.6	13.5±8.7	15.7±4.8	14.3±2.7	4.2±1.2	2.7±0.0	2.7±0.0
CI 95% (mg L^{-1})[h]	-	-	-	7.8-30.2	-	-	2.6-2.8	2.7-2.7
R^2	0.96	0.98	0.97	0.99	0.98	0.99	1.0	1.0

[a] *B. xenovorans* LB400 was growing using succinate (10 mM, 1,180 mg L^{-1}) as the carbon source.

[b] *B. xenovorans* LB400 was growing using biphenyl (5 mM, 770 mg L^{-1}) as the carbon source.

[c] 2,5-DCB and OH-2,5-DCBs were dosed at concentration ranging from 0 to 20 mg L^{-1}.

[d] 2,4,6-TCB and OH-2,4,6-TCBs were dosed at concentration ranging from 0 to 50 mg$L^{-1.}$

[e] Half-maximum inhibitory concentrations (IC50) were estimated using a first-order inactivation model with shoulder. R^2 is the coefficient of determination of the curve fitting.

[f] The IC50 of these compounds was not determined (N.D.) because they did not exhibited [a] significant toxicity.

[g] The standard deviation could not be calculated for several compounds because the first-order decay moiety of the curve did not include enough data points.

[h] The confidence intervals (CI 95%) of IC50 could not be calculated for several compounds because the first-order decay moiety of the curve did not include enough data points.

Table 6.2 Transformation of 2,5-dichlorobiphenyl (2,5-DCB), 2,4,6-trichlorobiophenyl (2,4,6-TCB) and their respective 2'-hydroxy- (2'-OH-), 3'-OH-, and 4'-OH- derivatives by *B. xenovorans* LB400 and formation of their potential metabolites, 2,5-dichlorobenzoic acid (2,5-DCBA) and 2,4,6-trichlorobenzoic acid (2,4,6-TCBA)

Compound	Succinate[a]			Biphenyl[b]		
	Initial[c] (%) (0 h)	Final[c] (%) (96 h)	CBA[d] (%) (96 h)	Initial[c] (%) (0 h)	Final[c] (%) (96 h)	CBA[d] (%) (96 h)
2,5-DCB	101.53±0.46	N.D.[e]	82.70±1.62	98.74±0.87	1.00±1.13	100.83±4.12
***P*-Value[f]**		*0.003*			*0.000*	
2'-OH-	99.71±1.35	53.40±0.64	47.07±0.18	98.94±0.42	N.D.[e]	99.01±3.4
***P*-Value[f]**		*0.000*			*0.000*	
3'-OH-	100.91±0.68	99.23±3.16	N.D.[e]	100.65±0.73	30.97±3.30	66.34±0.29
***P*-Value[f]**		0.377			*0.004*	
4'-OH-	100.13±0.19	80.41±5.34	N.D.[e]	100.20±1.09	39.33±0.92	60.33±2.41
***P*-Value[f]**		*0.022*			*0.007*	
2,4,6-TCB	98.09±3.00	90.01±5.80	N.D.[e]	100.62±0.86	104.16±4.17	N.D.[e]
***P*-Value[f]**		0.098			0.220	
2'-OH-	101.10±5.62	94.27±6.73	N.D.[e]	105.98±1.38	94.63±3.16	N.D.[e]
***P*-Value[f]**		0.269			0.379	
3'-OH-	105.65±3.26	87.69±2.68	N.D.[e]	103.84±1.40	94.74±5.39	N.D.[e]
***P*-Value[f]**		*0.020*			0.754	
4'-OH-	102.38±4.09	90.72±4.34	N.D.[e]	107.15±0.25	94.73±2.01	N.D.[e]
***P*-Value[f]**		0.075			0.498	

[a] *B. xenovorans* LB400 was growing using succinate (10 mM, 1,180 mg L^{-1}) as the carbon source.

[b] *B. xenovorans* LB400 was growing using biphenyl (5 mM, 770 mg L^{-1}) as the carbon source.

[c] 2,5-DCB, 2,4,6-TCB, and their hydroxylated derivatives are expressed as percentage of the initial concentration (5 mg L^{-1}). Results are presented as mean and standard deviation of three replicates.

[d] Chlorobenzoic acids (CBAs) are expressed as percentage of the maximum theoretical amount that could be produced given the initial concentration of PCBs and OH-PCBs. 2,5-DCBA and 2,4,6-TCBA are produced from the transformation of 2,5-DCB and OH-2,5-DCBs, and 2,4,6-TCB and OH-2,4,6-TCBs, respectively.

[e] Not detected (N.D.).

[f] The statistical significance of the transformations was assessed by comparing the initial and final concentrations using unpaired *t*-tests at 95% confidence level. Not detected (N.D.) concentrations were counted as zero.

Table 6.3 Transformation of 2,5-dichlorobenzoic acid (2,5-DCBA) by *B. xenovorans* LB400

Concentration	Succinate[a]		Biphenyl[b]	
	Initial (0 h)[c]	Final (96 h)[c]	Initial (0 h)[c]	Final (96 h)[c]
20 mg L^{-1}	99.88±4.84	102.75±2.17	113.10±0.99	103.50±0.70
10 mg L^{-1}	103.05±3.18	88.55±3.18	102.75±8.31	98.50±9.61
5 mg L^{-1}	98.09±4.38	91.49±4.95	105.80±6.50	101.99±2.83
2.5 mg L^{-1}	111.80±2.54	56.80±1.69	116.18±4.77	115.80±4.26

[a] *B. xenovorans* LB400 was growing using succinate (10 mM, 1,180 mg L^{-1}) as the carbon source.

[b] *B. xenovorans* LB400 was growing using biphenyl (5 mM, 770 mg L^{-1}) as the carbon source.

[c] 2,5-DCBA is expressed as percentage of the initial concentration. Results are presented as mean and standard deviation of three replicates.

Table 6.4 Level of expression of genes of the biphenyl pathway, bphA, bphB, bphC, and bphD, in *B. xenovorans* LB400 exposed to biphenyl (BP), 2,5-dichlorobiphenyl (2,5-DCB), and its hydroxylated derivatives, 2'-hydroxy- (2'-OH-), 3'-OH-, and 4'-OH-DCB.

	Biphenyl[a]	**Succinate**[b]				
Genes	**BP**[c] **(770 mg L^{-1})**	**BP**[c,d] **(5 mg L^{-1})**	**2,5-DCB**[c,d] **(5 mg L^{-1})**	**2'-OH-**[c,d] **(5 mg L^{-1})**	**3'-OH-**[c,d] **(5 mg L^{-1})**	**4'-OH-**[c,d] **(5 mg L^{-1})**
bphA	6.50±0.53	2.70±0.91	2.23±0.10	2.03±0.39	0.40±0.10	0.20±0.03
***P*-Value**[e]	*0.001*	*0.003*	*0.001*	*0.014*	*0.011*	*0.001*
bphB	6.81±1.97	5.11±3.41	2.53±0.53	1.98±0.40	0.39±0.13	0.25±0.08
***P*-Value**[e]	*0.005*	*0.027*	*0.009*	*0.015*	*0.018*	*0.018*
bphC	7.55±0.14	5.66±2.32	2.25±0.38	1.89±0.17	0.46±0.17	0.25±0.06
***P*-Value**[e]	*0.001*	*0.014*	*0.015*	*0.033*	*0.05*	*0.013*
bphD	8.79±1.18	4.79±1.87	1.86±0.04	1.78±0.27	0.52±0.10	0.20±0.11
***P*-Value**[e]	*0.001*	0.089	0.286	0.312	0.253	0.154

[a] *B. xenovorans* LB400 was growing using biphenyl (5 mM, 770 mg L^{-1}) as the carbon source.

[b] *B. xenovorans* LB400 was growing using succinate (10 mM, 1,180 mg L^{-1}) as the carbon source.

[c] Gene expression levels were recorded after 18 hours of exposure and were normalized by the expression level of 16S ribosomal DNA (rDNA) gene. Results are presented as mean and standard deviation of three replicates.

[d] Biphenyl (BP), 2,5-DCB, 2'-OH-, 3'-OH-, and 4'-OH-2,5-DCB were dosed at 5 mg L^{-1}.

[e] The statistical significance was assessed by comparing the genes expression levels in exposed and non-exposed cells using unpaired *t*-tests at 95% confidence level.

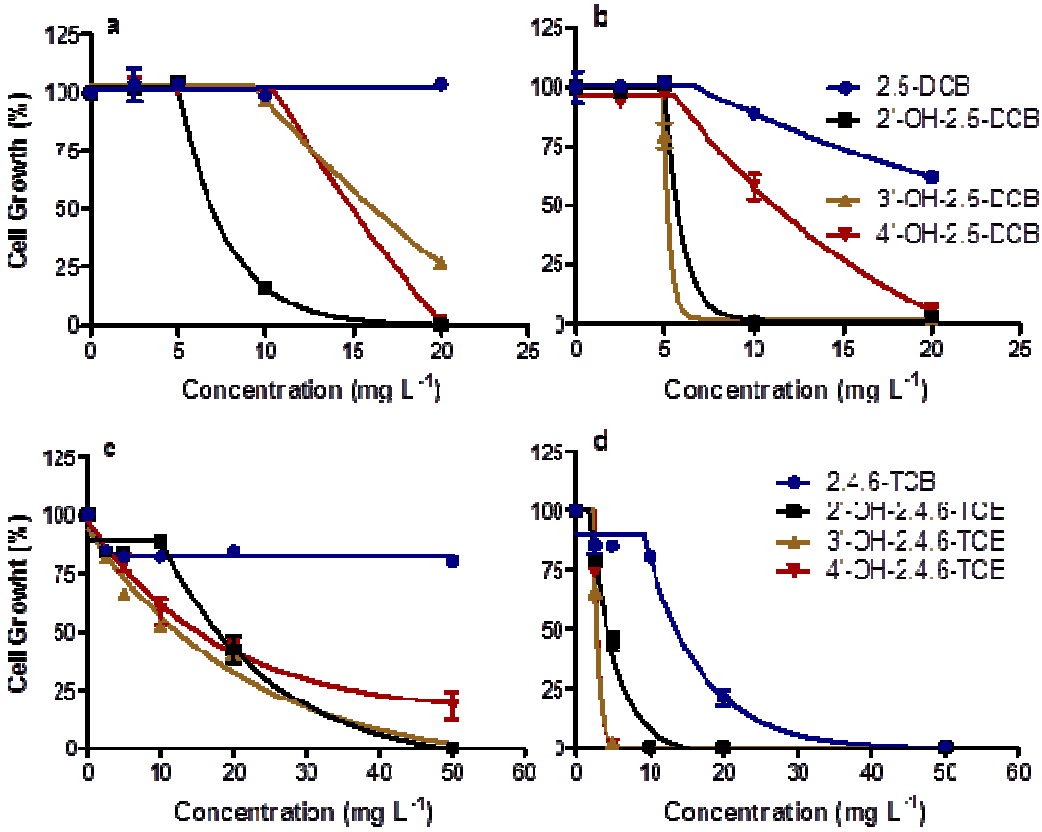

Figure 6.1 Dose-response curves showing the effect of 2,5-dichlorobiphenyl (2,5-DCB), 2,4,6-trichlorobiphenyl (2,4,6-TCB), and their hydroxylated derivatives on the growth of *B. xenovorans* LB400. The endpoint used for the measurement of growth inhibition was the cell concentration (expressed by the optical density at 600 nm - OD600) that was reached in the early stationary phase. Solid lines show fitting to a first-order inactivation model with shoulder. Panel a: Exposure to 2,5-DCB, 2'-hydroxy- (2'-OH-), 3'-OH-, and 4'-OH-2,5-DCB (0.0 to 20 mg L^{-1}) with succinate (1,180 mg L^{-1}) as the carbon source. Panel b: Exposure to 2,5-DCB, 2'-OH-, 3'-OH-, and 4'-OH-2,5-DCB (0.0 to 20 mg L^{-1}) with biphenyl (770 mg L^{-1}) as the carbon source. Panel c: Exposure to 2,4,6-TCB, 2'-OH-, 3'-OH-, and 4'-OH-2,4,6-TCB (0.0 to 50 mg L^{-1}) with succinate (1,180 mg L^{-1}) as the carbon source. Panel d: Exposure to 2,4,6-TCB, 2'-OH-, 3'-OH-, and 4'-OH-2,4,6-TCB (0.0 to 50 mg L^{-1}) with biphenyl (770 mg L^{-1}) as the carbon source. Errors bars show the standard deviation between three replicates.

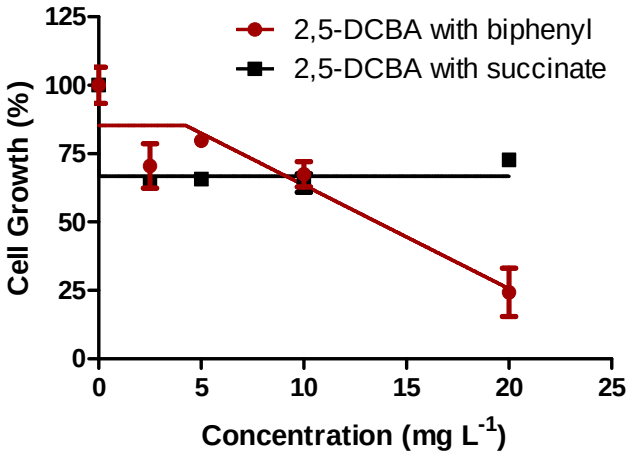

Figure 6.2 Dose-response curves showing the effect of 2,5-dichlorobenzoic acid (2,5-DCBA) (0.0 to 50 mg L^{-1}) on the growth of *B. xenovorans* LB400 growing on succinate (1,180 mg L^{-1}) and biphenyl (770 mg L^{-1}). Bacterial growth was expressed by the optical density at 600 nm (OD600) that was reached in the early stationary phase. Solid lines show fitting to a first-order inactivation model with shoulder. Errors bars show the standard deviation between three replicates.

CHAPTER 7

GENOME-WIDE TRANSCRIPTIONAL RESPONSE OF *BURKHOLDERIA XENOVORANS* LB400 EXPOSED TO MONO-HYDROXYLATED 2,5-DICHLOROBIPHENYL

7.1 Abstract

To have a deeper understanding of *B. xenovorans* capability to degrade OH-PCBs, genome-wide gene expression experiments were conducted using a custom-designed DNA expression microarray. Gene expression analyses were conducted to compare the transcriptional response of *B. xenovorans* LB400 cells exposed to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB to non-exposed cells (growing on succinate). After filtering, normalization, and data processing, 134 genes were showed to be differentially expressed upon exposure to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB as compared to non-exposed cells. A large proportion of differentially expressed genes were simultaneously up- or downregulated by biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB, suggesting that these three compounds induce similar transcriptional responses in *B. xenovorans* LB400, which is explained by their similarity of structures. Distribution of genes into ontology (or functional) categories showed that a larger number of genes differentially expressed are involved in metabolic processes, suggesting that biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB induced significant metabolic changes in exposed cells. Genes involved in oxido-reduction reactions were also well represented, which is consistent with active biodegradation of biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB. Other categories of

differentially expressed genes involved in response to stress, abiotic, and biotic stimuli and binding and metabolism of small molecules, which suggest that biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB exert toxic effects on the cells and that their degradation generates one-carbon molecules. Analysis of individual genes differentially expressed upon exposure to the target compounds showed overexpression of a few key genes involved in the lower biphenyl pathway.

7.2 Introduction

Although significant research has been conducted on degradation of PCBs by *B. xenovorans* LB400, very little is known about the molecular basis and biochemistry underlying on the PCB metabolism. Although in the previous chapters, I conducted gene expression analysis to support biodegradation rates of PCB and OH-PCBs, these analyses were entirely based on genes known to be involved directly in the breakdown of biphenyl and PCB molecules. These analyses were focused on preexisting knowledge of the metabolism of PCBs by *B. xonovorans* LB400. However, it is very likely that other genes and, possibly, entire metabolic pathways play an important role on biotransformation of PCBs in the model bacterium. Moreover, today very little is known about the regulation of the PCB metabolic pathway in *B. xenovorans* LB400. Because most cell activities are regulated at the transcriptional level, gene expression analysis using microarrays is a powerful means to study the metabolism of xenobiotic compounds. A microarray is a slide onto which a large number of oligonucleotide probes specific to a set of genes (or the entire genome) are spotted. Messenger RNA (mRNA) is extracted from bacterial tissues exposed to increasing concentrations of PCB or OH-PCBs and from non-exposed

cells. The mRNA is labeled with a fluorescent dye and hybridized with array probes. The slide is then scanned using laser emission. Computer-assisted analysis of the relative fluorescence intensities of the spots allows identification of the genes that are induced, repressed, or unaffected by exposure to the target compounds (a basic explanation of the microarray design and analysis has been provided in Chapter 3, Materials and Methods section).

The goal of this experiment was to design a custom microarray (there is currently no commercially-available microarrays for *B. xenovorans* LB400) that was printed by Agilent (e-Array platform) and to use it for the analysis of the genome-wide response of *B. xenovorans* LB400 to exposure to a model OH-PCB, 2'-OH-2,5-DCB. This congener was chosen because it was the hydroxylated derivative of 2,5-DCB that was the most significantly transformed by the bacterium (see Chapter 6). Together with 2'-OH-2,5-DCB, cells were also exposed to the parent PCB, 2,5-DCB, and the natural substrate of the biphenyl pathway, biphenyl. Gene expression levels in cells exposed to 2'-OH-2,5-DCB, 2,5-DCB, and biphenyl were then compared with non-exposed control cells.

7.3 Results and Discussion

7.3.1 Overall Gene Expression Levels

The transcriptional response of *B. xenovorans* cells exposed to 5 mg L^{-1} biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB was then investigated using genome-wide gene expression DNA microarrays. Analyses were performed by comparing individually the gene expression levels of each treatment, exposure to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB

against the gene expression levels of to non-exposed cells (growing on succinate). After filtering out the genes with low or low-quality signals, normalization by the average signal across arrays, and conducting class comparison using univariate t-tests ($p < 0.005$), 134 genes were showed to be differentially expressed upon exposure to 2,5-DCB, and 2'-OH-2,5-DCB as compared to non-exposed cells. Among them, 63, 72, and 75 were significantly upregulated (> 2.0) by exposure to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB, respectively. 15, 11, and 11 genes were significantly downregulated (< 0.5) by exposure to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB, respectively. A significant proportion of differentially expressed genes were simultaneously overexpressed or downregulated by biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB, respectively. In other words, high and significant correlations were observed between expression levels in cells exposed to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB (Pearson's correlation coefficients for 2'-OH-2,5-DCB versus biphenyl was 0.91, for 2'-OH-2,5-DCB versus 2,5-DCB was 0.99, for 2,5-DCB versus biphenyl was 0.92). While no significant correlation was observed between expression levels in cells exposed to any of the target compound and in non-exposed control cells (Pearson's correlation coefficients, for 2'-OH-2,5-DCB versus succinate was 0.32, for 2,5-DCB versus succinate was 0.31) (

Figure 7.2). All the 50 genes the most upgulated upon exposure to 2-OH-2,5-DCB are also the 50 genes the most upregulated upon exposure to 2,5-DCB. All the 50 genes the most upregulated upon exposure to 2-OH-2,5-DCB and 2,5-DCB, except 4 genes, are also among the 50 genes the most upregulated upon exposure to biphenyl. All the 20 genes the most downregulated upon exposure to 2-OH-2,5-DCB, except 3 genes, are also the 20 genes the most downregulated upon exposure to 2,5-DCB. Nine genes among the

20 genes that are the most downregulated upon exposure to 2-OH-2,5-DCB and 2,5-DCB are also among the 20 genes the most downregulated upon exposure to biphenyl. These observations suggest that the three target compounds used, biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB, induce similar transcriptional responses in *B. xenovorans* LB400. The difference in expression of genes of the biphenyl pathway (*bph* genes) that we observed between the compounds (Chapter 6), and that eventually explained differences in biodegradation rates, were likely not expressed at level high enough to be captured by microarray analysis. Indeed, the RT-qPCR method used for the detection of expression levels of *bph* genes is more sensitive and quantitative than microarray analysis, which should be referred to as a semi-quantitative method.

In order to get an overall picture of the effect of exposure of *B. xenovorans* cells to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB at the whole genome level, the distribution of genes differentially expressed at significant level were classified in gene ontology classes (or functional categories) using the on-line application BLAST2GO (http://www.blast2go.com/b2ghome). Results are presented in Figures 7.2, 7.3, and 7.4 for all the genes differentially expressed upon exposure to 2-OH-2,5-DCB. Only exposure to this compound was considered here since a large degree of similarity was observed across the three target compounds under study. Figure 7.2a showed that a larger number of genes differentially expressed fall in the category of metabolic processes (58 %), suggesting that cells exposed to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB were active and in exponential growth phase. Another important category includes genes involved in oxido-reduction reactions (

Figure 7.2a; 11 %), which is consistent with active biodegradation of biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB. Other categories well represented include genes involved in response to stress, abiotic, and biotic stimuli (

Figure 7.2a, 4 %), which suggest that biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB may exert toxic effects toward the cells. One category that also contains significant number of differentially expressed genes related to binding and metabolism of small molecules (Figure 7.2b, 58 % and 15 %. respectively), which suggest that degradation of biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB generates one-carbon molecules. Figure 7.3 (a and b) and Figure 7.4 (a, and b) showed similar diagrams comparing the genes that are the most upregulated (> 2 fold) and downregulated (< 0.8 fold) upon exposure to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB. Comparison between biological process categories of genes up- and down regulated is consistent with an increase of the induction of genes involved in the energy metabolism upon exposure to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB (Figure 7.3a and Figure 7.4a). More genes involved in the categories of cellular metabolism, primary metabolism, and macromolecule metabolism, were upregulated than downregulated (46 and 35%, respectively). Similarly, more genes involved in the categories of small molecules metabolism, were upregulated than downregulated (11 and 6%, respectively). On the contrary, more genes involved in biosynthetic processes were downregulated than upregulated (16 and 11%, respectively).

Comparison between molecular function categories of genes up- and down regulated shows more remarkable differences (Figure 7.3b and Figure 7.4b). More genes involved in the category of small molecule binding were upregulated than downregulated (25 and 14%, respectively). Also, more genes involved in the ion binding function were

upregulated than downregulated (17 and 9%, respectively). On the contrary, more genes involved in transferase activities were downregulated than upregulated (27 and 17%, respectively).

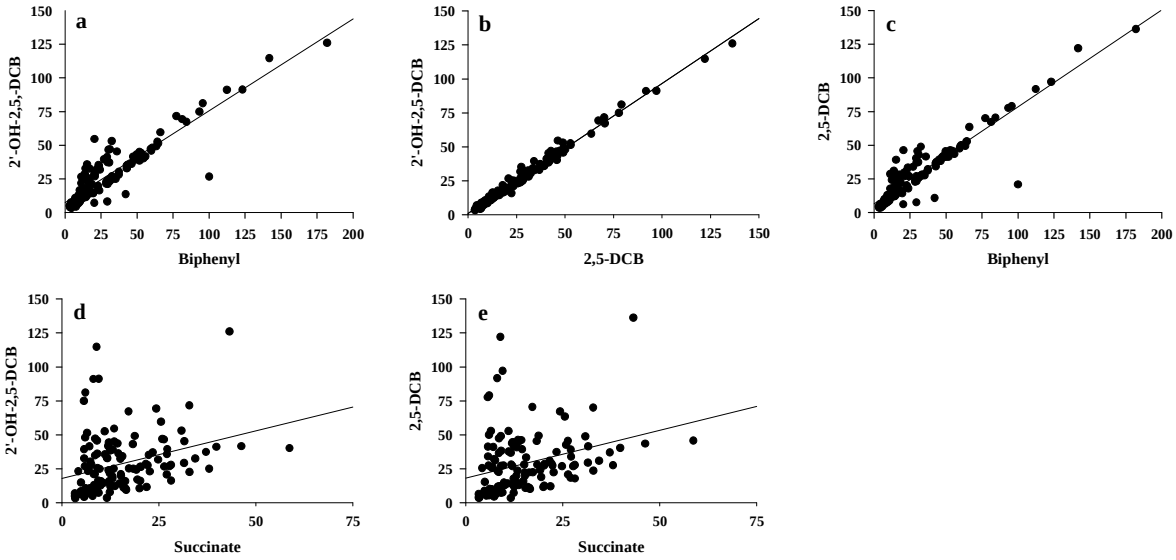

Figure 7.1. Correlation between the signal intensity of genes differentially expressed: a) 2'-OH-2,5-DCB vs. biphenyl, b) 2'-OH-2,5-DCB vs. 2,5-DCB, c) 2,5-DCB vs. biphenyl, d) 2'-OH-2,5-DCB vs. succinate, and e) 2'-OH-2,5-DCB vs. succinate *B. xenovorans* LB400 cells were growing on succinate as primary carbon source and were individually exposed to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB (5 mg L^{-1}). Control cells were not exposed to target compounds.

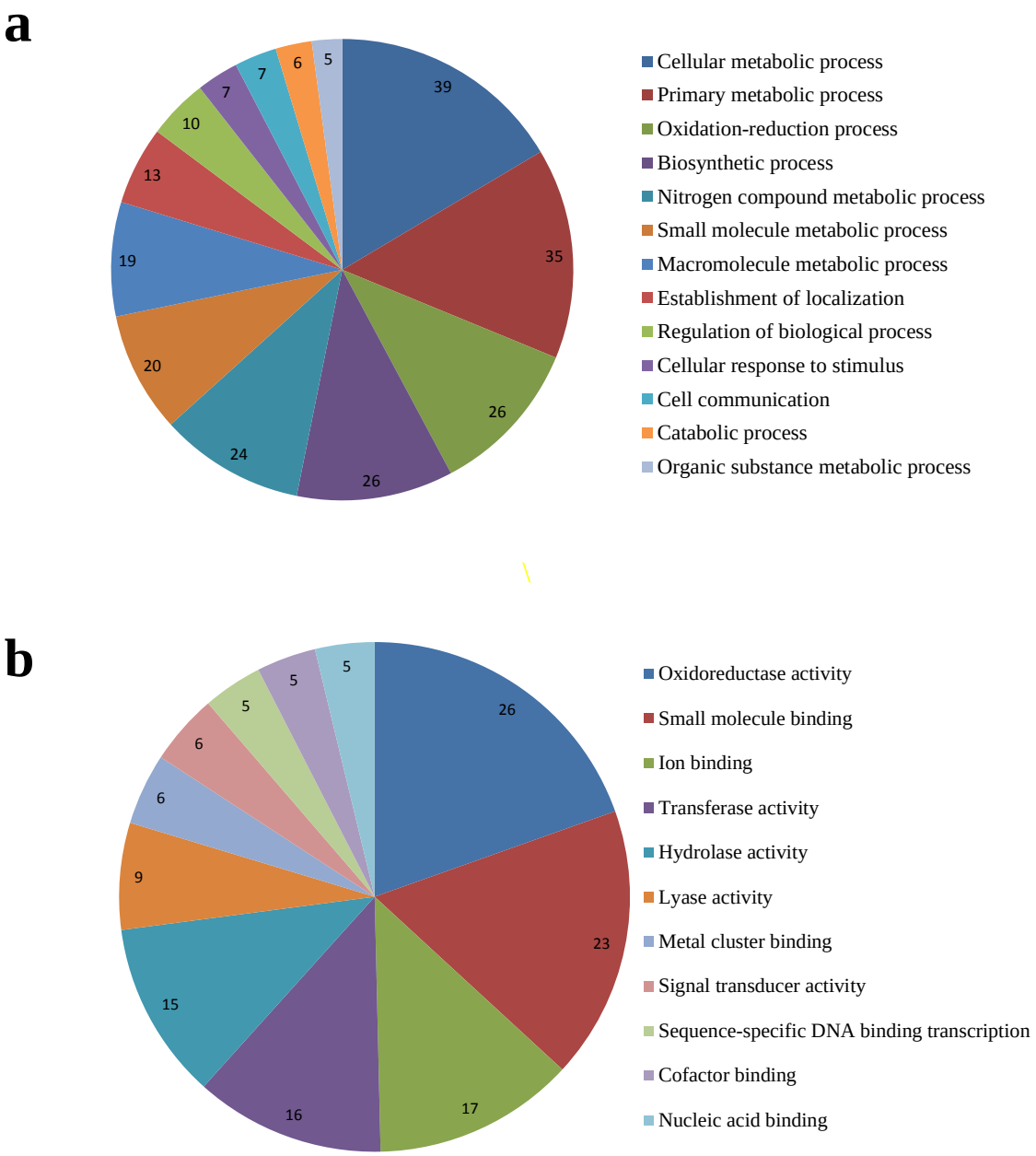

Figure 7.2. Distribution of all 134 differentially expressed genes in *B. xenovorans* LB400 cells exposure to 2'-OH-2,5-DCB (5 mg L^{-1}) into different gene ontology (functional) categories. **Panel a**: cellular processes, **panel b**: molecular function. Gene classification was performed using BLAST2GO on-line application. The numbers displayed represent the number of genes falling in each category. Categories including less than 5 genes were not included in the graph.

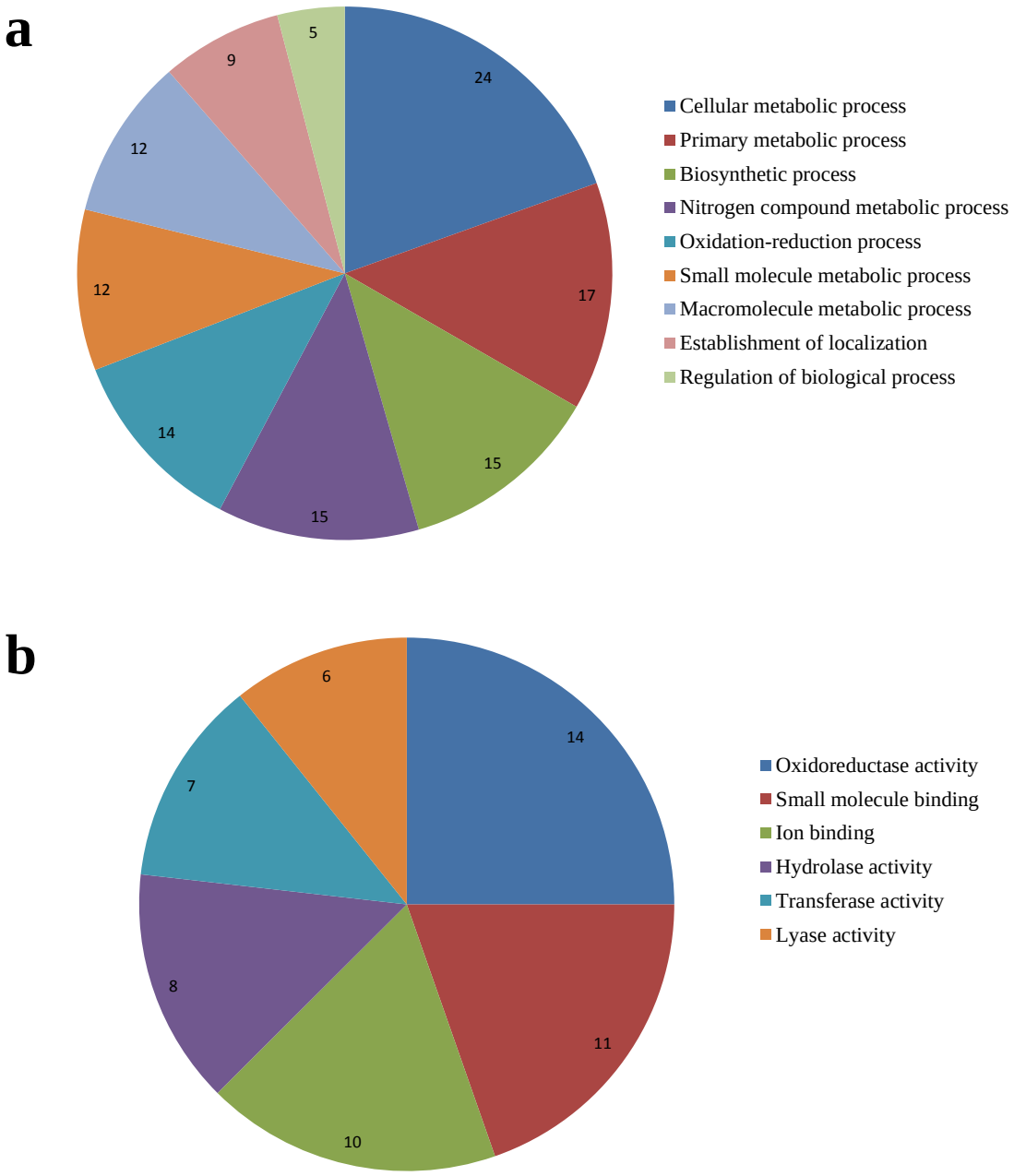

Figure 7.3. Distribution of 28 genes significantly overexpressed (> 2-fold) in *B. xenovorans* LB400 cells exposure to 2'-OH-2,5-DCB (5 mg L^{-1}) into different gene ontology (functional) categories. **Panel a:** cellular processes, **panel b:** molecular function. Gene classification was performed using BLAST2GO on-line application. The numbers displayed represent the number of genes falling in each category. Categories including less than 5 genes were not included in the graph.

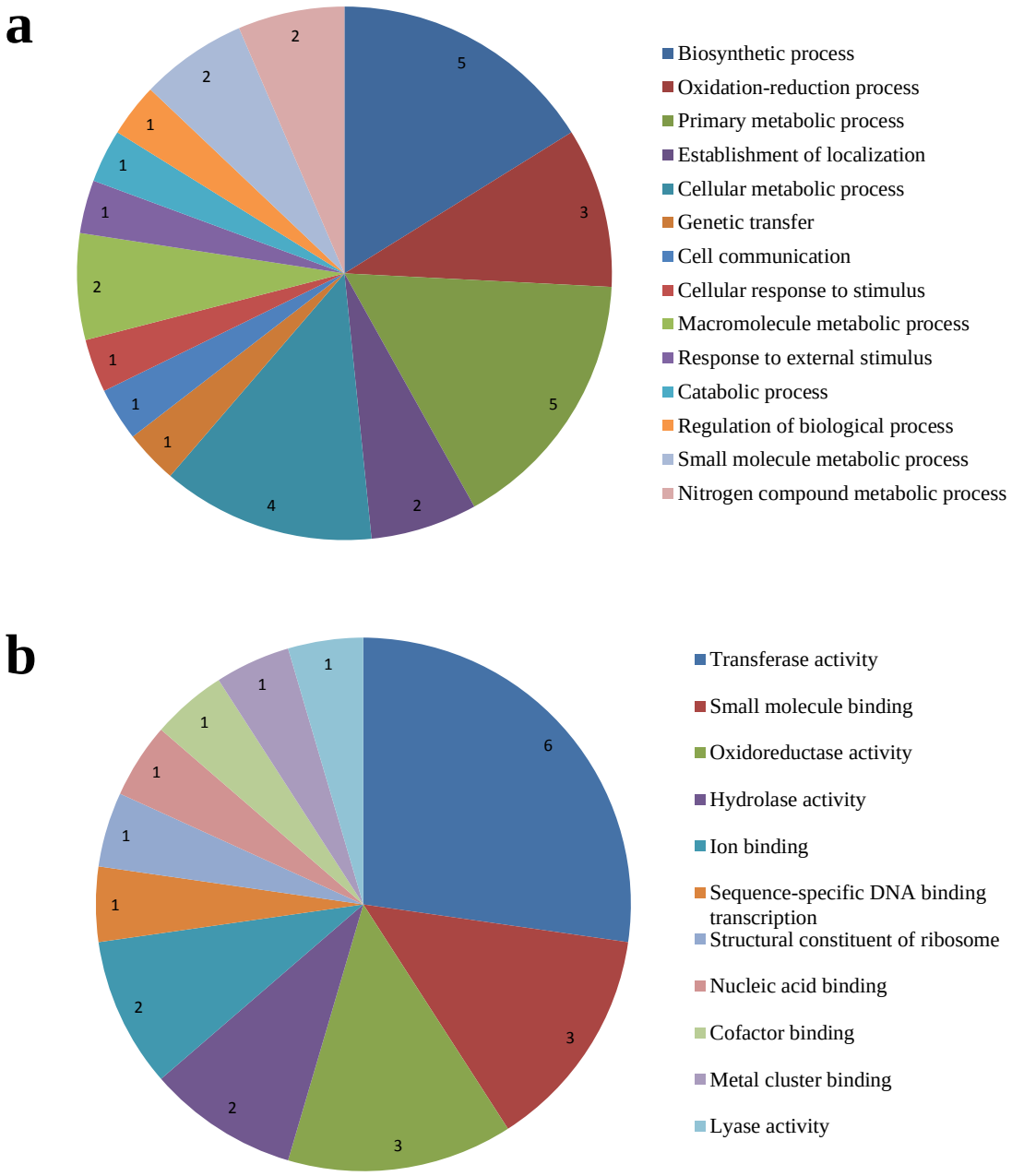

Figure 7.4. Distribution of 18 genes significantly downregulated (< 0.8 fold) in *B. xenovorans* LB400 cells exposure to 2'-OH-2,5-DCB (5 mg L^{-1}) into different gene ontology (functional) categories. **Panel a:** cellular processes, **panel b:** molecular function. Gene classification was performed using BLAST2GO on-line application. The numbers displayed represent the number of genes falling in each category.

7.3.2 Individual Gene Expression Levels

Table 7.1 and 7.2 presented the list of genes the most upregulated (> 2 fold) and the most downregulated (< 0.8 fold) upon exposure biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB. Analysis of individual genes showing significant differences in gene expression levels upon exposure to target compounds showed overexpression of a few key genes involved in the lower biphenyl pathway (Table 7.3). Interestingly, some of these genes, i.e., benzoyl-CoA dihydrodiol lyase (boxC), benzoyl-CoA-dihydrodiol lyase (boxC), benzoyl-CoA oxygenase component A (boxA), and benzoyl-CoA oxygenase component B (boxB) were induced by exposure to biphenyl, but not by 2,5-DCB and 2'-OH-2,5-DCB. The BoxA/BoxB enzymatic complexes are known to catalyze the aerobic reduction/oxygenation of the aromatic ring of benzoyl-CoA to form 2,3-dihydro-2,3-dihydroxybenzoyl-CoA. BoxA enzyme acts as a reductase that uses NADPH to reduce the oxygenase component BoxB. Both genes are known to be induced by benzoate (Zaar et al. 2004). The benzoyl-CoA oxygenase component A gene, Bxe_A1424, was expressed (relative expression level =7.10) when *B.xenovorans* cells were exposed to biphenyl, but not to 2'-OH-2,5-DCB and 2,5-DCB (gene expression levels = 0.88 and 0.65). The same trend was observed for the benzoyl-CoA oxygenase component B gene, Bxe_C0891, which was overexpressed 14.39 fold in cells exposed to biphenyl and not significantly overexpressed or downregulated in cells exposed to 2'-OH-2,5-DCB and 2,5-DCB (gene expression level = 1.02 and 0.62). The benzoyl-CoA-dihydrodiol lyase (BoxC) genes, Bxe_C0892 and Bxe_A1422, which encode enzymes involved in cleavage of the ring of 2,3-dihydro-2,3-dihydroxybenzoyl-CoA and transformation in to 6-hydroxy-3-hexenoyl-CoA were up-regulated (7.48 and 10.54 fold) in *B. xenovorans* cells

exposed to biphenyl. On contrary, these genes were downregualted in cells exposed to 2'-OH-2,5-DCB and 2,5-DCB (gene expression = 0.79 and 0.50 for Bxe_C0892, and 0.83 and 0.69 for Bxe_A1422, respectively). As stated above, the absence of detection of differential expression of genes of the upper pathway (*bph* genes) is consistent with the relatively low expression levels observed with RT-qPCR. Interestingly, genes of the chlorobenzoic acid pathway represent 4 out of the only 5 genes that are regulated by exposure to biphenyl and downregulated (or not differentially expressed) by exposure to 2-OH-2,5-DCB and 2,5-DCB.

In summary, whole-genome transcriptional analysis of *B. xenovorans* LB400 cells exposed to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB, showed that a large proportion of differentially expressed genes were simultaneously up- or downregulated by exposure to biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB, suggesting that these three compounds induce similar transcriptional responses. Gene classification by functional categories showed that genes differentially expressed are largely involved in metabolic processes, suggesting that biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB induced significant metabolic changes in exposed cells. In addition, significant differences in gene expression levels were observed for a few key genes involved in the lower biphenyl pathway. Further analyses will involve confirmation of microarray results using RT-qPCR. Indeed, although microarray is attractive as a high-throughput method, potentially allowing the identification of new genes and enzymes involved in metabolic and stress response of microorganisms, microarray results have poor quantitative values. Expression of genes identified using microarray analysis will have to be further confirmed using RT-qPCR.

Table 7.1. Genes upregulated more than 10-fold upon exposure to 5 mg L^{-1} 2'-OH-2,5-DCB, 2,5-DCB, and biphenyl. The genes have been sorted based on the level of expression in cells exposed to 2'-OH-2,5-DCB.

Annotation	Relative expression for different treatments[a]			Gene ID	FRD[e]	*p*-Values
	2'-OH-[b]	2,5-DCB[c]	BP[d]			
Formaldehyde-activating enzyme	182.20	172.72	252.89	Bxe_B2436	2.81E-04	6.00E-07
ABC transporter, periplasmic ligand binding	176.10	189.52	273.09	Bxe_B2425	6.54E-04	1.11E-05
Conserved hypothetical protein	166.14	188.18	254.07	Bxe_B2437	3.83E-04	3.50E-06
Methanol dehydrogenase-like protein (xoxF)	127.15	128.86	193.45	Bxe_B2427	2.81E-04	4.00E-07
Putative methanol dehydrogenase-like protein,	92.82	105.00	168.91	Bxe_B2426	1.21E-03	2.76E-05
Transcriptional regulator, LysR family	64.87	70.21	102.69	Bxe_B2423	4.52E-04	4.50E-06
Methylene-tetrahydromethanopterin	63.41	66.86	99.88	Bxe_B2431	3.83E-04	2.80E-06
Formyltransferase/hydrolase complex subunit	48.11	53.08	77.61	Bxe_B2464	2.81E-04	1.10E-06
Conserved hypothetical protein	34.44	33.69	44.61	Bxe_B2422	3.83E-04	3.40E-06
Formyltransferase/hydrolase complex subunit	32.66	35.64	56.12	Bxe_B2462	4.60E-04	6.40E-06
tetrahydromethanopterin biosynthesis protein,	31.02	37.43	53.59	Bxe_B2434	2.30E-03	6.94E-05
Beta-ribofuranosylaminobenzene 5'- phosphate	30.53	30.76	48.25	Bxe_B2430	1.04E-03	2.24E-05
Dihydroneopterin aldolase	25.88	29.65	44.07	Bxe_B2438	6.25E-04	1.02E-05
Formyltransferase/hydrolase complex subunit	22.82	23.02	33.92	Bxe_B2463	2.82E-03	9.02E-05
putative triphosphoribosyl- dephospho-CoA	21.08	22.02	31.00	Bxe_B2435	3.14E-03	0.0001066

Annotation	Relative expression for different treatments[a]			Gene ID	FRD[e]	*p*-Values
	2'-OH-[b]	2,5-DCB[c]	BP[d]			
Putative MxaS-like protein	17.31	19.47	26.27	Bxe_B2420	4.74E-03	0.0001951
Putative TonB-dependent siderophore receptor	16.83	18.53	25.41	Bxe_B2473	1.51E-03	3.54E-05
Hypothetical protein	16.54	11.90	2.31	Bxe_A3388	5.32E-03	0.00025
tetrahydromethanopterin biosynthesis protein,	15.87	17.77	25.93	Bxe_B2458	4.52E-04	5.60E-06
hypothetical protein	15.39	16.89	24.17	Bxe_B2424	5.95E-03	0.0002936
Conserved hypothetical protein	14.02	17.21	24.57	Bxe_B2432	1.93E-03	5.16E-05
Putative MxaR-like protein	13.51	13.67	19.08	Bxe_B2421	5.03E-03	0.0002232
Hypothetical protein	12.84	14.49	19.78	Bxe_B2418a	3.85E-03	0.0001458
Pyrroloquinoline quinone biosynthesis protein B	12.36	13.53	18.02	Bxe_B2467	6.97E-03	0.0004015
Conserved hypothetical protein (orf1)	12.24	14.96	21.81	Bxe_B2459	3.83E-04	3.10E-06
Putative proline iminopeptidase	11.17	10.92	14.66	Bxe_B2466	6.35E-03	0.0003463
Putative cytochrome b561	10.42	11.70	15.54	Bxe_B2465	7.50E-04	1.37E-05
Pyrroloquinoline quinone synthesis E	10.16	11.43	15.06	Bxe_B2470	7.71E-04	1.51E-05
Putative pterin-4-alpha-carbinolamine	-	11.09	17.25	Bxe_B2429	6.76E-03	0.0003798
Pyrroloquinoline quinone synthesis C	-	10.40	13.65	Bxe_B2468	2.12E-03	5.96E-05
Putative MxaK-like protein	-	-	14.28	Bxe_B2417	4.57E-03	0.0001849
Pyrroloquinoline quinone synthesis D	-	-	13.41	Bxe_B2469	2.02E-03	5.55E-05

Annotation	Relative expression for different treatments[a]			Gene ID	FRD[e]	*p*-Values
	2'-OH-[b]	2,5-DCB[c]	BP[d]			
Conserved hypothetical protein	-	-	17.76	Bxe_B2455	2.30E-03	6.85E-05
tetrahydromethanopterin biosynthesis protein,	-	10.14	13.82	Bxe_B2457	5.95E-03	3.00E-04
TonB like protein	-	-	11.21	Bxe_B2475	6.01E-03	0.0003122
Conserved hypothetical protein	-	-	12.88	Bxe_B2456	2.78E-03	8.71E-05
Conserved hypothetical protein	-	-	11.42	Bxe_B2474	2.30E-03	7.05E-05
HAD-superfamily hydrolase, subfamily IA,	-	-	13.09	Bxe_B2478	1.14E-02	0.0007981
Biopolymer transport protein ExbD/TolR	-	-	12.91	Bxe_B2477	4.60E-04	6.60E-06
Putative bipolymer transport protein,	-	-	11.39	Bxe_B2476	1.81E-03	4.62E-05

[a] Relative expression levels of genes of *B. xenovorans* cells growing on succinate and exposed to 2'-OH-2,5-DCB**,** 2,5-DCB, and biphenyl (BP)

[b] Cells were grown on succinate as primary carbon source and exposed to 5 mg L^{-1} of 2'-OH-2,5-DCB

[c] Cells were grown on succinate as primary carbon source and exposed to 5 mg L^{-1} of 2,5-DCB

[d] Cells were grown on succinate as primary carbon source and exposed to 5 mg L^{-1} of biphenyl (BP)

[e] FDR: False detection rate

Table 7.2. Genes downregulated lower than 0.8-fold upon exposure to 5 mg L^{-1} 2'-OH-2,5-DCB, 2,5-DCB, and biphenyl. The genes have been sorted based on the level of expression in cells exposed to 2'-OH-2,5-DCB.

Annotation	Relative expression for different treatment [a]			Gene ID	FRD	P Values
	2'-OH-[b]	2,5-DCB[c]	Bph[d]			
Putative acetoin dehydrogenase	0.10	0.10	0.10	Bxe_B0315	4.16E-02	4.67E-03
Major facilitator superfamily (MFS) tartrate/H+	0.28	0.31	0.41	Bxe_B1526	1.50E-02	1.13E-03
Short-chain dehydrogenase/reductase SDR	0.29	0.34	0.39	Bxe_B1525	9.45E-03	6.35E-04
Putative dehydrogenase	0.33	0.38	0.42	Bxe_B1523	2.84E-03	9.47E-05
Putative glycosyltransferase	0.34	0.41	0.72	Bxe_A0113	2.17E-02	1.85E-03
Major facilitator superfamily (MFS) metabolite	0.40	0.35	0.36	Bxe_B1522	2.01E-02	1.63E-03
Dihydroxy-acid dehydratase	0.41	0.45	0.45	Bxe_B1524	4.99E-03	2.16E-04
Hypothetical protein	0.42	0.76	0.71	Bxe_A3853	1.60E-03	3.87E-05
Putative membrane protein	0.44	0.53	0.78	Bxe_A1560	3.62E-02	3.90E-03
Hypothetical protein	0.47	0.61	0.86	Bxe_A2828	2.88E-02	2.86E-03
Sugar transferase, glycosyltransferase, group 1	0.47	0.52	0.76	Bxe_A2254	3.09E-02	3.19E-03
Putative short-chain dehydrogenase/reductase	0.50	0.55	0.63	Bxe_C0900	1.32E-02	9.42E-04
Conserved hypothetical protein	0.58	1.22	1.74	Bxe_B1159	2.17E-02	1.85E-03
ComEC/Rec2-related protein	0.59	0.46	0.38	Bxe_A1569	3.26E-02	3.38E-03
Transcriptional regulator, LysR family	0.63	0.65	0.66	Bxe_B0362	6.76E-03	3.76E-04

Annotation	Relative expression for different treatment [a]			Gene ID	FRD	P Values
	2'-OH-[b]	2,5-DCB[c]	Bph[d]			
Unknown protein (box pathway)	0.64	0.40	0.28	Bxe_C0894	3.33E-02	3.52E-03
Putative acetyltransferase	0.78	0.96	0.49	Bxe_A0094	4.31E-02	4.92E-03
Benzoyl-CoA-dihydrodiol lyase (boxC)	0.79	0.50	7.48	Bxe_C0892	1.86E-02	1.43E-03

[a] Relative expression levels of genes of *B. xenovorans* cells growing on succinate and exposed to 2'-OH-2,5-DCB**,** 2,5-DCB, and biphenyl (BP)

[b] Cells were grown on succinate as primary carbon source and exposed to 5 mg L^{-1} of 2'-OH-2,5-DCB

[c] Cells were grown on succinate as primary carbon source and exposed to 5 mg L^{-1} of 2,5-DCB

[d] Cells were grown on succinate as primary carbon source and exposed to 5 mg L^{-1} of biphenyl (BP)

[e] FDR: False detection rate

Table 7.3. Relative expression of the 4 genes not regulated by exposure to 2'-OH-2,5-DCB and 2,5-DCB. These genes were all well upregulated by exposure to biphenyl.

Annotation	Gene ID	2'-OH-	2,5-DCB	Biphenyl	p-Value
Benzoyl-CoA-dihydrodiol lyase (boxC)	Bxe_C0892	0.79	0.50	7.48	1.43E-03
Benzoyl-CoA-dihydrodiol lyase (boxC)	Bxe_A1422	0.83	0.69	10.54	3.48E-04
Benzoyl-CoA oxygenase component A (boxA)	Bxe_A1424	0.88	0.65	7.10	1.01E-03
Benzoyl-CoA oxygenase component B (boxB)	Bxe_C0891	1.02	0.62	14.39	2.08E-04

CHAPTER 8

CONCLUSION

8.1 Summary of Major Results

8.1.1 Specific Aim #1: Inhibitory Effects of OH-PCBs on *B. xenovorans* LB400

Prior to studying the capability of *B. xenovorans* LB400 to metabolize hydroxylated derivatives of lesser-chlorinated PCBs, the potential toxicity of these congeners toward the bacterium was evaluated with the cells growing on both succinate and biphenyl. Generally speaking, my results showed that OH-PCBs were more toxic than the corresponding parent PCBs and that toxicity (of PCBs and OH-PCBs) was higher when biphenyl was used as the primary carbon source (i.e., condition inductive of the biphenyl pathway) as compared with succinate (i.e., condition non-inductive of the biphenyl pathway). When *B. xenovorans* cells were growing on succinate, exposure to the tested PCBs did not result in significant growth inhibition even at high concentrations. On the contrary, exposure to PCBs while cells were growing on biphenyl resulted in significant growth inhibition at the high concentrations tested. On the other hand, exposure to hydroxylated derivatives of PCBs resulted in growth inhibitions at lower concentrations when compared to PCBs in the presence of both carbon sources. These results are in agreement with prior publications which have showed hydroxylation of PCBs by bacteria resulted in a higher toxicity (Camara et al. 2004; Sondossi et al. 2004; Parnell et al. 2006). Hydroxylation of organic compounds is known to result in an increase of solubility and bioavailability of the molecules susceptible to explain a higher toxic effect

on cells (Camara et al. 2004; Kitamura et al. 2008). However, as it was observed in mammals, it is likely that OH-PCBs would exert more specific toxicity on bacteria related to the change in the molecule stereochemistry introduced by the presence of the hydroxyl group. Structure-activity relationship of OH-PCB toxicity in bacteria has never been investigated. This toxicity may have important impacts on the biodegradation of PCBs and OH-PCBs in the environment.

8.1.2 Specific Aim #2: Degradation of OH-PCBs by *B. xenovorans* LB400

Bacterial aerobic degradation of PCBs is a major sink of lesser-chlorinated PCBs in the environment. (It is noteworthy that lesser-chlorinated PCBs often originate from anaerobic dechlorination of high-chlorinated congeners.) OH-PCBs are major PCB metabolites and abiotic transformation products of PCBs and they are notoriously more toxic and mobile than the parent PCBs. It is therefore relevant for bioremediation and natural attenuation of PCBs to investigate the potential bacterial transformation of OH-PCBs. Because aerobic degradation of PCBs occurs primarily with lesser-chlorinated PCBs, we selected hydroxylated derivatives of mono-, di-, and tri-chlorinated PCBs. In addition, because both metabolism and abiotic reactions generate primarily congeners carrying the hydroxyl group on the non (or lesser) chlorinated ring, we selected for each parent PCB the three hydroxylated isomers (*ortho*, *meta*, and *para*) with the phenolic group on the non-chlorinated ring. My results showed that 4-CB and 2,5-DCB were partially transformed under either condition inductive and non-inductive of the biphenyl pathway with the generation of the corresponding CBAs. When cells were growing on biphenyl and exposed to 4-CB, 4-CBA was not detected, suggesting further degradation of 4-CBA. 2,4,6-TCB was not significantly transformed under either conditions, neither

did its hydroxylated derivatives. When cells were growing on succinate as primary carbon source only 2'-OH-4-CB and 2'-OH-2,5-DCB were transformed and their corresponding CBAs were detected. For cells growing on biphenyl and exposed to subinhibitory concentrations of hydroxylated isomers of 4-CB and 2,5-DCB, all hydroxylated PCBs were transformed.

8.1.3 Specific Aim #3: Molecular Basis of the Transformation of OH-PCBs and Gene Expression Results

To have a better understating of molecular basis of degradation of OH-PCBs tested. RT-qPCR and micro array analyses were conducted. Since, CBAs were detected in cell extract as metabolites of PCBs and OH-PCBs degradation. The expression of four key genes involved in biphenyl pathway was measured. When cells were growing on succinate and exposed to 4-CB, 2'-OH-4-CB, 2,5-DCB, and 2'-OH-2,5-DCB the expression of four genes involved in upper biphenyl pathway was observed. This observation was in agreement with the degradation results. Cells growing on succinate as primary carbon source and exposed to 3'-OH-, 4'-OH-4-CB, 3'-OH-, and 4'-OH-2,5-DCB were unable to transform these target compounds and they did not show any expression of genes involved in upper biphenyl pathway. Gene classification by functional categories showed that genes differentially expressed are largely involved in metabolic processes, suggesting that biphenyl, 2,5-DCB, and 2'-OH-2,5-DCB induced significant metabolic changes in exposed cells. Also, significant differences in gene expression levels were observed for a few key genes involved in the lower biphenyl pathway.

8.2 Relevance of the Results

8.2.1 Implication of the Environmental Fate of OH-PCBs for Public Health

Metabolism of xenobiotic compounds commonly results in an increase of solubility and bioavailability when compared to the parent compounds. In addition, metabolites of xenobiotic pollutants have the potential to interfere with many biological systems and they typically exhibit specific activities that are not shown by the parent compounds (Letcher et al. 2000; Kitamura et al. 2008). This observation has been reflected in the regulations issued by the European Medicine Agency that requires monitoring of pharmaceuticals and their metabolites entering the environment. Under this guideline, any accumulation of metabolites that is predicted to be greater than 10% of the accumulation of the parent compound has to be monitored (Celiz et al. 2009). Because of the higher solubility and lower volatility resulting from the hydroxyl group, OH-PCBs also exhibit environmental behaviors that are expected to be different from PCBs. Although production and use of PCBs have been banned sine 1970s, similar guidelines should therefore be set regarding the release and potential accumulation of PCBs metabolites, including OH-PCBs, in view of the remediation strategies. For instance, it has been reported that addition of a hydroxyl group on an aryl core in a *para* position of a bulky hydrophobic group (such as the biphenyl core of PCBs) can transform non-estrogenic compounds (called proestrogens) to estrogenic ones (Kitamura et al. 2008). The U.S. Safe Drinking Water and Food Control Protection Acts mandate screening of estrogenic substances in drinking water. The act does not currently include OH-PCBs, as OH-PCBs are not formally listed as estrogenic contaminants (Wiegel et al. 1999).

8.2.2 Potential Formation of OH-PCBs during Biological and Abiotic Remediation of PCBs

Although production of PCBs has been banned in many countries since 1970s, PCBs are still found in every compartment of the environment. This implies that, despite multiple reports of the aerobic and anaerobic degradation of PCBs by microorganisms, natural attenuation of PCBs is very slow and/or incomplete and has failed to completely remove PCBs from the environment.

One puzzling aspect in natural attenuation of PCBs is that bioremediation seems to be slower at more heavily contaminated sites, which may be explain by the generation of toxic PCB metabolites that would impair further transformation. Another potential reason explaining the persistence of PCBs in the environment is that there is today no-evidence that PCBs could be fully mineralized by most organisms, including bacteria, fungi, plants, or mammals, therefore potentially resulting in the generation of toxic, dead-end metabolites. Another problem with the biodegradation of PCBs is that PCBs are usually found in the environment in the form of complex mixtures (e.g., Aroclor) that requires the presence of complex bacterial communities. Different species are responsible for the anaerobic dechlorination and aerobic oxidation of PCBs, and each PCB-degrading species seem to be capable of metabolizing only a narrow range of congeners, which severely limits the potential for a global PCB attenuation.

8.2.3 Monitoring PCBs, OH-PCBs, and other PCBs Metabolites in the Environment

As it is the case with most environmental contaminants, the toxicology, biodegradation, and environmental fate of metabolites of PCBs have received little attention. Only a few reports have been published about the detection of OH-PCBs in the environment. On the other hand, biodegradation, toxicity, and environmental fate of parent PCBs have been a topic of many literatures. This lack of environmental and toxicological information regarding OH-PCBs may be explained by several factors. First, OH-PCBs exhibit a large variety of structures (more than 800 congeners for the mono-hydroxylated group only) which each is characterized by unique chemicals and physical properties. Second, a large number of OH-PCBs are not today commercially available and are difficult or impossible to synthesize in the laboratory. Finally different extraction, preparation, and instrumental methods are required for the analysis of PCBs and OH-PCBs.

Based on the toxicity of OH-PCBs for animals and humans, and their potential impact on natural attenuation of PCBs, there is today a need to develop more advanced methods and perform more systematic analysis of OH-PCBs in sensitive environments, such as recreational waters and water supplies.

8.2.4 Incomplete Natural Attenuation of PCBs and OH-PCBs in the Environment

Because PCBs are not naturally occurring in the environment, it raises the question of the evolutionary bases of bacterial biodegradation pathways. Enzymes involved in the biodegradation of xenobiotics compound have been suggested to originate from the mutation of preexisting catabolic enzymes in response to contamination of the

environment by these pollutants. Living organisms are constantly exposed to numerous toxic metabolites (referred to as allelochemicals) that are released by plants, microorganisms, and insects and have synergistic and antagonistic effects on their relationships with their ecosystem (Singer et al. 2003). Living organisms exposed to a diversity of these allelochemicals have developed a large range of catabolic enzymes capable to degrade such compounds. Besides biphenyl, which is a naturally occurring compound (although not commonly found in the environment), different lignin phenolic monomers, were suspected to be natural substrates of biphenyl dioxygenase enzymes involved in the bacterial degradation of PCBs (Van Aken et al. 2010).

Natural transformation of PCBs in the environment has been the topic of several investigations since natural attenuation was often considered as a treatment option for PCB-contaminated sediments, a mechanism referred to as monitored natural attenuation (MNA). Reductive Dechlorination of PCBs is a process that occurs in the environment and has been reported in a range of PCB-contaminated sediments, including the Hudson River (NY), Silver Lake (MA), and Waukegan Harbor (IL) (Bedard and Quensen 1995; Pakdeesusuk et al. 2003; Gomes et al.2013). Even though reduction of PCB has been recorded, most of the investigations reported a limited success of the remediation process. Decades after PCBs were released on the sites, a significant contamination is still observed. For instance, Lake Hartwell site was subject to MNA, with the hope that deposition of clean sediment on the contaminated ones will prevent further spread of the contamination (Pakdeesusuk et al., 2003). However, two decades after monitoring started, significant PCB contamination still persist, even though evidence of *in situ* reductive dechlorination has been reported (Sivey and Lee 2007). In another study,

Kaštánek et al. (1999) monitored natural attenuation at an industrial PCB-contaminated site with a long history of contamination. The authors reported that the profile of PCB congeners underwent only limited changes over two decades, even though some aerobic oxidative biodegradation of lesser-chlorinated congeners may have occurred in the surface layers of the soil (Kaštánek et al. 1999).

8.2.5 Induction of Biphenyl Pathway in the Environment

Based on my observations, adding biphenyl as a primary substrate for microbial growth and an inducer of biphenyl pathway may enhance PCBs and OH-PCBs degradation. However, I have also observed that induction of biphenyl pathway by biphenyl has growth inhibitory effects. In addition, biphenyl has low water solubility (1.83 mgL^{-1}, SPARC calculator) and it is difficult to disperse biphenyl uniformly and efficiently in a PCB contaminated site. Neither groundwater infiltration nor geoaccumulation of biphenyl has been reported. (WHO 1999). Because of these complications, other compounds have been studied for induction of biphenyl pathway in the environment (Kown et al. 2008; Master and Mohn 2001). The feasibility of using non-toxic natural and synthetic inducers of the biphenyl pathway should be further studied. Non-toxic inducers that have higher water solubility and can be homogenously spread in contaminated sites may effectively increase the chance of natural attenuation of PCBs and OH-PCBs in the environment.

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