

**BIOLOGICAL EFFECTS OF HYDROXYLATED METABOLITES
OF POLYCHLORINATED BIPHENYLS**

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ABSTRACT

Polychlorinated biphenyls (PCBs) are widespread persistent organic pollutants. The metabolism of PCBs by various organisms involves many steps that can lead to the formation of a wide range of metabolites. These metabolites frequently exhibit a toxicity and biodegradability different than the parent compounds. There is currently little information available about the biological effects of PCB hydroxylated metabolites that can be generated by various organisms and potentially released into the environment.

The objective of the present research is to compare the toxicity of selected PCB congeners and their corresponding mono-hydroxylated metabolites. To achieve this objective, the following specific aims were performed: (1) to determine the effect of selected PCBs and PCB hydroxylated metabolites on the growth rate of a model PCB-degrading bacterium, *Burkholderia xenovorans* LB 400, (2) to determine the microbial toxicity of PCBs and PCB metabolites using the bioluminescent assay Microtox[®], and (3) to determine the estrogenicity of PCBs and PCB metabolites using the Yeast Estrogen Screen assay (YES).

The effects of a range of PCBs (PCB-2, -3, -8, -9, -30, -35, -36, -39, -61, -68, and -79) and their mono-hydroxylated metabolites on the growth rate of the PCB degrader, *Burkholderia xenovorans* LB400, were recorded. The results showed that the parent PCBs (50 mg L⁻¹) did not affect the growth rate of LB400 although their hydroxylated metabolites strongly inhibited microbial growth. Using Microtox[®] assay, Parent PCBs (50 mg L⁻¹) did not exhibit observable toxicity, while their hydroxylated metabolites showed a high level of toxicity (EC₅₀ ranges from 2 mg L⁻¹ to 46 mg L⁻¹).

Results using the YES assay also showed that the estrogenicity of hydroxylated metabolites of PCBs (50 mg L^{-1}) was higher than the parent PCBs.

The results obtained from the present study show that mono-hydroxylated metabolites of PCBs are more toxic than the corresponding parent PCBs. Because hydroxylated PCB derivatives are produced by a range of organisms and potentially released into the environment, this work raises new concerns associated with the environmental fate of PCBs.

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RENU BHALLA,

Temple University,

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DEDICATION

This Thesis is dedicated to

My Parents Mr. M. L. Bhalla and Mrs. Swarn Bhalla

*For teaching me to persevere & preparing me to face
challenges with faith and humility.*

They are my constant source of inspiration.

My loving Husband, Mr. Vikram Kaku

*For his unconditional love and support &
for being my constant strength.*

and Almighty God

For showering his blessings on me

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CHAPTER 1 INTRODUCTION

Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) are man-made toxic organic compounds that are persistent in the environment. They are a class of 209 congeners with a biphenyl ring and chlorines atoms attached at 1 to 10 positions. PCBs have high thermal stability, chemical inertness, and have high dielectric constant. Because of this, PCBs were used widely for a variety of industrial applications including dielectric fluids for capacitors and transformers, lubricating oils, adhesives, plastics and sealants (Borja *et al.*, 2005). The commercial usage of PCBs started in 1929 and was banned all over the world by the late 1970s, since they were largely reported to have high toxicity and persistence in environment.

Since PCBs were widely used in industries, they were released into the environment through industrial wastage, improper disposal, leakage, and spills. It is estimated that 1.5 million tons of PCBs have been produced worldwide, of which a large fraction has been released into the environment as a consequence of their manufacture, utilization, spill and improper disposal. Because of their high volatility and stability, PCBs have been widely dispersed by atmospheric routes.

PCBs are highly hydrophobic in nature and are chemically stable, so they bioaccumulate in organisms and they persist in the environment. PCBs can travel long distances in air and can be deposited in areas far away from where they were released (ATSDR, 1993). Once PCBs are in blood, they are carried by lipoproteins, and because PCBs are lipophilic, they accumulate preferentially in lipid-rich tissues. In human, PCBs

have been detected in breast milk and blood. It has been reported that exposures to PCBs may lead to serious immunological, neurological and endocrine disorders in children. Chloracne (skin disease) has also been reported due to chronic exposure to PCBs.

Biotransformation of PCBs

Even though PCBs are very stable chemically, they can be transformed by most organisms, which can result in the formation of toxic and persistent metabolites, such as hydroxylated PCBs (OH-PCBs). Following PCB exposure, approximately 40 different hydroxylated PCBs have been identified in human plasma (Hovander *et al.*, 2006). Exposure to hydroxylated PCBs has resulted in thyroid hormone disturbances, altered vitamin A levels and inhibition of phase II sulfation and glucuronidation in experimental organisms (e.g. rats, guinea pigs) (Brouwer *et al.*, 1998; Vanden *et al.*, 2002; Ghisari and Bonefeld-Jorgensen, 2005; Verreault *et al.*, 2006). This suggests that biotransformation of PCBs potentially increases toxicity in exposed organisms.

In mammals, PCBs undergo biotransformation by cytochrome P-450 (CYP) enzymes. The initial step in the metabolism of PCBs is a monooxygenase –catalyzed reaction, which generates a hydroxy group at *meta*- position by direct insertion or via formation of an arene oxide that rearranges to form a hydroxylated compound (Schnellmann *et al.*, 1985; Clevenger *et al.*, 1989). The hydroxylated PCBs have been selectively retained in human plasma and blood from Baltic grey seals (Bergman *et al.*, 1994). Studies on rats, hamsters and guinea pigs showed that 2,4,5-trichlorobiphenyl was metabolized to form hydroxylated PCBs in serum via direct insertion of a hydroxyl group

or via epoxidation and subsequent 1,2-chlorine shift at the 4-position (Haraguchi *et al.*, 2005).

PCBs constitute one class of the endocrine disrupting compounds (EDCs) that are present in environment, as EDCs can alter normal endocrine signaling in human and other species and they are known as estrogen mimics (xenoestrogen). The modulation of thyroid and estrogen hormone levels, the alteration of the biotransformation enzymes sulfo- and glucuronosyl-transferases, and binding of xenoestrogens to the thyroxine transport protein have all been suggested as contributing significantly to the endocrine disrupting effects of PCBs and their metabolites (Brouwer *et al.*, 1998; Kester *et al.*, 2000; Vanden *et al.*, 2002).

White-rot fungi have shown the ability to degrade many PCBs in complex mixtures and as single congeners. Degradation of PCBs is mediated by lignin-degrading enzyme system of the fungus that is secreted in the extracellular environment. Major components of the lignin-degrading enzyme system include lignin peroxidases (LiPs), Mn –dependent peroxidases (MnPs) and H₂O₂-producing system (Reddy, 1993). These are induced during secondary metabolism under nutrient-limiting culture conditions. Studies showed that the metabolism of 4,4'-dichlorobiphenyl by white-rot fungi *Phanerochaete* sp. MZ142 leads to the formation 2-hydroxy-4,4'-dichlorobiphenyl and 3-hydroxy-4,4'-dichlorobiphenyl (Kamei *et al.*, 2006). Biotransformation of five PCB congeners, 3,3',4,4'-tetrachlorobiphenyl, 2,3,3',4,4'-pentachlorobiphenyl, 2,3',4,4',5-pentachlorobiphenyl, 3,3',4,4',5-pentachlorobiphenyl and 2,3',4,4',5,5'-hexachlorobiphenyl, were degraded by the white-rot fungus *Phlebia brevispora* (Kamei *et al.*, 2006). Hydroxylated intermediates were formed as a result of initial hydroxylation.

These Hydroxylated intermediates were converted to monomethoxylated chlorobiphenyls.

In plants, biotransformation by PCBs is part of a three-phase process known as the green liver model (Sandermann, 1994). Phase I, the initial activation, consists of the oxidation of PCBs to produce various hydroxylated products that are characterized by a higher solubility and reactivity. Phase II involves conjugation of Phase-I activated compounds with glutathione or amino acids to form the products that are less toxic and more soluble than the parent PCBs. Phase III involves the sequestration of the conjugates in plant organelles or incorporation into plant structures (e.g. cell wall) (VanAken *et al.*, 2010). Harms *et al.* demonstrated that 3,3',4,4'-tetrachlorobiphenyl could be oxidized to several mono-hydroxylated intermediates by plant cell cultures of *Rosa* sp. and *Lactuca sativa* (Harms *et al.*, 2003). Studies on poplar plants showed that the metabolism of 4-monochlorobiphenyl gave three different mono-OH metabolites, including 2'-OH, 3'-OH and 4'-OH-4-chlorobiphenyl (Zhai *et al.*, 2010). Different plant species, including Paul's Scarlet rose, tobacco, *Solanum* sp., horseradish and alfalfa have been shown to transform PCBs into hydroxylated metabolites (Kucerova *et al.*, 2000). In another study on *S. nigrum*, the metabolism of di-, tri-, tetra-, and penta-chlorobiphenyls showed the formation of mono-hydroxylated PCBs (Rezek J. *et al.*, 2007).

Problem Statement

Metabolism of PCBs in mammals, plants and fungi frequently results in the formation of mono-hydroxylated PCB metabolites. These mono- hydroxylated PCBs have been detected in the environment, and as conjugated products in excreta (Jansson *et*

al., 1975; Sundström and Jansson, 1975; Moir *et al.*, 1996; Sinjari *et al.*, 1998). Although hydroxylated PCB metabolites are likely to be less stable than their parent compounds, they can be released and accumulate in the environment. For instance, mono-hydroxylated PCBs may undergo oxidation if exposed to air but only after prolonged periods of time. In mammals, mono-hydroxylated PCBs have been detected in liver, lung, kidney and adipose tissue (Klasson-Wehler E., 1994; Klasson-Wehler Eva *et al.*, 1998; Sinjari *et al.*, 1998). Due to their affinity for a plasma protein, the mono-hydroxylated PCBs are preferentially retained in the blood.

The published evidence summarized above shows that a variety of organisms are capable to metabolize PCBs into hydroxylated metabolites that could be potentially released into the environment. Although significant literature exists about the toxicity of PCBs and, to a lesser extent, their hydroxylated metabolites for human and mammalian models, there is currently very little information about the toxicity of hydroxylated PCBs metabolites for bacteria.

The hypothesis underlying this study is that mono-hydroxylated metabolites of PCBs exhibit a higher toxicity for bacteria than their parent PCB congeners. This question is relevant because biodegradation is the major sink of PCBs in the environment and bacteria are the most important PCB degraders in the environment. This hypothesis has been developed partly based on similar observations made with various organic pollutants. It has been observed that metabolism of many chemicals may lead to formation of metabolite more toxic than the parent compound. For example, tetrachloroethylene, a colorless liquid widely used for dry cleaning of fabrics, when it

undergoes anaerobic microbial dechlorination forms vinyl chloride, which is highly toxic and carcinogenic (Vogel *et al.*, 1985).

The overall objective of the current study is to determine the toxicity of selected mono- hydroxylated PCBs and to compare it with their corresponding parent PCBs.

To achieve this objective, we performed the following specific aims:

- (1) To compare the effect of selected PCBs and PCB metabolites on the growth rate of a bacterium relevant for PCB transformation

Working hypothesis: The inhibition of bacterial growth of the strain *Burkholderia xenovorans* LB400 when exposed to mono- hydroxylated metabolites will be greater than w their corresponding parent PCBs.

- (2) To compare the microbial toxicity of PCBs and PCB metabolites using the bioluminescent assay Microtox[®]

Working hypothesis: The toxicity endpoint of mono- hydroxylated metabolites using the Microtox[®] assay will be higher than of their corresponding parent PCBs.

- (3) To compare the estrogenicity of PCBs and PCB metabolites using the Yeast Estrogen Screen assay (YES).

Working hypothesis: The estrogenicity of mono- hydroxylated metabolites will be higher than that of their corresponding parent PCBs.

The part of this thesis has been or will be published in the following documents:

- (1) Bhalla, R., and B. Van Aken (2010). “Toxicity of polychlorinated biphenyls and their metabolites from bacterial transformation” *Proc. 85th Annual Meeting of the West Virginia Academy of Science*: p-43

- (2) Van Aken, B. and R. Bhalla (2010). “Microbial Degradation of Polychlorinated Biphenyls” In Comprehensive Biotechnology, Chapter 378. 2nd Edition, Elsevier. *In Press*
- (3) Bhalla, R., R. Suri, H.-J. Lehlmer, J. L. Schnoor, and B. Van Aken (2010). “Determination of the Toxicity of Hydroxylated Polychlorinated Biphenyls using Microtox[®] Test. *In Preparation.*

CHAPTER 2

LITERATURE REVIEW

Chemistry and Environmental Fate of PCBs

PCBs are toxic and persistent xenobiotic compounds that constitute today one of the major classes of environmental pollutants. They form a class of 209 discrete chemical compounds made of a biphenyl core with 1 to 10 chlorine atoms attached to it. PCBs were produced commercially as mixtures from 1920s to 1970s, and were marketed in US under the trade name Aroclor (Kuhre, 1995). PCBs have a high thermal and chemical stability, flame resistance and high dielectric constants. These properties made PCBs valuable for variety of industrial applications, including dielectric fluids for capacitors and transformers, heat transfer fluids, hydraulic fluids, lubricating and cutting oils, adhesives, sealants and plastics (Kuhre, 1995). In the early 1970s, the manufacture, use and importation of PCBs were banned all over the world, because PCBs were found to be toxic and highly persistent in the environment. In the United States, the Toxic Substances Control Act has banned the production and distribution of PCBs since 1976, but due to improper handling, spill and illegal disposal, there has been extensive environmental contamination by PCBs in soil and water.

Toxicity of PCBs

Toxicity of PCBs has been reported since the beginning of 1930's. PCBs enter the body through ingestion, inhalation and dermal contact. Since they are highly hydrophobic, PCBs tend to accumulate in liver and adipose tissues and in blood they are frequently associated with lipoproteins. Chronic exposure to PCBs in adult humans

results in fatigue, liver damage, skin disease (known as chloracne) and various detrimental effects on reproductive, immune and central nervous systems (Furukawa *et al.*, 2004). PCBs are also assumed to be endocrine disruptors. Studies have demonstrated that PCBs have negative effect on phytoplanktons, and have potentially harmful effects on oceanic food chain, oxygen production, and carbon dioxide mitigation (Furukawa *et al.*, 2004). The negative effects of PCBs have been shown in animals and humans, especially in newborn infants (Patandin *et al.*, 1998; Winneke *et al.*, 2002). Reported effects of background exposures in infants include reduced birth weight, less postnatal growth, impaired development, impaired immune response (Weisglas-Kuperus *et al.*, 1995; Weisglas-Kuperus, 1998), and lower thyroid hormone levels (Koopman-Esseboom *et al.*, 1994; Brouwer *et al.*, 1998; Osius *et al.*, 1999). The modulation of thyroid and estrogen hormone levels, the alteration of the biotransformation enzymes sulfo- and glucouronosyl-transferases, and their binding to the thyroxine transport protein have all been suggested as contributing significantly to the endocrine disrupting effects of PCBs and their metabolites (Brouwer *et al.*, 1998; Kester *et al.*, 2000; Vanden *et al.*, 2002).

Metabolism of PCBs by Higher Organisms

Exposure to PCBs is known to have numerous health effects including reproductive effects, toxicity in embryos, and estrogenic endocrine disruption as well as probable human carcinogenicity. PCB congeners undergo biotransformation and can result in the formation of toxic hydroxylated metabolites. These hydroxylated metabolites may have adverse effects in mammals. Several hydroxylated metabolites are strongly and selectively accumulated in mammalian tissues, including human blood. Hydroxylated

PCBs, which have been detected in human organs, blood, fatty tissue, and milk as well as in fish and wildlife have estrogenic effects, can disrupt thyroxin and vitamin A transport, and might lead to adverse neurodevelopmental effects. Therefore, explains why biotransformation of PCBs by mammals, bacteria, fungi, and plants has been extensively studied.

Metabolism by Mammals

In mammals, the metabolism of PCBs occurs primarily in the liver and it is a three-step process involving the following sequence: metabolic activation (Phase I), conjugation (Phase II), and sequestration or excretion (Phase III).

The initial step in the metabolism of PCBs is frequently catalyzed by the CYP-450 monooxygenase system, which generates an arene oxide intermediate often at *meta*- and *para*- positions that rearranges to form a mono- hydroxylated PCB metabolite (Figure-1) (Schnellmann *et al.*, 1985; Clevenger *et al.*, 1989). PCB arene oxides can also generate dihydrodiols via a hydrolytic pathway mediated by microsomal epoxide hydrolase, although the metabolism to mono- hydroxylated PCBs is more commonly observed.

The resulting mono- hydroxylated PCBs typically undergo a conjugation reaction with glucuronic acid or sulfate, which increases the water solubility and facilitates excretion. PCB arene oxide intermediates may also conjugate directly with the endogenous tripeptide glutathione (GSH) (Bakke, 1990). The GSH conjugation is catalyzed by glutathione-*S*-transferase mediation (Vermeulen, 1996). After dehydration, the resulting glutathionyl-PCB conjugate undergoes peptidase hydrolysis via the

mercapturic acid pathway (MAP) to form cysteine conjugate, which can be excreted either before or after acetylation to a mercapturic acid (Bakke *et al.*, 1982; Bakke and Gustafsson, 1986). The resulting PCB cysteine or mercapturic acid conjugate is subjected to a biliary excretion into the gastrointestinal tract and is converted to a thiol (-SH) via C-S β -lyase activity of the intestinal microflora (Larsen, 1985). The SH-PCB conjugate can be excreted as a free thiol or after conjugation with glucuronic acid to form S-glucuronides (Bakke *et al.*, 1982; Bakke, 1990).

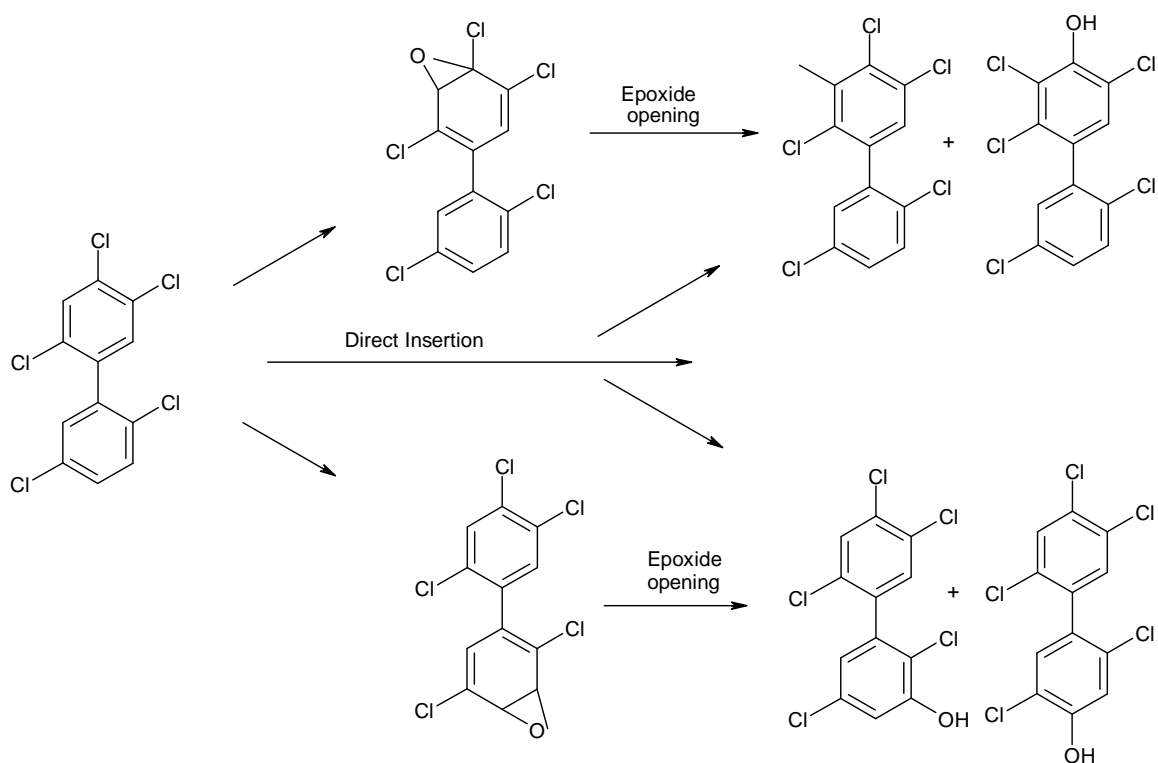


Figure 1: Formation of hydroxylated metabolites of 2,2',4,5,5'-pentachlorobiphenyl. Adapted from Letcher *et al.* (2000).

In the mid-1990s, hydroxylated PCBs with specific localization in the blood were identified in humans at relatively high concentrations in comparison to their PCB

precursors (Browner *et al.*, 1990; Bergman *et al.*, 1994; Klasson-Wehler E., 1994; Klasson-Wehler Eva *et al.*, 1998). Hydroxylated PCBs have now been detected in the blood of fishes, birds, and mammals (Bergman *et al.*, 1994; Klasson-Wehler E., 1994; Sandau and Norstrom, 1996; Asplund *et al.*, 1999). Hydroxylated PCBs were detected in livers of harbor seals from San Francisco bay and Gulf of Main (Park *et al.*, 2009).

Metabolism by Fungi

White-rot fungi have shown their ability to degrade complex mixtures of PCBs and single congeners in nitrogen-deficient (ligninolytic) culture. The ligninolytic enzymes of white-rot fungi catalyze the degradation of PCBs by using non-specific free radical mechanisms (Reddy, 1993). Major components of lignin-degrading enzyme system include lignin peroxidases (LiPs), Mn-dependent peroxidases (MnPs), and H₂O₂-producing system.

Metabolism of 4-chlorobiphenyl by the filamentous fungus *Paecilomyces lilacinus* results in the formation of the hydroxylated derivative (Sietmann *et al.*, 2006). In a second step, which is catalyzed by a monooxygenase, this hydroxylated derivative is oxidized to a 4-chloro-dihydroxy biphenyl with further cleavage by dioxygenase. In white-rot fungi *Phanerochaete chrysosporium*, two mechanisms appear to be responsible for the metabolism of PCBs (Hiratsukaa *et al.*, 2005): the LiP-catalyzed one-electron oxidation of the aromatic ring producing the aryl cation radical, and hydroxylation reactions of the aromatic ring by CYP-450 enzymes. Degradation of 4,4'-dichlorobiphenyl by *Phanerochaete* sp. MZ142 was shown to initially generate 2-hydroxy-4,4'-dichlorobiphenyl (II) and 3-hydroxy-4,4'-dichlorobiphenyl (Figure 2). Also

4-chlorobenzoic acid (V), 4-chlorobenzaldehyde (VI) and 4-chlorobenzyl alcohol (VII) were produced from 4,4'-dichlorobiphenyl via the formation of hydroxylated metabolites (Figure 2) (Kamei *et al.*, 2006).

Degradation by *Phanerochaete chrysosporium* was shown to produce, 4,4'-dichlorobiphenyl to 3-hydroxy-4,4'-dichlorobiphenyl and 4-hydroxy-4,4'-dichlorobiphenyl (Kamei *et al.*, 2006).

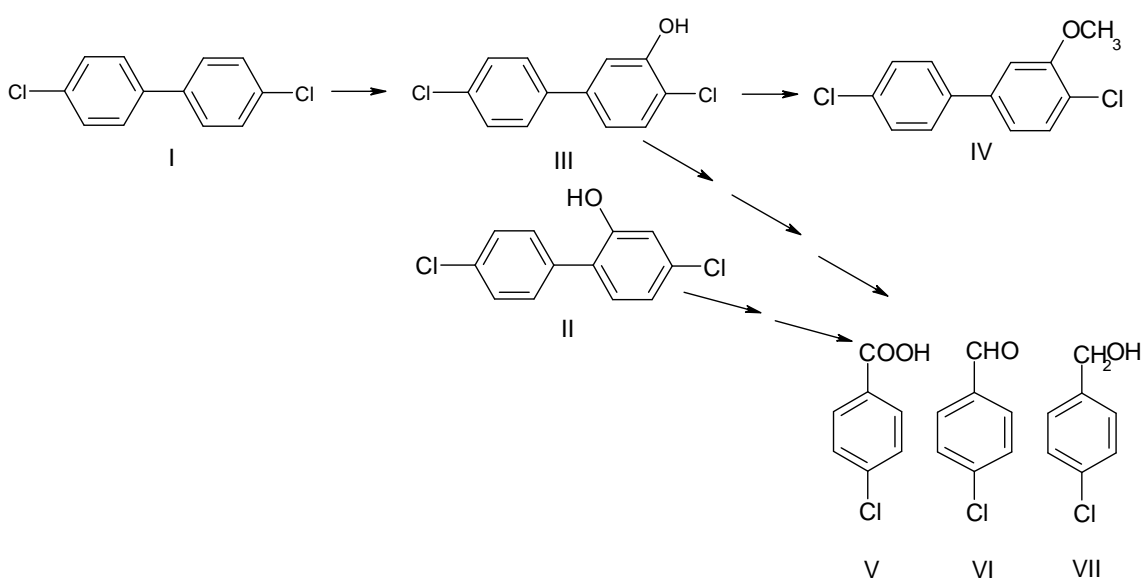


Figure 2: Metabolism of 4,4'-dichlorobiphenyl by *Phanerochaete* sp. MZ142. Adapted from Kamei *et al.* (2006).

Metabolism by Plants

Plant metabolism of PCBs follows a three-phase process known as the “green liver” model. According to “green liver” model, the metabolism of xenobiotics in plants is similar to the detoxification mechanism that occurs in the animal livers because of the presence of similar enzyme systems (Sandermann, 1994). In Phase I, the initial activation consists of oxidation of PCBs to produce various hydroxylated products, characterized by

a higher solubility and reactivity (Figure 3). Phase II involves conjugation of Phase I activated compounds with molecules of plant origin (e.g. glutathione or amino-acids) forming adducts less toxic and more soluble than the parent PCBs. Phase III involves sequestration of the conjugates in plant organelles (e.g. vacuole) or incorporation into plant structures (e.g. cell wall) (VanAken *et al.*, 2010). CYP-450 was suggested as the key enzyme responsible for plant activation of PCBs by Lee and Fletcher (Lee and Fletcher, 1992), who studied the involvement of mixed function oxidase systems in plant PCB metabolism. Other studies indicated that peroxidases are also involved in PCB metabolism in plants (Kucerova *et al.*, 2000).

Harms *et al.* demonstrated that 3, 3', 4, 4'- tetrachlorobiphenyl could be oxidized to several mono-hydroxylated intermediates by plant cell cultures of *rosa* sp. and *Lactuca sativa* (Harms *et al.*, 2003). In study on *Solanum nigrum*, the metabolism of di-, tri-, tetra-, and penta-chlorobiphenyls showed the formation of mono- hydroxylated PCBs (Kucerova *et al.*, 2000). Metabolism of 4-monochlorobiphenyl by poplar plants showed the formation of three hydroxylated metabolites, including 2'-OH, 3'-OH and 4'-OH 4chlorobiphenyls (Zhai *et al.*, 2010). In another study, the poplar plant (*Populus deltoides*) and switch grass (*Panicum virgatum*) were exposed to 3,3',4,4'- tetrachlorobiphenyl, whose metabolism resulted in the formation of 6-OH-3,3',4,4'- tetrachlorobiphenyl.

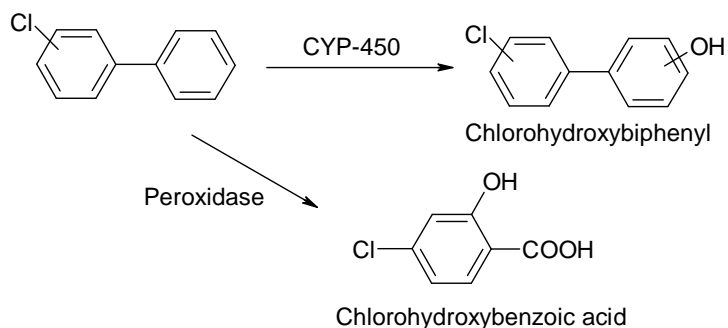


Figure 3: Pathway for metabolism of PCBs in plants

Metabolism by Bacteria

Anaerobic Biodegradation

Highly chlorinated congeners (having four or more chlorine atoms) of PCBs are recalcitrant to aerobic degradation and preferentially undergo anaerobic reductive dechlorination. Reductive dechlorination is involved in energy metabolism process. Microorganisms that do the reductive dechlorination of PCBs are present everywhere in anaerobic sediments and they involve various species from genus *Dehalococcoides* (Abraham *et al.*, 2002; Bedard *et al.*, 2006).

Aerobic Biodegradation

Lower chlorinated PCB congeners (with three or less chlorine atoms) which may be produced by anaerobic dechlorination, undergo co-metabolic aerobic oxidation mediated by dioxygenases, resulting in ring opening, and potentially complete mineralization of PCBs (Ohtsubo *et al.*, 2004; Field and Sierra-Alvarez, 2008; Furukawa and Fujihara, 2008).

Many bacterial strains are capable of oxidative degradation of PCBs, including many members of the genus *Pseudomonas*, *Burkholderia*, *Rhodococcus*, and *Bacillus*. Aerobic degradation of PCBs typically involves two clusters of genes, the first one is

responsible for the transformation of PCBs into chlorobenzoates and chlorinated aliphatic acids, known as *biphenyl upper pathway* and the second cluster of genes is responsible for the further mineralization of chlorobenzoates and chlorinated aliphatic acids, *known as biphenyl lower pathway* (Furukawa and Fujihara, 2008; Pieper and Seeger, 2008). The upper pathway involves a multistep 1,2-dioxygenase-mediated ring cleavage. Resulting ring-opening products, chlorobenzoate and organic acids, will be further transformed through the biphenyl lower pathway that is variable depending on the bacterial strain involved. Aerobic PCB-degrading bacteria (e.g., *Pseudomonas* sp.) commonly use biphenyl or mono-chlorinated biphenyl as growth substrate. Higher-chlorinated congeners (di- to tetrachlorinated biphenyls), are less susceptible to aerobic degradation and are only transformed through co-metabolic processes, i.e., they require an additional carbon source to support microbial metabolism, (Komancova *et al.*, 2003).

Estrogenicity of PCBs

Endocrine disruptors are chemicals which interfere with one or more function of the endocrine system (Cooke *et al.*). The endocrine system is present in all animals, including mammals, non-mammalian vertebrates, and invertebrates. The endocrine system consists of glands and the hormones they produce. Hormones are chemical messengers in the body that guide the development, growth, reproduction, and behavior of animals (Damstra *et al.*, 2002). Many industrial and environmental chemicals mimic, antagonize or indirectly alter the activity of hormones. These chemicals are known as endocrine disruptors, and they are capable to bind to estrogen or other hormone receptor, either imitating the action of the hormone or blocking its activity.

Estrogens are small lipophilic hormones that act as endocrine regulator in reproductive system in females. The predominant biological effects of estrogens occur through two intracellular estrogen receptors (ER α and ER β) (Kuiper *et al.*, 1996). By interfering with the normal functioning of these receptors, endocrine disruptors can perturb the growth and development of exposed organism. PCBs constitute one class of the endocrine disrupting compounds that are present in environment, as they act as estrogen mimics (xenoestrogens).

The first evidence that PCBs were estrogenic was reported in the early 1970's (Bitman and Cecil, 1970). Estrogenicity in both individual PCB congeners and mixtures has been reported. Aroclor 1242 increased uterine weight in immature rats (Jansen *et al.*, 1993; Soontornchat *et al.*, 1994) and Arcolor 1221 and Aroclor 1248 also have estrogenic effects (Geyer *et al.*, 2000) .

A convenient way to determine the estrogenicity of PCBs and their mono-hydroxylated metabolites is to use- recombinant yeast assays which are fast and inexpensive. The recombinant yeast assays represent *in vitro* models for studying the estrogenicity of environmental contaminants. Using these *in vitro* models, chemicals from different source showing estrogen modulating activity can be screened. In such a system, molecular interactions may be evaluated without organismal interactions that can cloud the identification of molecular structure or properties that are related to the actual mechanism of action. Recombinant yeast assays allow for reproductive biological data to be obtained in a time- and cost-effective manner (Schultz *et al.*, 1998).

Selection of PCB Metabolites

Metabolism of PCBs through hydroxylation mechanism may produce various hydroxylated metabolites. Hydroxylation is not always a specific mechanism and it may proceed through sequential reaction producing several mono- or di- hydroxylated metabolites, however mono- hydroxylated metabolites are usually the first to be produced as a result of oxidative metabolism in higher organism. These mono- hydroxylated metabolite, if not further metabolized may be released into the environment and may have potential harmful impact on various species. In the present study, we decided to focus on mono- hydroxylated metabolites only. These mono- hydroxylated metabolites of PCBs have no known anthropogenic source but have been reported to be found in many species and habitat. Table 1 shows a list of mono- hydroxylated metabolites found in various species and that were used in the present study.

Table 1: Hydroxylated metabolites of PCBs used in this study and their reported formation in different organisms

Metabolite	IUPAC Name	Parent Compound	Species found	Reference
2'-Hydroxy-4-Chlorobiphenyl	2'OH PCB-3	PCB-3	Poplar Plant	(Zhai <i>et al.</i> , 2010)
3'-Hydroxy-4-Chlorobiphenyl	3'OH PCB-3	PCB-3	Poplar Plant	(Zhai <i>et al.</i> , 2010)
4'-Hydroxy-4-Chlorobiphenyl	4'OH PCB-3	PCB-3	Poplar Plant	(Zhai <i>et al.</i> , 2010)
4'-Hydroxy-2,4'-Dichlorobiphenyl	4'-OH PCB-8	PCB-8	Rat	(Begum <i>et al.</i> , 1977)
2'-Hydroxy-2,5-Dichlorobiphenyl	2'-OH PCB-9	PCB-9	<i>Nicotina tabaccum</i>	(Rezek Jan <i>et al.</i> , 2008)
3'-Hydroxy-2,5-Dichlorobiphenyl	3'-OH PCB-9	PCB-9	<i>Nicotina tabaccum</i>	(Rezek Jan <i>et al.</i> , 2008)
4'-Hydroxy-2,5-Dichlorobiphenyl	4'-OH PCB-9	PCB-9	<i>Nicotina tabaccum</i>	(Rezek Jan <i>et al.</i> , 2008)
4'-Hydroxy-2,4,6-Trichlorobiphenyl	4'-OH PCB-30	PCB-30	Rainbow trout	(Carlson and Williams, 2001)
4'-Hydroxy-3,4',5-Trichlorobiphenyl	4'-OH PCB-39	PCB-39	Rat	(Hong <i>et al.</i> , 2007)
4'-Hydroxy-2,3,4,5-tetrachlorobiphenyl	4'-OH PCB-61	PCB-61	Rainbow trout	(Carlson and Williams, 2001)
4'-Hydroxy-3,3',4,5-Tetrachlorobiphenyl	4'-OH PCB-79	PCB-79	Rat	(Morse <i>et al.</i> , 1995)

CHAPTER 3 RESEARCH METHODOLOGY

Growth Analysis of Bacteria

Growth analysis of bacterial strain *Burkholderia xenovorans* (LB400) B-18064 was done with a range of PCBs and their corresponding mono- hydroxylated metabolites. All manipulations were performed under sterile conditions.

Terrific broth (10.16g/1000ml) purchased from Fisher Scientific Pittsburgh-PA was prepared and the solution was autoclaved at 121°C for 15 minutes. *Burkholderia xenovorans* strain LB400 was obtained from ARS Culture Collection (NRRL) (Peoria,IL). Strain was inoculated in 15mL tube containing terrific broth and the tubes were incubated at 28°C and 165 rpm in a shaking incubator.

PCBs were added under sterile conditions in the laminar hood in glass flasks. Two controls were prepared, one with PCBs and terrific broth without bacteria and the other with *B. xenovorans* LB400 and terrific broth without PCB. All tubes were kept in inclined position on shaking incubator at 120 rpm at room temperature.

Just before starting to take sample readings, 50µl of *B. xenovorans* B-18064 strain was added into triplicates except in control PCBs and terrific broth. Optical density on single wavelength at 600nm was measured using Hach-DR1 4000U spectrophotometer and reading was taken at the interval of every two hours in first 24 hrs and then at the 48th hour.

Statistical analysis of growth trend was done based on first-order exponential growth model:

$$N = N_0 e^{kt} \quad (1)$$

Where, N_0 = the initial bacterial number

N = bacterial number at time t

k = exponential growth rate

t = Time

This equation can be expressed in linear form as follows:

$$\text{Log } N = \text{Log } N_0 + kt \quad (2)$$

Where, k is the slope of the line, and $\text{Log}N_0$ is the y intercept of the line.

The exponential growth rate of bacteria *B. xenovorans* LB400 is hypothesized to be impacted differently by the PCBs and their corresponding mono- hydroxylated metabolites. Thus to compare the effect of PCBs and their corresponding mono- hydroxylated metabolites on the growth rate of bacteria, we conducted hypothesis test with the following Null and Alternative Hypotheses:

Null Hypothesis: Exponential growth rate of bacteria *B. xenovorans* LB400 is influenced by the PCBs and their corresponding mono- hydroxylated metabolites in a same manner (i.e. exponential growth rate are equal).

Alternate hypothesis: Exponential growth rate of bacteria *B. xenovorans* LB400 is influenced by the PCBs and their corresponding mono- hydroxylated metabolites in a different manner (i.e. exponential growth rate are different).

If the p-value for comparing slopes is less than 0.05, conclude that slopes or exponential growth rate are significantly different with 95% confidence level.

Microtox® Toxicity Test

In order to determine the toxicity of PCB and their metabolites, Microtox® toxicity protocol was used using Microtox® 500 analyzer. The Microtox® test uses the nonpathogenic, naturally luminescent strain of marine bacterium *Vibrio fischeri* (formerly *Photobacterium phosphoreum*) (Chu *et al.*, 1997). The end point of the test is the effective concentration 50% (EC₅₀), which is the concentration of a compound that causes a 50% decrease in the light output of the test organisms under controlled experimental conditions (usually after 5 and 15 minutes.). The EC₅₀ is calculated by log-linear plotting of sample concentration versus percentage of light decrease (response) or more precisely by log-log plotting of the gamma factor versus sample concentration. The gamma factor (Γ) is defined as the ratio of light lost at time *t*, to the light remaining at time *t*, for a given sample concentration and when compared to the non-exposed control (blank) (Blaise and Féraud, 2007).

In the present study, the marine bacteria *V.fischeri* were exposed to PCBs and their corresponding mono- hydroxylated metabolites. When the bacteria undergo a toxic stress, one may expect decreases of luminescence in a dose-dependent manner. The reduction in the light output over the time was measured after 5 minutes and 15 minutes. The changes in luminescence measured in the concentration series was compared to a reagent control (2% NaCl and bacteria, without toxic compounds) and, EC₅₀ is calculated from a linearized derivation of the dose-response curve.

Gamma factor (Γ) is calculated using following two equations:

$$\Gamma_t = \left[R_t \times \left(\frac{I_0}{I_t} \right) \right] - 1 \quad (3)$$

Where, I_0 = Intensity of light produced by sample at initial time

I_t = Intensity of light produced by sample at time t

R_t = Ratio of light output of Microtox[®] control sample (blank) after time t to the initial light output of the control. R_t corrects for any inhibition induced by negative control.

$$R_t = \frac{I_{ct}}{I_{ci}} \quad (4)$$

Where, I_{ct} = absolute light intensity produced by negative control at time t

I_{ci} = absolute light intensity produced by negative control at initial time

Gamma (Γ) is converted to percentage effect (inhibition) using following equation:

$$\% \text{ effect} = \left[\frac{\Gamma_t}{(1 + \Gamma_t)} \right] \times 100 \quad (5)$$

The rate theory of biological inhibition suggests a simple mathematical relationship between the concentration of a toxic substance and the response of the susceptible organism when the response is measured in terms of Γ values.

$$\Gamma = k \cdot C^p \quad (6)$$

Where, C is the concentration, p is the number of toxic molecules per target site and k is the composite factor relating to free energy changes and volume changes made during reaction. This equation may be expressed in a linear form which allows for the calculation of a concentration based on the Γ value.

$$\log C = b \times \log \Gamma + \log a \quad (7)$$

Where, b is the slope of the line fitted to data and $\log a$ is the y-intercept of that line.

The concentration of PCBs that result in a 50% reduction of light emission by bacteria in the Microtox[®] reagent (EC_{50}) was estimated from the equations (5) and (7). At

50% effect, Γ is equal to 1. Substituting value of Γ in equation (7), we can obtain value of concentration at 50% effect as shown in equation (8).

$$\log C = \log a \quad (8)$$

YES Assay

In the Yeast Estrogen Screen (YES) assay, a human estrogen receptor (hER) is expressed in the yeast *Saccharomyces cerevisiae* in a form that can bind with the estrogen-responsive sequences (ERE) (Routledge and Sumpter, 1996). The estrogen receptor is generally not present in yeast cells, so a recombinant DNA sequence of hER was stably integrated into the main chromosome of the yeast and it encodes for the estrogen receptor (protein). Expression plasmids with *lac-Z* gene (reporter gene) were also introduced in the yeast cells. The *lac-Z* gene encodes the enzyme β -galactosidase. The estrogen responsive sequences (ERE) are situated inside a strong promoter sequence on the expression plasmids of yeast cells. When hER protein binds with an active ligand i.e., an endocrine disrupting compound, it can bind the ERE and interacts with the transcription factors and other transcriptional components to initiate the gene transcription. This results in the expression of the reporter gene, *lac-Z* (Routledge and Sumpter, 1996). As a result, the enzyme β -galactosidase is secreted into the medium. The presence of β -galactosidase in the medium results in the transformation of the yellow chromogenic substrate, chlorophenol red β -D-galactopyranoside (CPRG) which is added in the growth medium, CPRG is metabolized by the enzyme β -galactosidase into a red product that can be measured by absorbance at 540nm.

For determining the concentration of a compound that produces half maximal response (EC_{50}), sigmoidal concentration-response curves are fitted to a symmetric logistic function by using the software Prism (Rutishauser *et al.*, 2004).

$$Response = a + \frac{(b-a)}{1 + 10^{[(\log EC_{50} - \log C) \times m]}} \quad (9)$$

The responses of the test systems toward the PCBs and hydroxylated metabolites tested are expressed as percent of the maximum response of the system represented by the response to estradiol (E_2). For each system, the recorded response is plotted versus the $\log C$ (logarithm of concentration). To determine the maximum value of the response, the concentration-response curve for E_2 is fitted to equation (9). From the curve fitting, the top (b) and the bottom (a) values are determined and the recorded values are expressed as percent of maximum response of E_2 . The fitted slope (m) of the concentration-response curves for E_2 is set as a constant and used for fitting the curves for each PCB and corresponding metabolite under study.

The relative potencies (RP_i) were calculated as the ratio of EC_{50} for estradiol (E_2) to EC_{50} for each PCB and metabolites tested (i) for estrogenicity.

$$RP_i = \frac{EC_{50}(E_2)}{EC_{50}(i)} \quad (10)$$

Concentration of PCBs

In North America, the concentration of PCBs in atmosphere is normally 10^{-6} - 10^{-5} $mg L^{-1}$ and that in the aquatic environment is less than 2×10^{-6} $mg L^{-1}$ (Qingyu *et al.*, 2001). The suspended particles in the water accumulate PCBs and investigations show that concentration of solid-combined PCBs is greater than 1×10^{-4} $mg L^{-1}$ (Qingyu *et al.*,

2001). For the Microtox[®] bioluminescent assay we used PCB concentration of 50, 5, 2.5 and 2 mg L⁻¹. For Growth analysis and YES assay 25mg L⁻¹ concentration of PCBs was used in present study. These concentrations are much higher than the actual concentration of PCBs found in the environment. In environmental toxicology it is a common approach to use higher concentrations than likely to be found in the environment, because we cannot test chronic toxicity under laboratory conditions. A latent and always controversial hypothesis is that exposure to higher dose for shorter exposure time (acute toxicity) is expected to reflect a comparable toxic effect as exposure to lower dose for longer period of time (chronic toxicity). Therefore, we used high doses of PCBs and hydroxylated metabolites in our present experimental approach.

CHAPTER 4

RESULTS AND DISCUSSIONS

Microtox[®] Basic Toxicity Test

The toxicity of twenty two mono-hydroxylated metabolites of polychlorinated biphenyls (PCBs) and their parent compounds, including mono- to tetra-chlorinated PCBs, were tested using the microbial bioluminescent toxicity test, Microtox[®]. As an endpoint of the testing, the EC₅₀ after 5 minutes and 15 minutes of exposure were recorded (Table 2). The EC₅₀ is the concentration of PCBs that causes 50% of decrease in light output of the test organism (*Vibrio fischeri*). Therefore a lower EC₅₀ values indicates higher toxicity of the compound. Results of the Microtox[®] toxicity assays are presented in Figures 4 to 6 for each parent compound and their corresponding mono-hydroxylated metabolites. The relative toxicity of each compound is expressed by normalized reduction of light emission after 5 min (in percent) as a function of the concentration (in mg L⁻¹). Table 2 summarized the assay end-point, the EC₅₀, and the corresponding R² of the regression data as recorded after 5 and 15 min of exposure (Flokstra *et al.*, 2008).

Table 2: List of PCB congeners and their hydroxylated metabolites used in this study. The Table shows the EC₅₀ values of the PCBs and hydroxylated metabolites and their corresponding coefficient of determination R² as recorded after 5 min and 15 min using Microtox[®] assay.

PCB*	CHEMICAL NAME	Conc. mg/l used	EC₅₀ mg/l (5min)	EC₅₀ mg/l (15min)	R² (5min)	R² (15min)
PCB 2	3-Chlorobiphenyl	50	13.07	45.69	0.9526	0.8117
<i>4-OH</i>	4-Hydroxy-3-Chlorobiphenyl	5	9.24	11.50	0.9955	0.9932
PCB 3	4-Chlorobiphenyl	50				
<i>2'OH</i>	2'-Hydroxy-4-Chlorobiphenyl	50	13.23	15.93	0.9931	0.9875
<i>3'OH</i>	3'-Hydroxy-4-Chlorobiphenyl	50	6.83	9.06	0.9963	0.9706
<i>4'OH</i>	4'-Hydroxy-4-Chlorobiphenyl	50	9.58	13.88	0.9988	0.9951
PCB 8	2,4'-Dichlorobiphenyl	50				
<i>4'-OH</i>	4'-Hydroxy-2,4'-Dichlorobiphenyl	2.5	1.42	1.88	0.9997	0.9945
PCB 9	2,5-Dichlorobiphenyl	50	14.54	28.52	0.8457	0.8966
<i>2'-OH</i>	2'-Hydroxy-2,5-Dichlorobiphenyl	50	3.82	6.86	0.9999	0.9202
<i>3'-OH</i>	3'-Hydroxy-2,5-Dichlorobiphenyl	50	3.01	2.92	0.8938	0.9991
<i>4'-OH</i>	4'-Hydroxy-2,5-Dichlorobiphenyl	2.5	3.56	5.06	0.9905	0.9592
PCB 12	3,4-Dichlorobiphenyl	50				
<i>4'-OH</i>	4'-Hydroxy-3,4-Dichlorobiphenyl	5	3.61	4.06	0.9953	0.9791
PCB 30	2',4',6'-Trichlorobiphenyl	50				
<i>2'-OH</i>	2'-Hydroxy-2,4,6-Trichlorobiphenyl	50	6.16	6.76	0.9821	0.9519
<i>3'-OH</i>	3'-Hydroxy-2,4,6-Trichlorobiphenyl	50	9.13	9.38	0.9842	0.9911
<i>4'-OH</i>	4'-Hydroxy-2,4,6-Trichlorobiphenyl	5	21.58	21.69	0.9885	0.9676
PCB 35	3,3',4'-Trichlorobiphenyl	50				
<i>2'-OH</i>	2'-Hydroxy-3,3',4'-Trichlorobiphenyl	2.5	6.76	5.86	0.9994	0.9783
<i>4'-OH</i>	4'-Hydroxy-3,3',4'-Trichlorobiphenyl	50	11.23	8.57	0.9931	0.9914
PCB 36	3,3',5'-Trichlorobiphenyl	50				
<i>2'-OH</i>	2'-Hydroxy-3,3',5'-Trichlorobiphenyl	2.5	9.55	7.13	0.9811	0.9991
<i>4'-OH</i>	4'-Hydroxy-3,3',5'-Trichlorobiphenyl	5	11.97	9.45	0.9870	0.9753
PCB 39	3,4',5'-Trichlorobiphenyl	50				
<i>4'-OH</i>	4'-Hydroxy-3,4',5'-Trichlorobiphenyl	5	25.74	12.87	0.9688	0.9451
PCB 61	2',3',4',5'-Tetrachlorobiphenyl	50				
<i>2'-OH</i>	2'-Hydroxy-2',3',4',5'-tetrachlorobiphenyl	2	26.31	22.62	0.9994	0.9386
<i>3'-OH</i>	3'-Hydroxy-2',3',4',5'-tetrachlorobiphenyl	50	33.25	18.31	0.8700	0.9888
<i>4'-OH</i>	4'-Hydroxy-2',3',4',5'-tetrachlorobiphenyl	50	139.90	64.40	0.4341	0.9918
PCB 68	2,3',4',5'-Tetrachlorobiphenyl	50				
<i>4'-OH</i>	4'-Hydroxy-2,3',4',5'-tetrachlorobiphenyl	50	30.99	10.33	0.9768	0.9813
PCB 79	3,3',4',5'-Tetrachlorobiphenyl	50				
<i>4'-OH</i>	4'-Hydroxy-3,3',4',5'-Tetrachlorobiphenyl	50	7.22	1.87	0.9349	0.8247

*Parent PCBs are in **bold** letter and their corresponding metabolites are in *italics*

For all parent PCBs (except PCB-2 and PCB-9), no EC_{50} was calculated as there was no observable reduction in luminescence of the bacteria, indicating that most of parent PCBs do not exhibit observable toxicity. For some parent PCBs, a reduction in light output was observed, but no EC_{50} could be calculated because the series of plots did not satisfy the fitting criteria imposed by the Microtox[®] data analysis Omni software. Hormesis, was detected with all parent PCBs (except PCB-2 and PCB-9), resulting in negative Γ values, which indicates a higher light emission with the samples exposed to PCBs as compared to the non-exposed controls. Hormesis, or the stimulatory effect of low concentrations of toxic chemicals, on the organismal metabolism, is commonly observed in luminescent bioassays (Shen *et al.*, 2009). Calabrese (1999) provided some evidence that hormesis is due to overcompensation in response to a disruption of the homeostasis.

Two parent PCBs showed a recordable toxic effect: PCB-2 with an EC_{50} of 13.07 $mg\ L^{-1}$ after 5 min and 45.69 $mg\ L^{-1}$ after 15 min and PCB-9 with an EC_{50} of 14.54 $mg\ L^{-1}$ after 5 min and 28.52 $mg\ L^{-1}$ after 15 min of time. Although these two parent PCBs showed recordable toxic effects, 4-OH PCB-2 was diluted 10 times and 4'-OH PCB-9 was diluted 20 times as they were highly toxic in comparison to their corresponding parent PCBs. For all mono-hydroxylated metabolites of PCBs tested, we observed a significant reduction in bioluminescence after 5 and 15 min of exposure, indicating that mono-hydroxylated metabolites were toxic to the test bacterium. The values of EC_{50} after 5 min and 15 min of exposure for the analyzed PCB metabolites varied from 1.4 to 139.9 $mg\ L^{-1}$ and from 1.9 to 64.4 $mg\ L^{-1}$ respectively (Table 1). The highest toxicities were recorded with hydroxylated derivatives of di-chlorinated biphenyls (2,4'-, 2,5- and 3,4-

dichlorinated biphenyls) with EC_{50} at 5 min ranging from 1.4 to 3.6 $mg L^{-1}$. All hydroxylated metabolites of trichlorinated biphenyl showed an EC_{50} ranging from 6.16 to 25.74 $mg L^{-1}$ after 5 min. For hydroxylated metabolites of tri- and tetrachlorinated PCBs (3,3',4-, 3,3',5-, 3,4',5- trichlorinated biphenyls and 2',3',4',5'-, 2,3',4,5'-, 3,3',4,5'- tetrachlorinated biphenyls), we observed a decrease in EC_{50} (i.e., an increase of toxicity) from exposure at 5 min to exposure at 15 min.

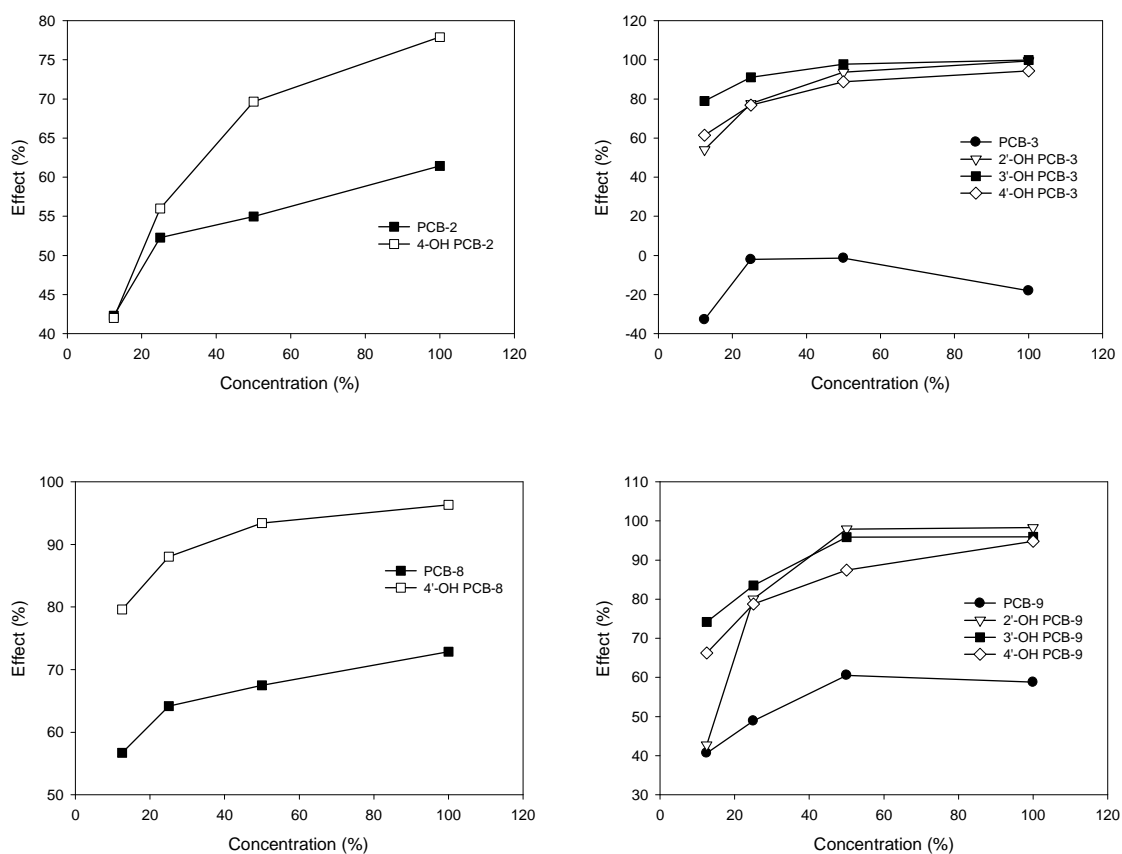


Figure 4: Percentage of effect vs. concentration for exposure to PCB-2, PCB-3, PCB-8, PCB-9 and their hydroxylated metabolites recorded using the Microtox[®] assay. The above data are presented for 5 minutes of exposure of *Vibrio fischeri* to PCBs and their corresponding hydroxylated metabolites. Concentrations used were 5 mg L⁻¹ for 4'-OH PCB-2, 2.5 mg L⁻¹ for 4'-OH PCB-8 and -9, and 50 mg L⁻¹ for all other compounds.

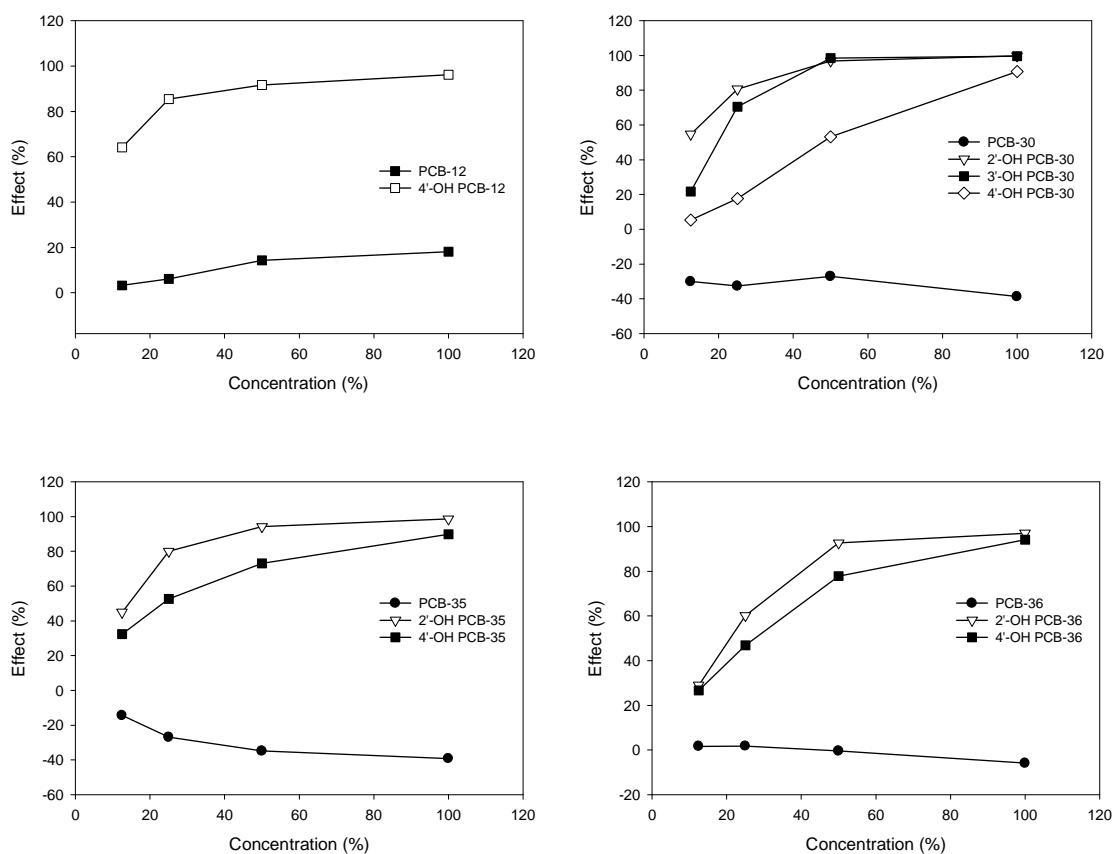


Figure 5: Percentage of effect vs. concentration for exposure to PCB-12, PCB-30, PCB-35, PCB-36 and their hydroxylated- metabolites recorded using the Microtox[®] assay. The above data are presented for 5 minutes of exposure of *Vibrio fischeri* to PCBs and their corresponding hydroxylated metabolites. Concentrations used were 5 mg L⁻¹ for 4'-OH PCB-12, -30 and -36, 2.5 mg L⁻¹ for 2'-OH PCB-35 and -36, and 50 mg L⁻¹ for all other compounds.

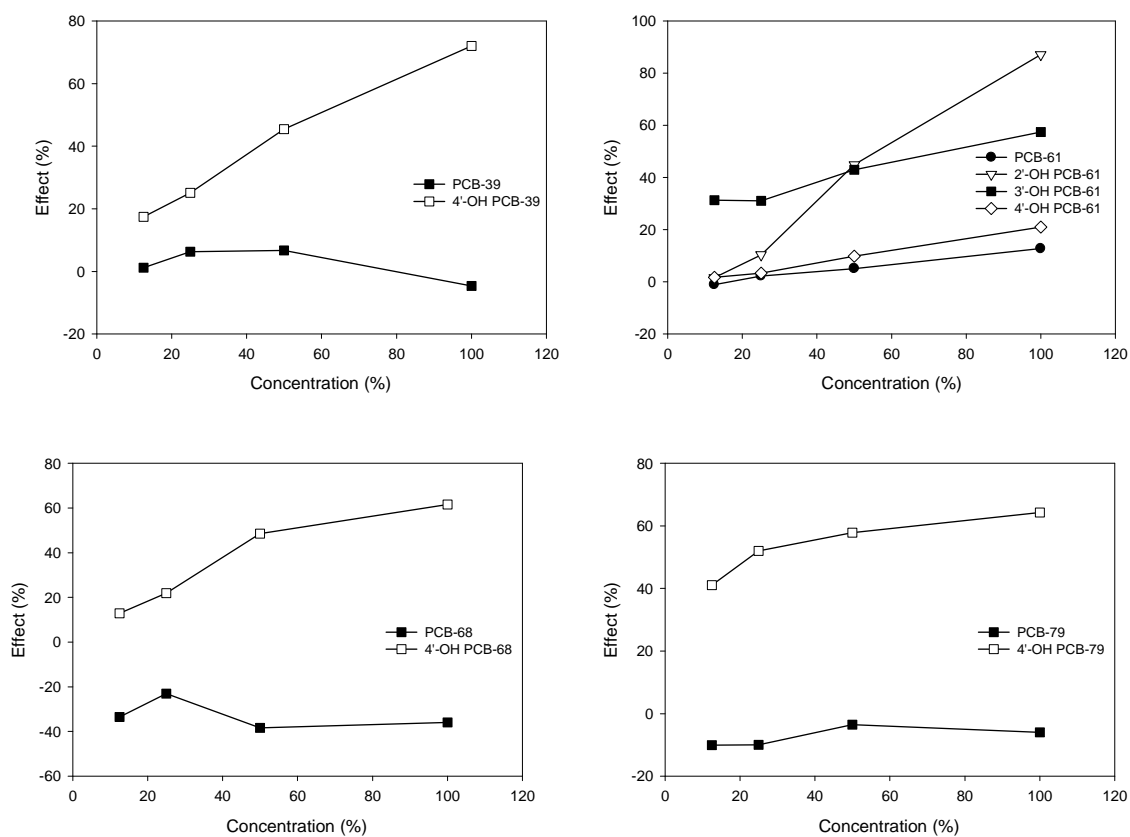


Figure 6: Percentage of effect vs. concentration for exposure to PCB-39, PCB-61, PCB-68, PCB-79 and their hydroxylated metabolites recorded using the Microtox[®] assay. The above data are presented for 5 minutes of exposure of *Vibrio fischeri* to PCBs and their corresponding hydroxylated metabolites. Concentrations used were 5 mg L⁻¹ for 4'-OH PCB-39, 2 mg L⁻¹ for 2'-OH PCB-61, and 50 mg L⁻¹ for all other compounds.

Figures 4-6 shows the percentage of effect (%) vs concentration (mg L^{-1}) for PCBs and their corresponding mono-hydroxylated metabolites. As higher percentage of effect corresponds to a reduction of light output by bacteria this is associated with a higher toxicity of the compound. For hydroxylated metabolites of lower PCBs (mono- and dichlorinated PCBs), we observed a slight decrease of the normalized toxicity expressed by the EC_{50} after the exposure at 15 min by comparison to exposure at 5 min.

Figure 7 shows the comparison of EC_{50} values of various mono-hydroxylated metabolites used in present study. The hydroxylated metabolites of mono- and dichlorobiphenyls (PCB-2 and -9) showed high EC_{50} values after 15 minutes of exposure, indicating decrease in toxicity of these hydroxylated metabolites after 15 minutes. The metabolites of higher chlorinated PCBs (PCB-35, -36, -39, -61, -68, and -79) showed decrease in EC_{50} values after 15 minutes exposure, indicating that toxicity was increased after 15 minutes for higher chlorinated hydroxylated PCB metabolites. The exceptional decrease in EC_{50} value was observed in 4'-OH PCB-61 (from $139.90 - 64.40 \text{ mg L}^{-1}$). On the other hand, hydroxylated derivatives of tri-chlorinated PCBs showed either no significant change or a decrease of EC_{50} values between exposure at 5 min and 15 min. For a few compounds, EC_{50} values at 15 min were higher than those obtained after 5 min of exposure.

It was reported that Microtox[®] is more sensitive to selected compounds after exposure 15min (Bundy *et al.*, 1997). Similarly Ruiz *et al.*, (1997) reported that the full toxicity cannot always be recorded after 5 min of exposure. For these reasons, the standard Microtox[®] test record toxicity after 5 and 15 min of exposure. Within a series of hydroxylated derivatives (i.e., originating from the same parent compound) of tri- and

tetra-chlorinated PCBs, the toxicity increased with the position of the hydroxyl group following the sequence *para*-, *meta*-, and *ortho*- substitution. However, the hydroxylated derivatives from 4-chlorobiphenyl showed an increasing toxicity following the sequence *ortho*-, *para*-, and *meta*- substitution.

Some PCB metabolites (metabolites of PCB-8, -9, -35 and -36) were found to be highly toxic, so they had to be diluted 20 times to determine their toxicity. Similarly, metabolites of PCB-2, -12, -30, -36, and -39 were diluted 10 times. Also, 2'-OH PCB-61 was highly toxic and was diluted 25 times in diluent. In hydroxylated metabolites of lower chlorinated PCBs (4-OH PCB-2, 2'-OH PCB-3, 3'-OH PCB-3, 4'-OH PCB-3, 2'-OH PCB-9 and 4'-OH PCB-9) increase in EC_{50} from 5 to 15 min was observed. EC_{50} values recorded in this study showed that most parent compounds themselves did not show any observable toxicity but their corresponding mono-hydroxylated metabolites were significantly toxic. This suggests that PCB metabolites may play an important role in the toxicity of PCBs.

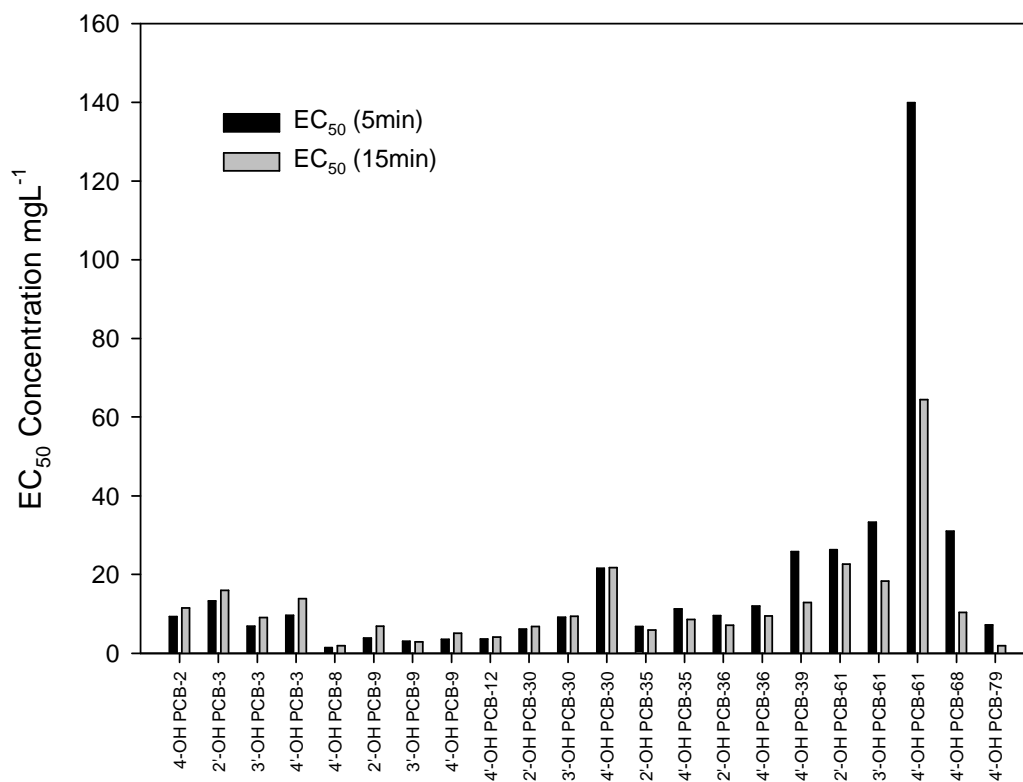


Figure 7: Comparison of EC₅₀ values of recorded using Microtox[®] assay for different hydroxylated metabolites of PCBs after 5 minutes and 15 minutes of exposure.

YES Assay

The estrogenicity of parent PCBs and their corresponding hydroxylated metabolites was determined using the YES assay. YES assay was performed by incubating PCBs and their mono-hydroxylated metabolites with recombinant yeast strain and 141 μ M CPRG at 30°C for 72 hours. Experiments were performed in triplicates. EC₅₀ values recorded for PCBs and their corresponding mono-hydroxylated metabolites are shown in Table 3. Higher EC₅₀ values correspond to a lower estrogenicity and lower EC₅₀ value corresponds to a higher estrogenicity of the compound. Figure 8 shows the estrogenic response of PCB-2, -3, and -8 and their corresponding mono-hydroxylated metabolites. The exposure to 4'-OH PCB-2 resulted to submaximal growth so that no full dose response curve was obtained. This was observed because the initial higher concentrations were very toxic to yeast cells and no growth was observed at these higher concentrations. Results also showed that PCB-3 (Figure 8) was less estrogenic by comparison to its hydroxylated metabolites. A full dose-response curve was only observed for the parent compound, PCB-3 up to a concentration of 25 mg L⁻¹. On the other hand, the metabolite, 2'-OH PCB-3 showed toxic effects at concentration greater than 6 mg L⁻¹ and no yeast growth was observed above that concentration. Therefore drawing a full dose-response curve was not possible with this compound.

Hydroxylated metabolites of PCB-9, -30 and -61 showed an increase of the estrogenic activity of an order of magnitude following the sequence *ortho*- to *meta*- and *para*- substitution (Figures 9 and 11).

Table 3. Estrogenic activity of PCBs and their mono-hydroxylated metabolites obtained using the standard 96-well microtitre plate YES assay

CHEMICAL NAME	EC ₅₀ of Estrogenic Activity (mg L ⁻¹)
3-Chlorobiphenyl	7.852
4-Hydroxy-3-Chlorobiphenyl	1.296
4-Chlorobiphenyl	8.030
2'-Hydroxy-4-Chlorobiphenyl	1.018
3'-Hydroxy-4-Chlorobiphenyl	455.3
4'-Hydroxy-4-Chlorobiphenyl	6.187
2,4'-Dichlorobiphenyl	9.124 x 10 ⁻³
4'-Hydroxy-2,4'-Dichlorobiphenyl	6.01 x 10 ⁻⁴
2,5-Dichlorobiphenyl	3.122
2'-Hydroxy-2,5-Dichlorobiphenyl	0.508
3'-Hydroxy-2,5-Dichlorobiphenyl	0.071
4'-Hydroxy-2,5-Dichlorobiphenyl	4.62 x 10 ⁻³
3,4-Dichlorobiphenyl	403.3
4'-Hydroxy-3,4-Dichlorobiphenyl	0.013
2,4,6-Trichlorobiphenyl	0.322
2'-Hydroxy-2,4,6-Trichlorobiphenyl	847.0
3'-Hydroxy-2,4,6-Trichlorobiphenyl	0.538
4'-Hydroxy-2,4,6-Trichlorobiphenyl	7.21 x 10 ⁻⁴
3,3',4-Trichlorobiphenyl	0.652
2'-Hydroxy-3,3',4-Trichlorobiphenyl	0.184
4'-Hydroxy-3,3',4-Trichlorobiphenyl	0.289
3,3',5-Trichlorobiphenyl	0.612
2'-Hydroxy-3,3',5-Trichlorobiphenyl	0.651
3,4',5-Trichlorobiphenyl	0.431
4'-Hydroxy-3,4',5-Trichlorobiphenyl	1.261
2,3,4,5-Tetrachlorobiphenyl	0.524
2'-Hydroxy-2,3,4,5-tetrachlorobiphenyl	3.95 x 10 ⁻²
3'-Hydroxy-2,3,4,5-tetrachlorobiphenyl	3.03 x 10 ⁻³
4'-Hydroxy-2,3,4,5-tetrachlorobiphenyl	30.25
2,3',4,5'-Tetrachlorobiphenyl	9.43 x 10 ⁻³
4'-Hydroxy-2,3',4,5'-tetrachlorobiphenyl	1.404 x 10 ⁻³

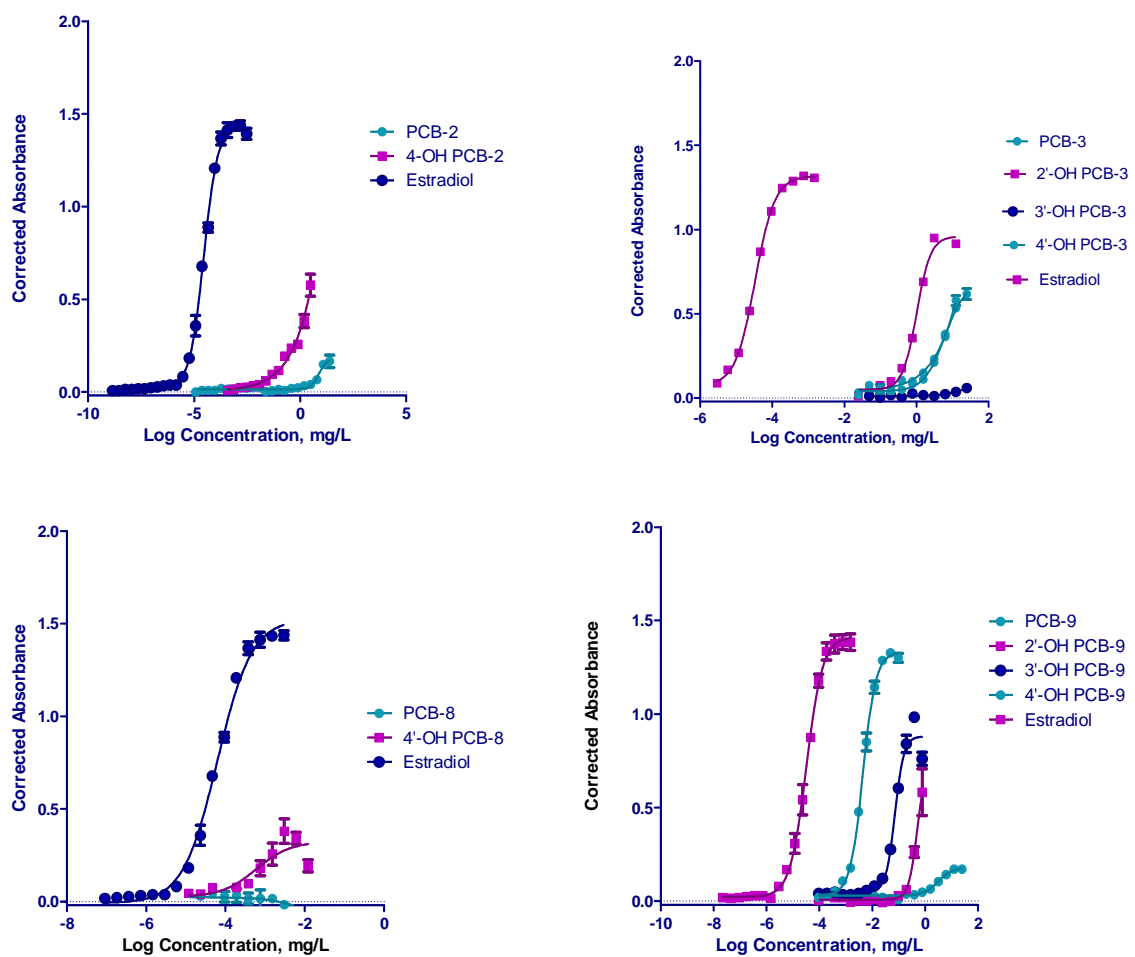


Figure 8: The estrogenic response of PCB-2, PCB-3, PCB-8, PCB-9 and their hydroxylated metabolites as measured using the YES assay. Experiments were done in triplicates and error bars show the standard deviation of triplicate measurements.

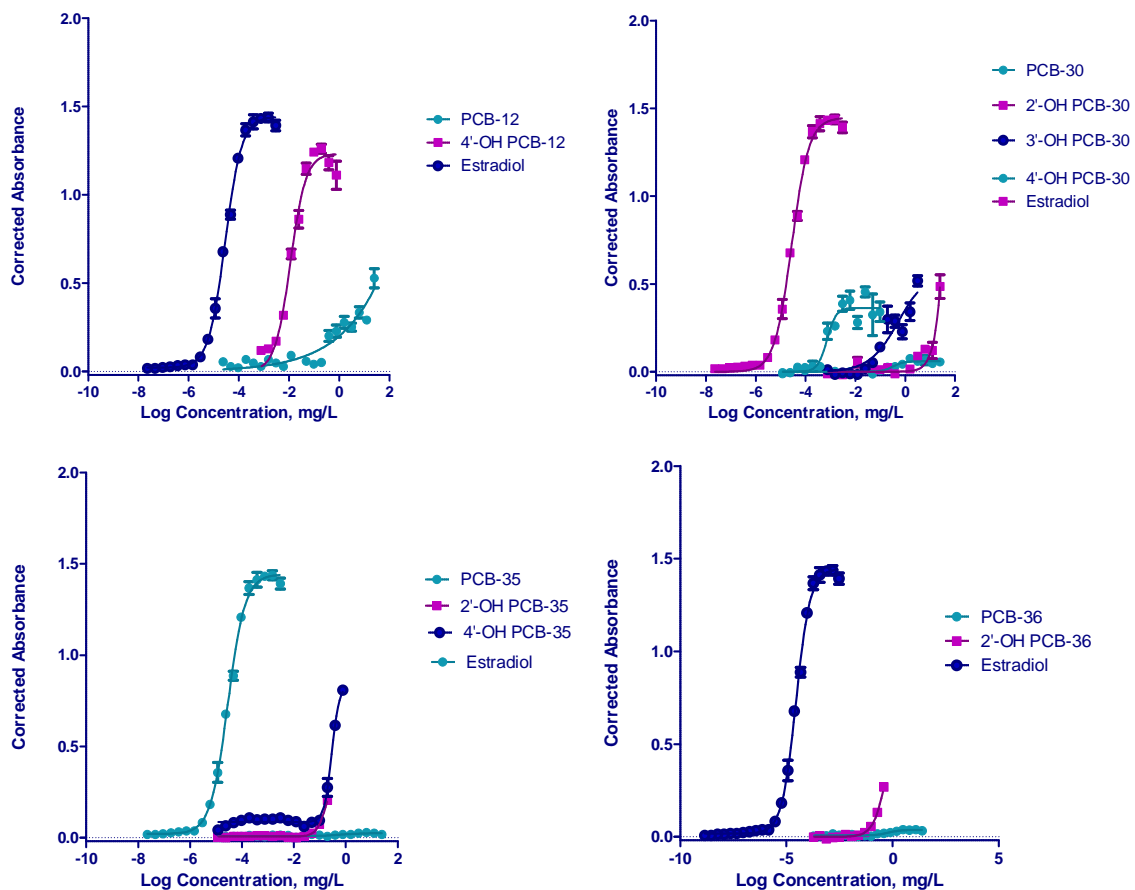


Figure 9: The estrogenic response of PCB-12, PCB-30, PCB-35, PCB-36 and their hydroxylated metabolites as measured using the YES assay. Experiments were done in triplicates and error bars shows the standard deviation of triplicate measurements.

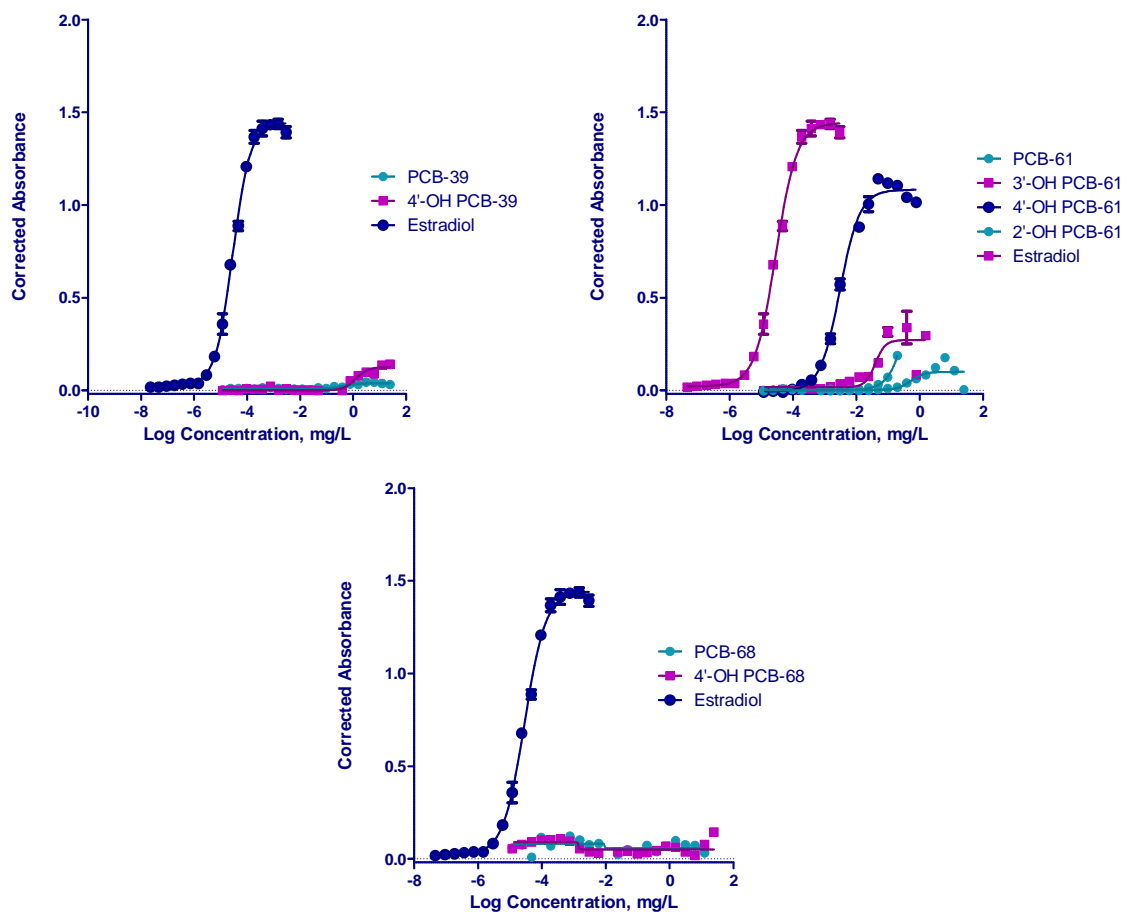


Figure 10: The estrogenic response of PCB-39, PCB-61, PCB-68, and their hydroxylated metabolites as measured using the YES assay. Experiments were done in triplicates and error bars shows the standard deviation of triplicate measurements.

Some parent PCBs and their mono-hydroxylated metabolites (PCB -39 and -68) showed no observable estrogenicity. For 2'-OH PCB-36, only estrogenicity at the lowest concentration (0.39 mg L^{-1}) could be recorded, because the higher concentrations (25 mg L^{-1} to 0.78 mg L^{-1}), resulted in highly toxic effect and no yeast could grow. Similarly it was observed in Microtox[®] assay that 2'-OH PCB-36 was highly toxic and had to be diluted 20 times in diluent to measure an observable toxicity.

Estrogenicity of PCBs is commonly attributed to the formation of hydroxylated metabolites (Korach *et al.*, 1988). Hydroxylated PCBs produced estrogenic effects at very low concentrations in rainbow trout hepatocytes (Andersson *et al.*, 1996; Blom *et al.*, 1998; Andersson *et al.*, 1999). The Results that we obtained using the YES assay showed that for tri- and tetrachlorinated PCB metabolites, there was an increase of the estrogenicity of mono-hydroxylated PCBs by an order of magnitude following the sequence *ortho-*, *meta-*, *para-* substitution (refer Appendix A for chemical structure of the different compounds). A similar trend was also observed while testing the toxicity of PCBs and their mono-hydroxylated metabolites using the Microtox[®] assay. Also, it was observed that most *para-* substituted mono-hydroxylated PCBs were highly toxic ; they had to be diluted 10 times (PCB-2, -12, -30, -36 and -39) or 20 times (PCB-8 and -9) more in diluents (2% NaCl) to obtain a measureable toxicity using Microtox[®] assay . Interestingly, it was observed that *para-* substituted mono-hydroxylated PCBs in YES assay showed higher estrogenicity.

Relative potencies (RP_i) were calculated for PCBs and their corresponding mono-hydroxylated metabolites. RP_i s are widely used to characterize and compare the potency of a wide variety of chemicals analyzed using *in vitro* bioassays. RP_i s are generally

calculated as a simple ratio: the EC₅₀ of a well characterized standard divided by the EC₅₀ of a target compound. However such estimates are valid only when the dose-response curves for the sample and standard are parallel and exhibit the same maximum achievable response (efficacy) (Villeneuve *et al.*, 2000). For PCB-9, -30, and -61 and their corresponding mono-hydroxylated metabolites, the same maximum achievable response was obtained as with the estradiol standard and their RP_i were calculated (Table 4).

Results obtained from PCB-39 and PCB-68, are in agreement with the statement that *para*-hydroxylated PCBs chlorinated at *ortho*-position on the same ring are weak binders to estrogen receptor but often exhibit anti-estrogenic activity (Korach *et al.*, 1988 ; Kramer *et al.*, 1997).

Table 4: Relative Potencies (RP_i) for selected PCBs and their corresponding hydroxylated metabolites

CHEMICAL NAME	RELATIVE POTENCY(RP _i)
3-Chlorobiphenyl	3.63 x 10 ^{-6*}
4-Hydroxy-3-Chlorobiphenyl	2.201 x 10 ^{-5*}
4-Chlorobiphenyl	4.173 x 10 ⁻⁷
2'-Hydroxy-4-Chlorobiphenyl	3.29 x 10 ⁻⁵
3'-Hydroxy-4-Chlorobiphenyl	7.35 x 10 ⁻⁸
4'-Hydroxy-4-Chlorobiphenyl	5.41 x 10 ⁻⁶
2,4'-Dichlorobiphenyl	6.87 x 10 ^{-3*}
4'-Hydroxy-2,4'-Dichlorobiphenyl	.104*
2,5-Dichlorobiphenyl	1.035 x 10 ⁻⁵
2'-Hydroxy-2,5-Dichlorobiphenyl	6.35 x 10 ⁻⁵
3'-Hydroxy-2,5-Dichlorobiphenyl	4.52 x 10 ⁻⁴
4'-Hydroxy-2,5-Dichlorobiphenyl	6.99 x 10 ⁻³
3,4-Dichlorobiphenyl	7.11 x 10 ⁻⁸
4'-Hydroxy-3,4-Dichlorobiphenyl	2.15 x 10 ⁻³
2,4,6-Trichlorobiphenyl	8.656 x 10 ⁻⁵
2'-Hydroxy-2,4,6-Trichlorobiphenyl	3.29 x 10 ⁻⁸
3'-Hydroxy-2,4,6-Trichlorobiphenyl	5.18 x 10 ⁻⁵
4'-Hydroxy-2,4,6-Trichlorobiphenyl	.0387
3,3',4-Trichlorobiphenyl	4.37 x 10 ^{-5*}
2'-Hydroxy-3,3',4-Trichlorobiphenyl	1.545 x 10 ^{-4*}
4'-Hydroxy-3,3',4-Trichlorobiphenyl	9.87 x 10 ^{-5*}
3,3',5-Trichlorobiphenyl	4.656 x 10 ^{-5*}
2'-Hydroxy-3,3',5-Trichlorobiphenyl	4.37 x 10 ^{-5*}
3,4',5-Trichlorobiphenyl	6.6 x 10 ^{-5*}
4'-Hydroxy-3,4',5-Trichlorobiphenyl	2.27 x 10 ^{-5*}
2,3,4,5-Tetrachlorobiphenyl	5.47 x 10 ⁻⁵
2'-Hydroxy-2,3,4,5-tetrachlorobiphenyl	7.26 x 10 ⁻⁴
3'-Hydroxy-2,3,4,5-tetrachlorobiphenyl	9.48 x 10 ⁻³
4'-Hydroxy-2,3,4,5-tetrachlorobiphenyl	9.48 x 10 ⁻⁷
2,3',4,5'-Tetrachlorobiphenyl	3.04 x 10 ^{-3*}
4'-Hydroxy-2,3',4,5'-tetrachlorobiphenyl	.020*

Values in asterisk (*) indicate that full dose-response curve for these PCBs and their corresponding hydroxylated metabolites was not obtained because of high toxicity of these compounds

Growth Analysis using *Burkholderia xenovorans* LB400

Inhibition of growth of a well known PCB-degrading bacterium *Burkholderia xenovorans* LB400 in the presence of PCBs and their hydroxylated metabolites was determined. It was found that growth rate of *B. xenovorans* LB400 decreased with exposure to hydroxylated metabolites. Figures 11 to 14 show the growth of *B. xenovorans* in presence of PCBs and their corresponding hydroxylated metabolites that were recorded over 24 hours. A higher absorbance value indicates higher concentration of the bacteria which in turn indicates a higher growth rate of the bacteria. Results obtained showed that PCBs themselves did not exhibit any recordable toxicity, which was previously observed using the Microtox[®] assay. Inhibition of the growth of *B. xenovorans* strain LB400 was primarily due to the presence of hydroxylated metabolites. Initial high absorbance at time zero was also observed with some of the parent PCBs because of precipitation of insoluble PCBs when added to growth medium. In case of PCB-39, the 4'-OH metabolite and the parent compound had similar high absorbance, suggesting that 4'-OH PCB-39 is not a growth inhibitor of *B. xenovorans*. This behavior may be related to the chemical structure of 4'-OH PCB-39 (Refer to Appendix A), as hydroxyl group is attached to *para*- position and chlorine substitution is on *ortho*-position on the same ring. Among all the PCBs (except PCB-39) 4'-OH metabolite showed very low absorbance and inhibited the growth of *B. xenovorans* (Figure 11-14), suggesting that they are highly toxic. Growth inhibition as functional response is an ecologically relevant parameter for the determination of environmental risks. Since toxicants affect all metabolic aspects of cell growth (Pillet *et al.*, 1991), growth inhibition is the metabolic test that always responds negatively to toxicants and that does not

provide misleading indications. Inhibition in growth of *B. xenovorans* by chlorobenzoates has also been observed. 2-chlorobenzoates (2-CBA) and 4-chlorobenzoates (4-CBA) are produced by the biphenyl pathway. Studies showed that, the presence of 2-CBA and 4-CBA inhibited the growth of bacterial strain *B. xenovorans* strains on glucose (Martinez *et al.*, 2007). These compounds can act as energy “uncouplers”, affecting negatively the growth of the cells. Results from growth analysis experiments in present study confirmed that PCBs themselves may not be very toxic but their mono-hydroxylated metabolites are highly toxic and inhibit the growth rate of bacteria.

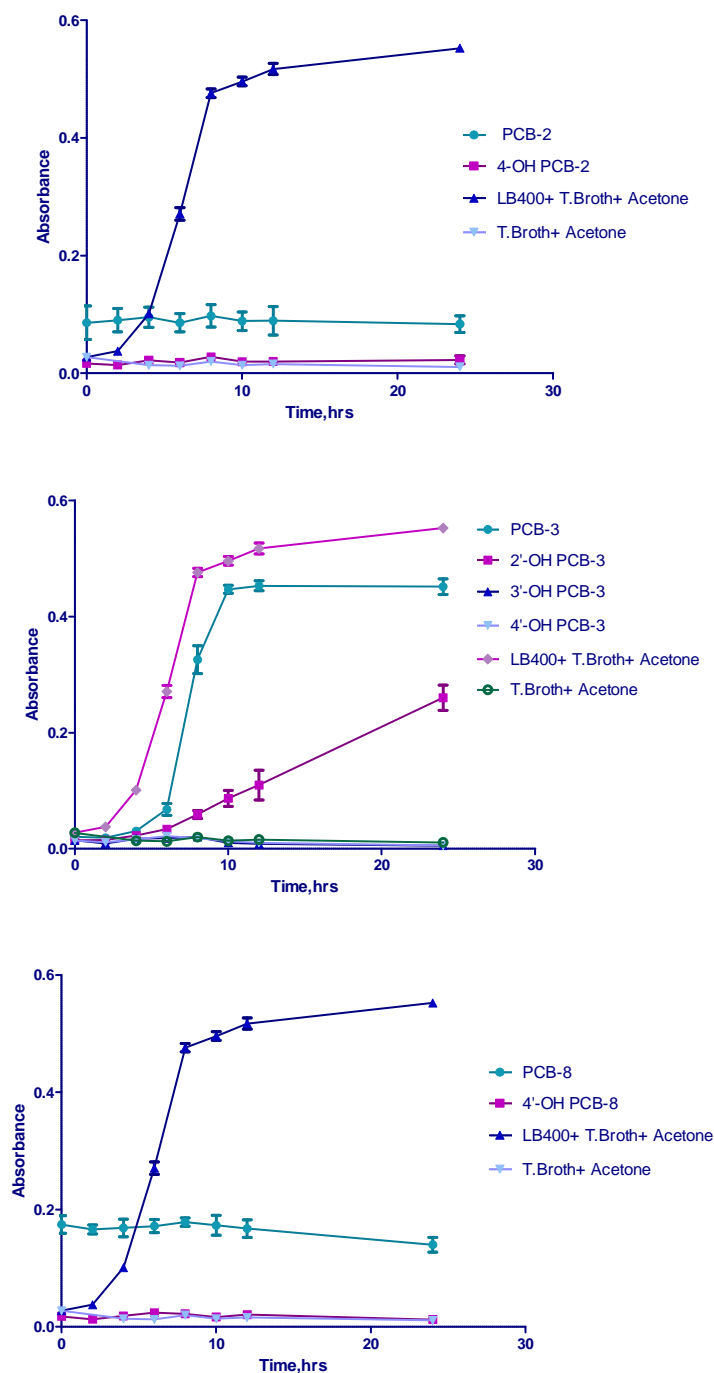


Figure 11: Effect on the growth rate of *Burkholderia xenovorans* in presence of PCB-2, PCB-3, PCB-8 and its hydroxylated metabolites. Experiments were performed in triplicates and error bars shows the standard deviation of triplicate measurements. Absorbance was measured at single wave-length of 600 nm.

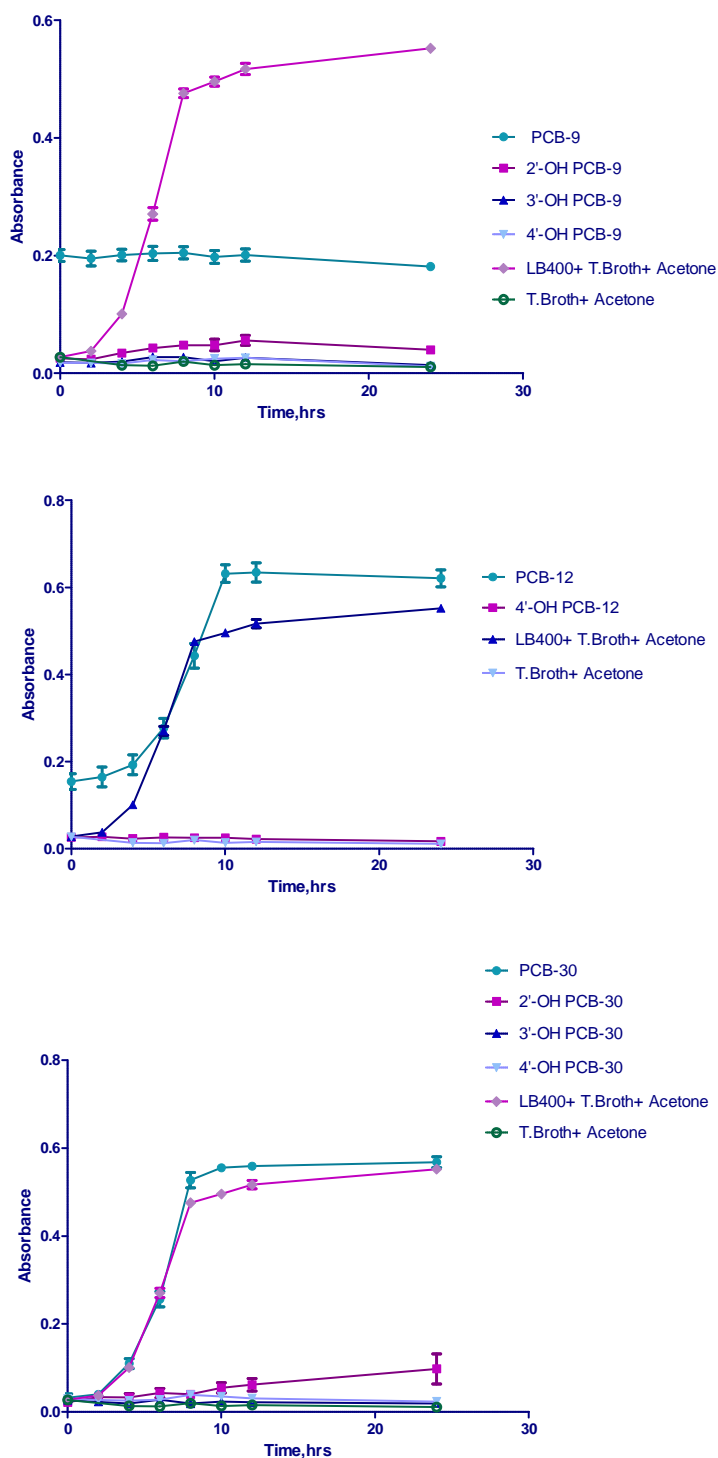


Figure 12: Effect on the growth rate of *Burkholderia xenovorans* in presence of PCB-9, PCB-12, PCB-30 and its hydroxylated metabolites. Experiments were performed in triplicates and error bars shows the standard deviation of triplicate measurements. Absorbance was measured at single wave-length of 600 nm

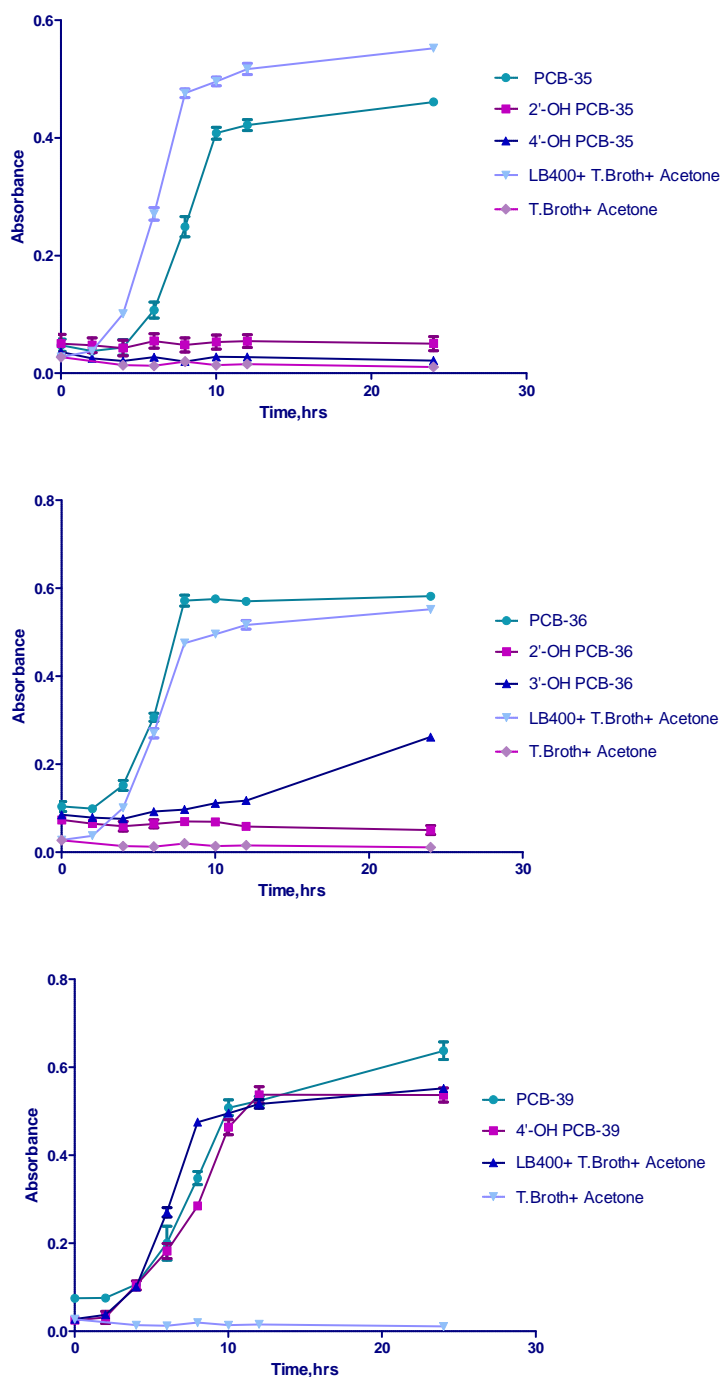


Figure 13: Effect on the growth rate of *Burkholderia xenovorans* in presence of PCB-35, PCB-36, PCB-39 and its hydroxylated metabolites. Experiments were performed in triplicates and error bars shows the standard deviation of triplicate measurements. Absorbance was measured at single wave-length of 600 nm.

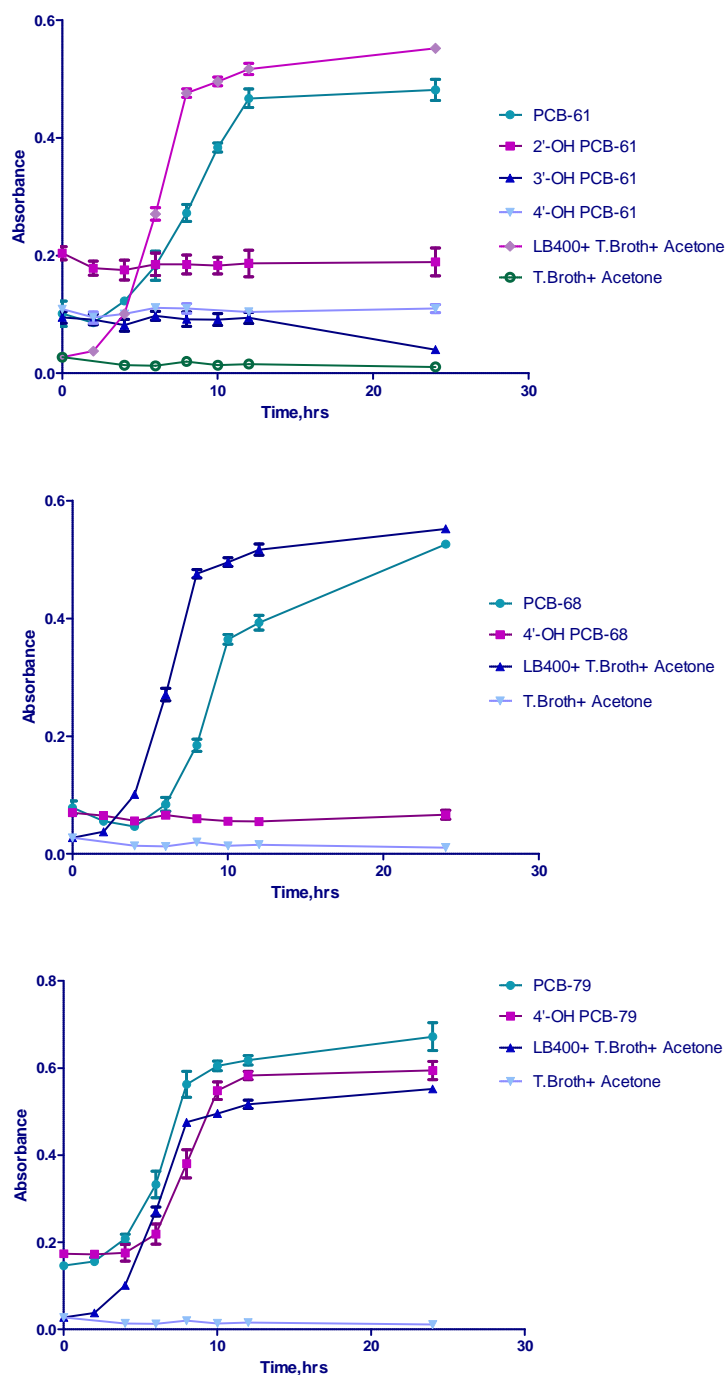


Figure 14: Effect on the growth rate of *Burkholderia xenovorans* in presence of PCB-61, PCB-68, PCB-79 and its hydroxylated metabolites. Experiments were performed in triplicates and error bars shows the standard deviation of triplicate measurements. Absorbance was measured at single wave-length of 600 nm

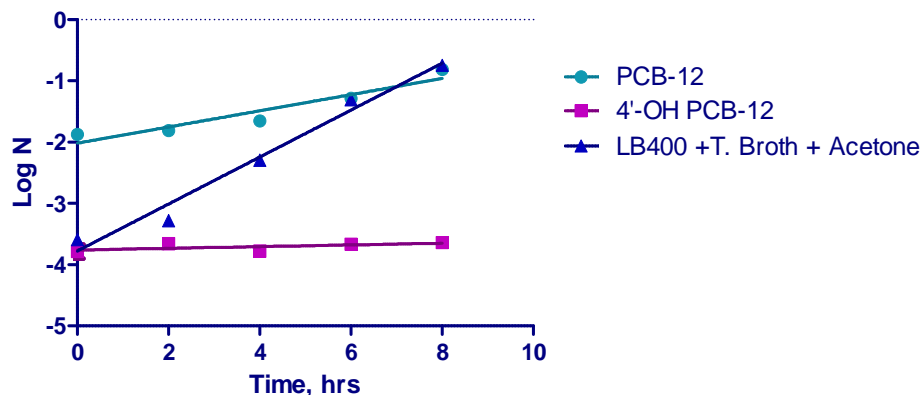


Figure 15: Statistical analysis of Growth data of *Burkholderia xenovorans* in presence of PCB-12 and its hydroxylated metabolite. Comparison of slopes yields p-value of 0.00001 and hence null hypothesis is rejected.

It can be seen that the bacterial growth as recorded by the absorbance at 600nm was generally higher than in the presence of the corresponding hydroxylated metabolites (Figures 11-14). Results obtained from the growth-analysis of a range of PCBs and their mono-hydroxylated metabolites were analyzed statistically in excel using bacterial growth model. Comparison of all the slopes yielded a p-value < 0.00001 , hence null hypothesis is rejected and the exponential growth rates are different. Figure 15 is a sample (exponential growth rate) of PCB-12 and 4'-OH PCB-12 showing slopes are significantly different and hence PCBs and their mono-hydroxylated metabolites influence growth rate of bacteria *B. xenovorans* LB400 in a different manner.

CHAPTER 5

CONCLUSIONS

PCBs in the environment undergo transformations to produce a variety of products, including hydroxylated metabolites. The objective of the present study was to compare the effect of PCBs and their mono-hydroxylated metabolites on the growth rate of bacteria and to compare the toxicity and estrogenicity of PCBs and their mono-hydroxylated metabolites. Our central hypothesis was that mono-hydroxylated metabolites of PCBs exhibit higher toxicity than their corresponding parent PCBs. Results from our three specific aims provide converging evidence that confirm our hypothesis. It is noteworthy that the major focus of the present research was to study the effect of hydroxylated metabolites on bacteria. Therefore, testing the estrogenicity study on PCBs and its hydroxylated metabolites was on the margin of the central scope of our work. We took the advantage of existing technical expertise in our department to conduct these estrogenicity assays.

Besides the observation that mono-hydroxylated metabolites of PCBs exhibit higher toxicity than their corresponding parent compounds, our results showed a recognizable pattern of toxicity. Hydroxylated metabolites from tri- and tetrachlorinated PCBs showed a constant trend: the toxicity increased by an order of magnitude following the sequence *ortho*-, *meta*-, and *para*- substitution of the hydroxyl group. Because structure-toxicity relationships are usually very difficult to establish in toxicity studies, this would call for further investigation of the origin of this observation.

In the past, the main focus on toxicity studies was the parent PCB compounds, as they were considered as highly toxic. New trends in risk assessment studies lead to focus on metabolites of xenobiotic compounds. The present study has demonstrated that mono-hydroxylated metabolites are highly toxic in comparison to their parent PCBs. The increase in toxicity of hydroxylated metabolites may be partially related to the increase in solubility. As PCBs are hydrophobic, they cannot be taken up by the cells whereas hydroxylated metabolites are more soluble and are more bioavailable. Similarly, the hydroxylated PCB metabolites are expected to be more mobile and susceptible to be transported over long distances, contaminating soils, and drinking water. Values of $\log K_{ow}$ (octanol-water partition coefficient) are very important in predicting pollutant mobility. Hydroxylated metabolites have lower $\log K_{ow}$ values than parent PCBs, suggesting that they are more soluble and therefore bioavailable. Various organisms, such as plants, crustaceans, algae, and fungi, can transform PCBs into mono-hydroxylated metabolites, which are more soluble and can be more toxic than the parent PCBs. The present study showed that hydroxylated metabolites of PCBs are highly toxic for bacteria that are the major agents responsible for PCB biodegradation in soils and sediments. Our findings about the toxicity of mono-hydroxylated PCB metabolites suggest that these compounds may inhibit bacterial growth of bacteria which in turn will affect the biodegradation efficiency. Besides parent PCB compounds, bioassays and other detection techniques should therefore focus on hydroxylated PCBs and other PCB metabolites.

Also, techniques used for the bioremediation of PCBs may not be efficient for removal of hydroxylated PCBs. Biodegradation performance could be affected by the toxicity and/or chemical and physical properties of hydroxylated metabolites. The

generation of toxic metabolites may be particularly significant if the PCB compounds are the sole available carbon and energy source. Accumulation of hydroxylated metabolites of PCBs in soil may affect the viability and therefore bacterial performance in the degradation of PCB congeners. Furthermore, the toxicity of hydroxylated metabolites generated during the oxidation of PCBs may partly explain the recalcitrance of these pollutants to biodegradation.

Another environmental consequence of the toxicity of hydroxylated metabolites produced as a result of PCB transformation is their accumulation by adsorption on the soil surface and the potential for many edible and agricultural plants growing on contaminated soil to adsorb PCB hydroxylated metabolites. There is therefore risk for exposure of these toxic hydroxylated metabolites to human through consumption of these edible plants.

Cloning of bacterial enzymes in plants can help plants to degrade various xenobiotic pollutants. The potential accumulation in plants of PCB hydroxylated metabolites raises the question of further metabolism inside plant tissues. Improving metabolic biodegradation capabilities of plants and microorganisms by genetic engineering would help overcome a major shortcoming of environmental biotechnology, i.e., the risk of production of toxic metabolites that could further contaminate the food chain.

Future Perspectives

Based on our results on the toxicity of PCB hydroxylated metabolites, there is a need to look for the presence of these metabolites in the environment. In addition, the

results obtained from the present study showed that hydroxylated metabolites affect the growth of bacteria and are highly toxic although the YES assay showed that the same compounds are highly estrogenic in comparison to the parent PCBs. We know that bacteria lack hormonal system. So the apparent correlation between bacterial toxicity and estrogenicity of hydroxylated metabolites is unclear. Also, the mechanism of toxicity of hydroxylated metabolites is not known. There is a need to understand this correlation between estrogenicity and bacterial toxicity. Also, suitable methods for the biodegradation of hydroxylated metabolites by bacteria should be developed. Microarrays technology could be used to test the effect of hydroxylated metabolites of PCBs on PCB degraders. Similarly, the mechanisms of toxicity of hydroxylated PCBs should be studied more extensively in order to be able to optimize efficient methods for the biodegradation of these toxic metabolites.

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GLOSSARY

BIOACCMULATION: Accumulation of substances, such as pesticides or other organic chemical in an organism. Bioaccumulation occurs when an organism absorbs a toxic substance at a rate greater than that at which the substance is lost.

BIOTRANSFORMATION: Biotransformation is the chemical modification made by an organism on a chemical compound.

CO-METABOLISM: Co-metabolism is defined as the simultaneous degradation of two compounds, in which the degradation of the second compound (the secondary substrate) depends on the presence of the first compound (the primary substrate). This process is essential for the biodegradation of certain organic pollutants that cannot be used as sole carbon or energy source.

ENDOCRINE DISRUPTORS (EDC): EDCs are exogenous substances that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of normal cell metabolism, reproduction, development and behavior.

EPOXIDATION: Any reaction that converts a compound (especially an alkene) into an epoxide.

LIPOPROTEIN: Lipoprotein is a biochemical assembly that contains both proteins and lipids whose function is to transport water-insoluble lipids in the water-based bloodstream.

PHYTOPLANKTON: Phytoplanktons are tiny, free-floating, photosynthetic organisms in aquatic systems. They contain chlorophyll needed for photosynthesis.

1, 2 SHIFT: 1, 2 shift is a chemical rearrangement where a hydrogen atom on an aromatic ring undergoes an intermolecular migration primarily during a hydroxylation reaction.

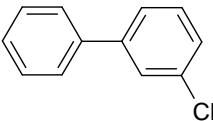
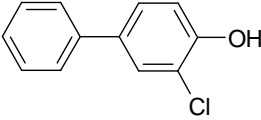
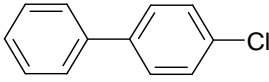
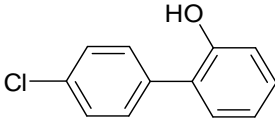
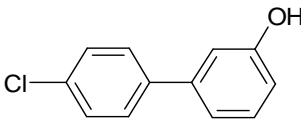
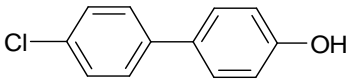
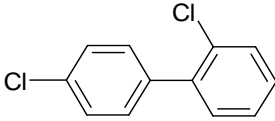
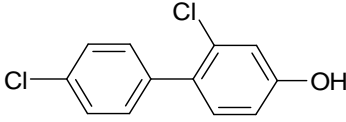
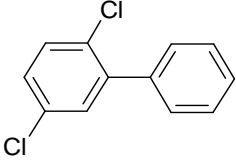
XENOESTROGENS: Xenoestrogens are novel, industrially made compounds that have estrogenic effects and differ from ancient naturally occurring estrogenic substances produced by living organisms. These compounds act as estrogen mimics.

DOSE-RESPONSE RELATIONSHIP: The dose-response relationship, or exposure-response relationship, describes the change in effect of an organism caused by differing levels of exposure or dose to a standard stressor after a certain exposure time. This may apply to individuals (a small amount has no observable effect, a large amount is fatal), or to populations (how many people or organisms are affected at different levels of exposure).

APPENDIX

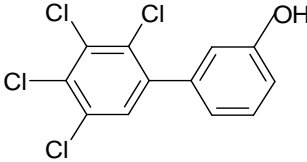
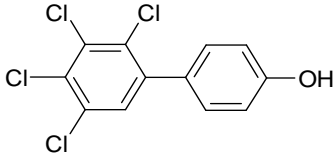
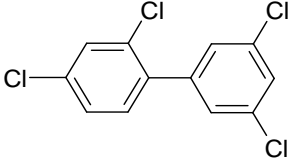
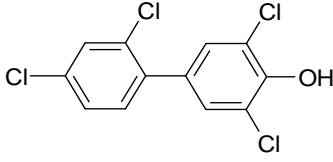
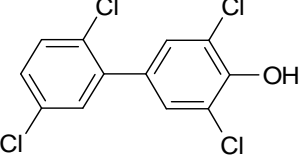
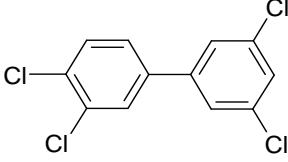
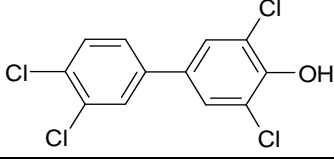
APPENDIX A
CHEMICAL STRUCTURE OF PCBs AND ITS METABOLITES

Table A-1: Chemical structure and source of PCBs and its Metabolites used in this study

CHEMICAL NAME	CHEMICAL FORMULA	SOURCE
3-chlorobiphenyl		Iowa University
4-Hydroxy-3-Chlorobiphenyl		Iowa University
4-chlorobiphenyl		Iowa University
2'-Hydroxy-4-Chlorobiphenyl		Iowa University
3'-Hydroxy-4-Chlorobiphenyl		Iowa University
4'-Hydroxy-4-Chlorobiphenyl		Iowa University
2,4'-Dichlorobiphenyl		Accustandard
4'-Hydroxy-2,4'-Dichlorobiphenyl		Iowa University
2,5-dichlorobiphenyl		Accustandard

CHEMICAL NAME	CHEMICAL FORMULA	SOURCE
2'-Hydroxy-2,5-dichlorobiphenyl		Accustandard
3'-Hydroxy-2,5-dichlorobiphenyl		Accustandard
4'-Hydroxy-2,5-dichlorobiphenyl		Accustandard
3,4-Dichlorobiphenyl		Iowa University
4'-Hydroxy-3,4-Dichlorobiphenyl		Iowa University
2,4,6-Trichlorobiphenyl		Accustandard
2'-Hydroxy-2,4,6-Trichlorobiphenyl		Accustandard
3'-Hydroxy-2,4,6-Trichlorobiphenyl		Accustandard
4'-Hydroxy-2,4,6-Trichlorobiphenyl		Accustandard
3,3',4-Trichlorobiphenyl		Accustandard

CHEMICAL NAME	CHEMICAL FORMULA	SOURCE
2'-Hydroxy-3,3',4-Trichlorobiphenyl		Iowa University
4'-Hydroxy-3,3',4-Trichlorobiphenyl		Iowa University
3,3',5-Trichlorobiphenyl		Iowa University
2'-Hydroxy-3,3',5-Trichlorobiphenyl		Iowa University
4'-Hydroxy-3,3',5-Trichlorobiphenyl		Iowa University
3,4',5-Trichlorobiphenyl		Iowa University
4'-Hydroxy-3,4',5-Trichlorobiphenyl		Iowa University
2,3,4,5-tetrachlorobiphenyl		Accustandard
2'-Hydroxy-2,3,4,5-tetrachlorobiphenyl		Accustandard

CHEMICAL NAME	CHEMICAL FORMULA	SOURCE
3'-Hydroxy-2,3,4,5-tetrachlorobiphenyl		Accustandard
4'-Hydroxy-2,3,4,5-tetrachlorobiphenyl		Accustandard
2,3',4,5'-tetrachlorobiphenyl		Accustandard
4'-Hydroxy-2,3',4,5'-tetrachlorobiphenyl		Iowa University
4'-Hydroxy-2,3',4,5'-tetrachlorobiphenyl		Accustandard
3,3',4,5'-Tetrachlorobiphenyl		Iowa University
4'-Hydroxy-3,3',4,5'-Tetrachlorobiphenyl		Iowa University