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Microbial Transformations of Tetrabromobisphenol A and Its Metabolites, and Their Impact on Toxicity to the Developing Zebrafish (*Danio rerio*) Embryo

by

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ABSTRACT OF THE DISSERTATION

Microbial Transformations of Tetrabromobisphenol A and Its Metabolites, and Their Impact on Toxicity to the Developing Zebrafish (*Danio rerio*) Embryo

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Anthropogenic chemicals are of concern because they are resistant to biodegradation, can accumulate in aquatic environments and sediments, and biomagnify in the food chain. One such compound, tetrabromobisphenol A (TBBPA) is the most widely used brominated flame retardant worldwide. TBBPA contamination has been detected in dust, sediments, and aquatic environments as well as in human serum, breast milk and other tissues of aquatic and terrestrial animals. Microorganisms utilize these chemicals by many mechanisms for degradation or transformation resulting in metabolites with different environmental fates. Microorganisms in the environment can transform TBBPA either by anaerobic dehalogenation to bisphenol A (BPA) or aerobic O-methylation to TBBPA dimethyl ether (TBBPA DME). *Mycobacterium* spp. were able to O-methylate TBBPA at a faster rate than BPA. Additionally, these data demonstrate that TBBPA O-methylation is a ubiquitous reaction in the environment. However, O-methylating organisms comprise only a minor portion of the total heterotrophic population. To determine whether microbial metabolism alters the toxicity of TBBPA,

zebrafish embryos were exposed to TBBPA and its metabolites. These data show that BPA and TBBPA DME exhibit lower potency that TBBPA, demonstrating that microbial metabolism results in products with reduced toxicity. In addition, while all three caused edema and hemorrhage, only TBBPA caused decreased heart rate, edema of the trunk, and tail malformations. Matrix metalloproteinase (MMP) expression was examined due to the role of these enzymes in the remodeling of the extracellular matrix during tissue morphogenesis, wound healing and cell migration. The trunk and tail phenotypes seen after TBBPA exposure could in part be due to alteration of proper MMP expression/activity. Unlike the O-methylation of TBBPA, transformation of BPA to BPA monomethyl and BPA dimethyl ether results in increased toxicity to the developing zebrafish embryo causing increased mortality at 5 and 28 days post fertilization and lower LC_{50} values than for TBBPA DME. Taken together, the data presented in this thesis indicate that microbial metabolism of brominated flame retardants results in compounds with differing toxicity. Further, these data illustrate a new mechanism for microbial transformation of BPA, producing metabolites warranting further study to understand their prevalence in the environment.

Dedication

To my mom and Ryan, you inspired me to find what I truly love to do. I carry your memory with me always…

To my family, for always being there…

I love you.

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1.0. General Introduction

Microorganisms display great versatility and adapt to many types of environmental conditions from extreme temperatures, pH levels and salt concentrations. Additionally, microorganisms are able to utilize a wide variety of substrates for use as carbon sources, electron donors and acceptors. This diversity is important and is key since microorganisms play a vital role in environments heavily contaminated with anthropogenic compounds.

Anthropogenic compounds number in the thousands and are used in every day life in every industry including the petrochemical industry, manufacturing of plastics, in commercial and household products, and dry cleaners, etc. These compounds include such chemicals as petroleum, aromatic hydrocarbons, methanol, chlorofluorocarbons (CFCs), tetra- and trichloroethylene, pesticides, polychlorinated biphenyls (PCBs), and flame-retardants (Swoboda-Coldberg, 1995). Several of these chemicals, such as PCBs and CFCs, have been banned due to deleterious effects on the environment and in exposed organisms. Anthropogenic chemicals can cause developmental, health and reproductive effects in humans, mammals and aquatic organisms.

Certain anthropogenic chemicals are of concern because they tend to be resistant to degradation, can accumulate in aquatic environments and sediments, and biomagnify up the food chain. Microorganisms are able to metabolize these chemicals in both aerobic and anaerobic environments, and use a variety of mechanisms for degradation or transformation. There are different characteristics important when considering the fate of such compounds in the environment including the physical and chemical characteristics of the compound, temperature, water availability, light, and oxygen levels (Swoboda-

Coldberg, 1995). The major environmental sinks for contaminants are the atmosphere, soils, sediments, oceans and biota. Additionally, an understanding of their fate and transport is essential for determining the likelihood as to where they will end up. Finally, the lipophilicity of these compounds affects the bioavailability for biodegradation and also for the probability of accumulation in fatty tissues and biomagnifications through the food chain (Swoboda-Coldberg, 1995).

Organohalides are an important class of anthropogenic compounds, and include compounds that contain halogen atoms, fluorine, chlorine and bromine. One of the most notable classes of organohalides are the PCBs, which are known for their environmental persistence, recalcitrance and toxicity to a number of organisms (Swoboda-Coldberg, 1995). In addition to the PCBs, chlorinated dioxins, which are produced as a byproduct of incineration, are another group of toxic and recalcitrant compounds, 2,3,78-tetrachlorodibenzo-*p*-dioxin, being the most notable. Further, there are also chlorinated and brominated phenolic compounds used in many industries, including the manufacture of plastics, in the paper industry and also for flame-retardants.

One group of organohalides, the brominated flame-retardants (BFR), will be the subject of this thesis, specifically tetrabromobisphenol A (TBBPA). BFRs are used in a wide range of consumer products and have been shown to exhibit endocrine disrupting potential, however, information regarding their toxicity during development is lacking. Additionally, in the environment, microorganisms can transform them by either reductive dehalogenation or O-methylation, resulting in novel compounds with unknown toxicological impact. Therefore, these studies will examine one such transformation, O-

methylation, of TBBPA and its anaerobic dehalogenation metabolite, bisphenol A (BPA) and the resulting toxicological effects on the developing zebrafish embryo.

1.1. Brominated Flame-Retardants

Flame-retardants are compounds used in commercial and household items to slow combustion in the event of a fire. Flame-retardants are classified into four groups: inorganic, organohalogen (chlorinated and brominated), organophosphorus and nitrogen-based flame-retardants (Alaee *et al.*, 2003; Segev *et al.*, 2009). The organohalogen flame-retardants are used because they are able to trap free radicals that are formed during the combustion process. The chlorinated and brominated compounds release the halogens and decompose at a temperature favorable to slowing combustion. Fluorinated compounds are overly stable and decompose and release their halogens at temperatures much higher than the burn temperature. Iodinated compounds, on the other hand, are not stable and therefore not used (Alaee *et al.*, 2003). Brominated flame-retardants are used more frequently than chlorinated forms due to their high efficacy and a decreased volatility at high temperatures (Segev *et al.*, 2009).

Brominated flame retardants (BFR) are composed of three major classes of compounds; aliphatic, cycloaliphatic, and aromatic, which are used to increase fire resistance by slowing the burn rate in a variety of consumer products, including textiles, computer equipment, furniture, plastics and wood (Alaee *et al.*, 2003; Segev *et al.*, 2009). BFRs are separated into additional groups based upon the incorporation of the compound into polymers, additive or reactive (Fig. 1.1). The additive BFRs include, hexabromocyclododecane (HBCD) and polybrominated diphenyl ethers (PBDE), which

are blended with the polymers and are more likely to leach out of the products into the environment. The reactive BFRs, such as tetrabromobisphenol A (TBBPA) are chemically bonded onto the polymer and are less likely to leach from products (Alaee *et al.*, 2003; Segev *et al.*, 2009). The bromine substituent on these compounds increases their lipophilicity and decreases water solubility, affecting the likelihood for persistence and bioaccumulation in the environment (Segev *et al.*, 2009).

Polybrominated biphenyls (PBB) were used as flame-retardants in the 1970's, but banned by the United States in the late 1970's and by Europe in 2000 (de Wit, 2006; Hakk and Letcher, 2003). Despite decreased production and a ban on use since the late 1970s, PBBs are still detected in serum and tissue samples (Hakk and Letcher, 2003; Terrell *et al.*, 2008). Exposure to PBBs may result in abdominal cramps, nausea, vomiting, and they are possible endocrine disruptors (Thomas *et al.*, 2001). Studies on the exposure/toxicity of PBBs are limited, and most of the knowledge of PBB exposure comes from a widespread contamination of cattle products in Michigan during 1973-74, where over 85% of the Michigan population was exposed to contaminated milk from cattle fed with food contaminated with PBB (Fries, 1985). There were no acute health effects, but an increased risk of digestive tract cancers and lymphoma has been suggested (Hoque *et al.*, 1998).

HBCD is an additive BFR that is used primarily in expanded and extruded polystyrene foams, used in building and home insulation and also in textile back coating in furniture (de Wit, 2006). Worldwide demand in 2003 was approximately 22,000 metric tons, with Europe being the dominant area for use (de Wit *et al.*, 2009). HBCD has been detected in air, soil and sediment samples (Abdallah *et al.*, 2008; Covaci *et al.*, 2007;

Morf *et al.*, 2005). Additionally, HBCD has been detected in breast milk and adipose tissue samples (Kuiper *et al.*, 2007a; Lam *et al.*, 2009; Thomsen *et al.*, 2010; van Leeuwen and de Boer, 2008). Research into the toxicological effects of HBCD are limited, but HBCD has been detected in many organisms and tissues in rats and is excreted in urine and feces (Germer *et al.*, 2006; Hakk and Letcher, 2003).

PBDEs are a class of BFRs that were in wide use and distribution, but due to toxicity concerns, several congeners, penta- and octaBDE, have been banned by the European Union (deWit 2009). There are 209 possible congeners, differing in the position of the bromine atoms. PBDEs are typically used as a mixture of congeners in polyurethane foam, hard plastics, textiles, electronic equipment and rubber casings for wires. The most commonly used congeners were the tetra-, penta-, hexa-, hepta-, octa- and decaBDEs (deWit 2009). PBDEs are endocrine disruptors, and can cause neurotoxic and reproductive effects in rodents (deWit 2009). PBDEs have been detected in air, serum, breast milk and in many organisms including humans, birds, dolphins, and other aquatic organisms (Gao *et al.*, 2009; Teuten *et al.*, 2005). PBDEs can be dehalogentated by abiotic processes, such as photochemical decomposition, or microbially mediated processes that have been previously characterized (Bastos *et al.*, 2008; He, 2006; Robrock *et al.*, 2009; Tokarz *et al.*, 2008). Additionally, hydroxylated and methoxylated PBDEs have been recently identified with the potential to bioaccumulate in the environment (Teuten *et al.*, 2006; Valters *et al.*, 2005). Recently, several studies have published PBDE occurrence levels in environmental samples from China. The use of PBDEs, and flame-retardants in China has been increasing due to rapid industrialization and increased manufacturing in many areas, particularly the Pearl River Delta (Table 1.1)

(Lam *et al.*, 2009; Shi *et al.*, 2009a; Zhang *et al.*, 2009). The increased BFR demand has prompted increased monitoring of the population and environment for BFRs, particularly PBDE contamination and exposure, which has led to identifiable differences in the levels of PBDE in breast milk, which is higher in the southern area of China than in the north (Table 1.1) (Gao *et al.*, 2009; Lam *et al.*, 2009; Li *et al.*, 2008a; Luo *et al.*, 2009; Ma *et al.*, 2009; Shi *et al.*, 2009a; Zhu *et al.*, 2009b). These differences correspond to the areas in China utilizing higher levels of PBDE, which is an important finding, linking increased demand and exposure potential.

Tetrabromobisphenol A ([TBBPA, 4,4'-isopropylidenebis(2,6-dibromophenol)]) is the most widely used and distributed BFR worldwide (Hakk and Letcher, 2003). TBBPA is produced by bromination of bisphenol A in an organic solvent, such as methanol, for use as a reactive flame retardant in epoxy resins and printed circuit boards, but derivatives are also produced for use in paper and textile adhesives (Alaee *et al.*, 2003). TBBPA can also be used as an additive BFR in conjunction with antimony oxide and as an alternative to octabromodiphenyl ether (Octa-BDE) in mixtures (Covaci *et al.*, 2009). As an additive BFR, TBBPA is commonly used in television and casings, printer components, fax machines, photocopiers, coffee makers and plugs/sockets (Covaci *et al.*, 2009). These items are recycled or found in landfills, and TBBPA can leach out into the environment. The studies in this thesis are focused on TBBPA, specifically on its environmental transformation and toxicity, thus this chapter will cover TBBPA and its metabolites in greater detail.

1.2. Environmental and Microbial Degradation

1.2.1 Polybrominated Diphenyl Ethers

PBDE contamination in the environment is widely detected in soils, sediments, water, and also blood and tissues of humans and other animals (Jakobsson *et al.*, 2002; Kawashiro *et al.*, 2008; Li *et al.*, 2008a; Miller *et al.*, 2009; Tokarz *et al.*, 2008; Zhu and Hites, 2006). The main congener used as a BFR is decabromodiphenyl ether (decaBDE), with the decaBDE congener comprising 97-99% of the technical mixture (Gerecke *et al.*, 2005). PBDEs, especially decaBDE, are highly lipophilic ($LogK_{ow}$= 8.7) and not readily bioavailable (Tokarz *et al.*, 2008). Despite this, the most predominant forms of PBDEs in the environment are the tetra- and pentabrominated congeners, which are known to be more toxic than decaBDE (Tokarz *et al.*, 2008). The discrepancy between the congeners used in manufacturing and the congeners detected in different matrices suggests that microorganisms, in addition to other factors, could play a role in the dehalogenation of the PBDEs in the environment. There have been several studies demonstrating the reductive dehalogenation of decaBDE and octaBDE to lower brominated congeners, specifically detecting the presence of tetra- and pentaBDE in these cultures (Gerecke, 2005; Gerecke *et al.*, 2006; He, 2006; Tokarz *et al.*, 2008). Additionally, the potential for degradation of lower brominated congeners has been examined and complete anaerobic dehalogenation of 4,4'-diphenyl ether to diphenyl ether in a fixed film plug flow reactor has been shown (Rayne *et al.*, 2003). These studies with PBDE illustrate that the flux of contaminants in the environment may not be due to waste streams or contamination from site sources, but may be a result of

biotransformation by indigenous microorganisms and as such, the levels in the environment of the metabolites may be higher than that of the parent compound.

1.2.2 Tetrabromobisphenol A

The environmental persistence of TBBPA is due to its high lipophilicity (log K_{ow}=5.9), low volatility (7.0 x 10^{-11} atm-m^3/mol), low water solubility (4.16mg/l at 25 °C in H_2O) and recalcitrance to biodegradation (Hakk and Letcher, 2003; Haneke, 2002; Kuramochi *et al.*, 2008). The widespread use of TBBPA, and its detection in dust, sediments and in terrestrial and aquatic organisms has led to increased concerns regarding its effects on wildlife and humans (Johnson-Restrepo *et al.*, 2008). Environmental contamination of TBBPA is affected by many factors, specifically the pH of the sediments and soils. At a basic pH, TBBPA is more water-soluble which increases the potential for groundwater contamination and mobility of TBBPA, whereas at neutral pH, TBBPA is not soluble and its mobility in sediments is minimized (Segev *et al.*, 2009). A recent review by Covaci *et al* (2009), highlights the best extraction and analytical methods for identifying TBBPA in water, air, soil/ sediment/ sludge and biological samples which should assist in future identification of TBBPA and TBBPA metabolites in environmental samples. This review also summarizes the literature reporting on the detection of TBBPA in various environmental matrices and provides a recent, comprehensive review of TBBPA (Covaci *et al.*, 2009).

TBBPA has been detected in a wide variety of matrices including dust, sediments, sewage, human serum, cord blood and breast milk samples as summarized in Table 1.1. Atmospheric testing for TBBPA has demonstrated concentrations ranging from 0.04-0.17

pg/m^{-3}, which suggests that TBBPA can be transported over great distances (Xie *et al.*, 2007). TBBPA has been detected in dust samples at 10 ng/g and 75 ng/g in both domestic and office settings, respectively (Geens *et al.*, 2009) and the exposure of individuals in electronics facilities has been well documented by detection of TBBPA in serum and on clothing samples (Mäkinen *et al.*, 2009). Recently, studies in Japan (Kawashiro *et al.*, 2008) and France (Cariou *et al.*, 2008) have detected TBBPA in maternal serum, cord blood, the umbilical cord and in breast milk at concentrations ranging from pg/g (pptr) to ng/g (ppb) levels (Johnson-Restrepo *et al.*, 2008).

TBBPA can be abiotically degraded in the atmosphere and in sediments. Photolytic degradation of TBBPA has been demonstrated but is affected by the pH of the matrix (Eriksson *et al.*, 2004). Specifically, if the pH is below the pK_a of TBBPA then the decomposition decreases as the pH decreases. However in matrices with pH higher than the pKa this degradation is independent of pH (Eriksson *et al.*, 2004). Additionally, reactive oxygen species and superoxide anions in the environment are also capable of degrading TBBPA (Han *et al.*, 2009). Photodecomposition of TBBPA results in halogenated phenols or quinones, which may also adversely affect the health of humans and natural terrestrial and aquatic ecosystems due to accumulation and exposure (Han *et al.*, 2009). Furthermore, thermal transformation of TBBPA has also been demonstrated, and incineration of TBBPA produces brominated dibenzo-*p*-dioxins and dibenzofurans (Lin *et al.*, 2009). Dehalogenation of TBBPA can occur under high temperature conditions with $NaNH_3$, ethyl acetate and zinc dust with NaOH and ammonium formate (Lin *et al.*, 2009).

In addition to abiotic transformation, TBBPA undergoes two different types of microbially mediated transformations (Fig. 1.1) by indigenous microorganisms in the environment: debromination to bisphenol A (BPA, 4,4'-isopropylidenediphenol) and O-methylation to TBBPA monomethyl ether (TBBPA MME) and TBBPA dimethyl ether [(TBBPA DME), 4,4'-isopropylidenebis(2,6-dibromo-1-methoxybenzene)] (Fig.1.1) (Arbeli *et al.*, 2006; George and Häggblom, 2008; Voordeckers *et al.*, 2002). TBBPA can be reductively sequentially dehalogenated to BPA under methanogenic and sulfate-reducing conditions found in anaerobic sediments, but the microorganisms responsible for this degradation are not yet known (Arbeli and Ronen, 2003; Arbeli *et al.*, 2006; Ravit *et al.*, 2005; Ronen and Abeliovich, 2000; Voordeckers *et al.*, 2002). Reductive dehalogenation of TBBPA has also been shown in sewage sludge in areas receiving leachate from contaminated landfills (Sellström and Jansson, 1995). Additionally, microbes in aerobic sediments have the ability to transform TBBPA by sequentially O-methylating the free hydroxyl groups to give the mono- and di-methyl ether forms (Allard *et al.*, 1987; George and Häggblom, 2008). The fate of TBBPA DME in aerobic soils is currently not known and no additional transformation or degradation of the TBBPA DME derivative has been reported. However the increased lipophilicity and decreased water solubility of this compound make it more likely to accumulate and persist in the environment. TBBPA DME has been detected in sediments near a plastic manufacturing plant in Sweden at 24 and 1500 ng/g dry weight compared to TBBPA levels at 34 and 270 ng/g (Sellström and Jansson, 1995).

1.2.2.1. Microbial O-methylation

O-methylation is a ubiquitous reaction, characterized by the transfer of a methyl group from S-adenosyl methionine (SAM) to an oxygen atom and has been identified in several bacteria, fungi and mammals. This transformation is thought to be an alternative pathway to degradation, however in many instances the function and purpose is not yet understood. O-methylation is catalyzed by O-methyltransferases, several of which have been characterized, but the majority of the bacterial enzymes mediating this reaction have not been studied in detail (Cho *et al.*, 2008; Coque *et al.*, 2003; Kim *et al.*, 2004; Lavid *et al.*, 2002; Wein *et al.*, 2002; Yang *et al.*, 2004).

The O-methylation reaction in aerobic soils and sediments has previously been studied with halogen-substituted (specifically chlorinated) phenols, thiophenols and chloroguaiacols. It has also been shown that *Mycobacterium sp*. strain CG-1 and CP-2 and *Mycobacterium fortuitum* strain CG-2 and other soil bacteria catalyze the O-methylation reaction (Allard *et al.*, 1987; Häggblom *et al.*, 1986; Häggblom *et al.*, 1989a; Häggblom *et al.*, 1989b; Häggblom *et al.*, 1988; Neilson *et al.*, 1988). O-methylation of TBBPA was previously demonstrated, but was only catalyzed by Gram-positive bacteria at a slower rate than 2,6-dibromophenol (Allard *et al.*, 1987; Valters *et al.*, 2005). Additionally, O-methylation of high molecular weigh PAHs has been detected as a step in the biodegradation pathway of these compounds (Hückelhoven *et al.*, 1997; Kim *et al.*, 2008; Kim *et al.*, 2004; Kweon *et al.*, 2007; Wunder *et al.*, 1997). O-methylated halogenated polybrominated diphenyl ethers (MeO-PBDE) similar in structure to industrial PBDEs, also exist naturally in the environment (Teuten *et al.*, 2006; Teuten *et al.*, 2005; Vetter *et al.*, 2007) and have been isolated from fish, whales,

dolphins, marine sponges, algae and acorn worms (Teuten *et al.*, 2005; Valters *et al.*, 2005). However, MeO-PBDE derivatives originating from PBDEs used as flame retardants have been identified (Valters *et al.*, 2005; Wan *et al.*, 2009).

O-methylated compounds are prevalent in the environment and can be produced by the transformation of chlorinated/brominated compounds in different matrices. Historically, O-methylation has been an unwanted reaction in wine production and in paper mills. In wine production, the presence of cork taint, which is caused by the O-methylation of chlorinated compounds, such as 2,4,6-trichlorophenol, on the cork by filamentous fungi in the cork can be a major problem (Alvarez-Rodriguez *et al.*, 2002; Coque *et al.*, 2003). This transformation results in an undesired off-taste of the wine and causes a negative impact on the economy of the wine industry. Additionally, O-methylation has been implicated in producing a musty taint in chickens, eggs, sake, and drinking water (Curtis *et al.*, 1974; Engel *et al.*, 1966; Miki *et al.*, 2005; Nyström *et al.*, 1992). In contrast, O-methyltransferases can also produce the pleasant, volatile phenolics in rose petals, and the aroma compounds of strawberries (Lavid *et al.*, 2002; Wein *et al.*, 2002). Paper mills use chlorine bleach products to lighten the pulp for papermaking (Neilson *et al.*, 1983). This creates low molecular weight chlorinated compounds in effluents, which can then be microbially transformed (Neilson *et al.*, 1994; Neilson *et al.*, 1983). Many microorganisms have been identified as capable of O-methylating halogenated phenols (chlorinated or brominated compounds) including; *Rhodococcus, Mycobacterium, Acinetobacter, Pseudomonas and Trichoderma species* (Allard *et al.*, 1987; Alvarez-Rodriguez *et al.*, 2002; Coque *et al.*, 2003; George and Häggblom, 2008; Häggblom *et al.*, 1986; Häggblom *et al.*, 1988).

It has been hypothesized that O-methylation of halogenated compounds is a detoxification mechanism (Allard *et al.*, 1985) and that Gram positive organisms display higher O-methylation activity than Gram negative bacteria (Allard *et al.*, 1985; Neilson *et al.*, 1988). However, several O-methylated chlorinated phenolic compounds have been tested in zebrafish and result in increased toxicity in these organisms (Neilson *et al.*, 1984). The exact reason for microbial O-methylation of halogenated phenolics has not been elucidated. Furthermore, studies have hypothesized that O-methylation of halogenated phenols is a constitutive process, but one inducible O-methyltransferase has been identified (Neilson *et al.*, 1988).

1.2.3. Bisphenol A

BPA is used as an additive in plastic manufacturing and is known to leach out of items such as baby bottles, reusable plastic containers, and water bottles (Kubwabo *et al.*, 2009; Vandenberg *et al.*, 2007). It is also used in the manufacturing of the epoxy coating in metal cans, resulting in leaching from the can material into the food products. Additionally, and in the interest of this project, it is a metabolite of the reductive dehalogenation of TBBPA by indigenous microorganisms in the environment (Voordeckers *et al.*, 2002).

Biodegradation of BPA has been studied in several environmental matrices. BPA degradation has been demonstrated in sea- and river water, sediments, rhizosphere sediments, compost leachate, and sewage sludge (Danzl *et al.*, 2009; Kang *et al.*, 2004; Masuda *et al.*, 2007; Toyama *et al.*, 2009; Zhang *et al.*, 2007).BPA is mineralized to CO_2 under aerobic conditions by *Pseudomonas monteilii* strain N-502, *Achrobacter*

xylosoxidans strain B-16, *Streptomyces* sp., *Novosphingobium* sp. strain TYA-1, *Sphingomonas* sp strain BP-7, *Sphingomonas yanoikuyae* BP-11R and a Gram negative bacterium designated strain MV-1 (Kang *et al.*, 2004; Lobos *et al.*, 1992; Masuda *et al.*, 2007; Toyama *et al.*, 2009; Yamanaka *et al.*, 2008; Zhang *et al.*, 2007). The degradation of BPA occurs through the same pathway with several of these organisms, the first identified metabolites are p-hydroxybenzoic acid (HBA) and p-hydroxyacetophenone (HAP) (Lobos *et al.*, 1992; Masuda *et al.*, 2007; Zhang *et al.*, 2007). Under anaerobic conditions, BPA is recalcitrant and persistent (Voordeckers *et al.*, 2002). Glycosylation of BPA by freshwater algae can occur, forming a metabolite more likely to accumulate in the plant and algae, thus are more likely to be taken up by other organisms (Nakajima *et al.*, 2007). Abiotic chlorination of BPA can be stimulated by irradiation under light conditions forming a monochlorinated BPA metabolite (Liu *et al.*, 2009).

1.3. TBBPA and BPA Toxicity

1.3.1. Overview of Toxic Mechanisms and Cell Response

Xenobiotic compounds are of concern because they can cause deleterious effects in organisms exposed to them (Klassen, 2008). Toxicants can impair function or cause death of a cell or of an organism by either an immediate/delayed toxicity that may or may not be reversible. Additionally, the effects from exposure can be localized to specific cells or tissues, or systemic due to effects on cellular processes present in all cells. Toxicants can cause carcinogenicity, genotoxicity, neurotoxicity, reproductive toxicity, developmental toxicity and immunotoxicity (Fig. 1.2) (Klassen, 2008). Further, many

xenobiotic compounds act as ligands for cell surface or nuclear receptors resulting in activation or repression of cellular signaling pathways important in normal cellular function (Fig 1.2.).

Specific regulation of gene expression, protein function, development and physiology in eukaryotes is regulated, in part, through a complex signaling network comprised of interconnecting signaling pathways (Klassen, 2008). Although the pathway network is complex, some basic principles exist for the signaling pathways. For example, cellular signaling is activated by a ligand binding to a receptor, either endogenous or exogenous, on the cell surface, which then activates intracellular signaling proteins and interacts with target proteins (Fig 1.3.) (Klassen, 2008). These target proteins are altered by the interaction with the intracellular signaling proteins and can result in altered metabolism, gene expression or cell shape and movement (Fig. 1.3.). There are four types of intercellular signaling: contact-dependent, paracrine, synaptic, and endocrine signaling (Klassen, 2008). Contact-dependent signaling requires the cells to be in direct membrane-membrane contact. Paracrine signaling involves the secretion of signals that once released from the cell act locally or at a distance on neighboring cells. Synaptic signaling is the type performed by neurons, transmitting signals from axons releasing neurotransmitters at synapses, located far from the cell body. Finally, endocrine signaling is dependent on endocrine cells, which secrete hormones into the bloodstream. These hormones are distributed throughout the body of an organism and are important in many cellular functions (Klassen, 2008).

TBBPA and BPA are known endocrine disruptors, capable of acting through the estrogen, progesterone and thyroid receptors and interfering with proper hormone binding

(Iso *et al.*, 2006; Kitamura *et al.*, 2005a; Kitamura *et al.*, 2005b; Legler and Brouwer, 2003; Meerts *et al.*, 2000). These receptors are part of the nuclear receptor superfamily, which are intracellular receptors also known as ligand activated transcription factors. This family also includes the aryl hydrocarbon receptor (AhR), retinoic acid receptor (RAR and RXR) and androgen receptor (AR) (Table 1.2.)(Klassen, 2008). These receptors are grouped into six subfamilies (McEwan, 2009). Generally, in the case of these transcription factors, once the ligand is bound to the receptor, the ligand/receptor complex dimerizes, either as homodimers or heterodimers, and bind to DNA elements in the upstream regulatory portion of the promoter and regulate transcription of these genes (Fig. 1.4.) (Aranda *et al.*, 2009; McEwan, 2009).

The nuclear receptors can also mediate effects through other cellular signaling pathways that are known to be responsible for proper development and cellular function, including the Wnt (wingless) and the mitogen activated protein kinase (MAPK) pathways (Fig. 1.3) (Mulholland *et al.*, 2005). In addition to the involvement of nuclear receptors, there is also evidence for cross regulation of the Wnt and MAPK pathways (Behrens, 2000). The Wnt pathway is responsible for the development of the anterior-posterior axis during embryonic development (Mulholland *et al.*, 2005; van Amerongen and Nusse, 2009). Additionally, this pathway is responsible for cell proliferation, cell fate decisions, regulating gene expression and cell migration (van Amerongen and Nusse, 2009; Verkade and Heath, 2008). The Wnt pathway is an example of a paracrine signaling pathway, with the glycoproteins acting on the neighboring cells mediating cellular function during development (Fig. 1.3) (Behrens, 2000). β−catenin is essential in the canonical Wnt pathway, requiring the Wnt proteins for stabilization, and acting in

complex as a transcriptional regulator (Behrens, 2000). In the absence of β–catenin, the Wnt pathway is known as the noncanonical Wnt pathway and functions in releasing Ca^{2+} from intracellular stores, activating the Jun N-terminal Kinase (JNK) signaling pathway, activating the Rho family of small GTPases, and regulating E-cadherin recycling (Verkade and Heath, 2008). The non-canonical Wnt pathway, is responsible for the integration of cell polarity and movement, specifically migration movements that drive narrowing and lengthening (convergence and extension) of the developing embryo (Verkade and Heath, 2008). Additionally, this pathway is involved in the proper formation of the posterior axis, and mutants within this pathway exhibit shortened anterior-posterior axes (Harrington *et al.*, 2007).

MAPK are serine/threonine kinases responsible for the mediation of cellular signals from the cell membrane to the nucleus (Westermarck and Kahari, 1999). There are several different pathways, including JNK, Erk, and p38 (Huang *et al.*, 2004). The JNK pathway responds to inflammatory cytokines, osmotic stress, and apoptotic signals, whereas the Erk pathway is activated by cytokines, growth factors and phorbol esters (Mook *et al.*, 2004). Both of these pathways are involved in the regulation of cellular migration but the mechanisms of activation and regulation are different (Huang *et al.*, 2004). Substrates for this pathway include paxillin, Jun and other molecules involved in regulating cell migration/adhesion (Huang *et al.*, 2004). p38 has only recently been implicated in cellular migration, historically its role was mainly in activating signaling in response to inflammation, apoptosis, and cell differentiation (Huang *et al.*, 2004). The Erk1/2 (motif of Thr-Glu-Tyr) pathway is activated by growth factors (such as epithelial growth factor and fibroblast growth factor) and its substrates include focal adhesion

kinase (FAK), paxillin and other kinases (Huang *et al.*, 2004). FAK is also a substrate of Erk, which under increasing times of activation, FAK will be over-expressed. In addition to growth factor stimulation of the MAPK pathway, this cascade can be activated by the production of reactive oxygen species (ROS) in the cells. These include peroxides, superoxides, singlet oxygens, and hydroxy radicals (Pan *et al.*, 2009). ROS can activate the Ras/Raf pathway and cause non-apoptotic, oxidative cell death, and tumorigenesis whereas the p38 pathway activation results in premature senescence and apoptosis (Pan *et al.*, 2009).

1.3.2. *In Vitro* and *In Vivo* Toxicity of TBBPA and BPA

The detection, accumulation, and persistence of TBBPA and BPA have resulted in increased attention regarding the toxicity of these compounds to humans and other organisms. Studies examining the toxicological effects of TBBPA exposure are compiled in Table 1.3. Specifically, TBBPA and BPA are known endocrine disruptors, acting through the estrogen and thyroid hormone signaling pathways, but they have differing results, mainly because of the difference in the presence/absence of bromine substituents (Hamers *et al.*, 2006; Kitamura *et al.*, 2005b; Meerts *et al.*, 2001; Meerts *et al.*, 2000). The estrogenic potentials of TBBPA and BPA differ. TBBPA has weaker estrogenic activity as compared to BPA, but exhibits both an agonist and anti-estrogenic activity to estradiol (E_2) in E_2 reporter assays in MCF-7 cells, whereas BPA exhibits only agonist activity (Kitamura *et al.*, 2005b; Samuelsen *et al.*, 2001). Binding studies examining the affinity for TBBPA, tribromobisphenol A, dibromobisphenol A, monobromobisphenol A, and BPA were performed in MCF-7 cells and determined that the estrogenic potential of

the brominated analogs of BPA (mono-,di- and tri-bromobispheno A) was increased as the number of bromines on the compound decreased (Samuelsen *et al.*, 2001). These data suggest that BPA preferentially acts through the estrogen-signaling pathway, and is a more potent xenoestrogen as compared to TBBPA.

1.3.2.1. TBBPA

Studies examining the effects of TBBPA exposure have increased in recent years, due in part to the demonstration of its endocrine disruption. *In vitro* binding studies have shown that TBBPA is a thyroid hormone agonist and competitively binds human transthyretin (TTR) 10 times stronger than thyroxine (T4), whereas BPA does not show any agonist or antagonist response (Kitamura *et al.*, 2002; Meerts *et al.*, 2000). Increased binding affinity for TTR correlated with increasing numbers of bromines, as determined by analysis of the brominated analogs of BPA (Meerts *et al.*, 2000). Recently, it has been shown that TBBPA causes oxidative damage by the formation of reactive oxygen species (ROS) in human neutrophil granulocytes by activating the NADPH oxidase via extracellular- signal regulated kinase (ERK) 1/2 phosphorylation (Reistad *et al.*, 2005). NADPH activation as well as increased cell death and increased cellular levels of calcium and glutamate has also been seen in rat cerebellar granule cells at concentrations of 10 mM and 20 mM TBBPA (Reistad *et al.*, 2007). The oxidative damage and production of ROS has been shown in several additional models including the earthworm, zebrafish, human cell culture and in plants (Kling and Forlin, 2009; Li *et al.*, 2008b; Reistad *et al.*, 2005; Xue *et al.*, 2009). TBBPA also interferes with cell signaling in human A549 epithelial alveolar lung cells and the untransformed rodent cell line NHK, by deactivating

the ERK thus affecting the MAP kinase pathway, (Strack *et al.*, 2007). Additionally, TBBPA exposure causes cytotoxicity in human cell culture, and neurological and reproductive effects in the mouse, rat and zebrafish animal models (Kuiper *et al.*, 2007a; Mariussen and Fonnum, 2003; Nakajima *et al.*, 2009; Reistad *et al.*, 2007; Strack *et al.*, 2007). Therefore, the presence of TBBPA in humans, specifically in developing fetus and cord blood samples, warrants investigation into the effects caused by TBBPA exposure during development.

In addition to the mammalian studies, there have been several studies examining TBBPA toxicity to aquatic organisms. Recent studies have shown that the lipophilicity of TBBPA contributes to the maternal transfer of TBBPA from mother to egg in the zebrafish (Nyholm *et al.*, 2008). Furthermore, a life cycle study demonstrated that adult exposure to TBBPA results in decreased egg production. Embryos that were viable, demonstrated decreased hatching at concentrations above 0.375 μM (Kuiper *et al.*, 2007b). Embryos exposed to TBBPA exhibited malformations in hindgut formation and pericardial fluid accumulation, decreased hatching and decreased long-term survival (Kuiper *et al.*, 2007b). These studies illustrate the sensitivity and the ease using zebrafish as a model system, which allows for an understanding of both how toxicity can relate to mammals, due to homologous systems, but also can contribute information as to environmental survival and fitness after exposure to TBBPA.

1.3.2.2. BPA

BPA is a known endocrine disruptor, exerting effects mainly through nuclear hormone receptors, most notably the estrogen receptor. BPA is an estrogen receptor agonist and

can have the potency of estradiol (Kang *et al.*, 2007; Kitamura *et al.*, 2005b; Li *et al.*, 2009; Samuelsen *et al.*, 2001; Vandenberg *et al.*, 2009; Wetherill *et al.*, 2007). BPA is also an androgen receptor antagonist, preventing androgen-dependent transcription once bound to the androgen receptor. Low dose BPA exposure *in utero* can result in increased prostate size and weight (Wetherill *et al.*, 2007). BPA exposure can also affect proper progesterone receptor signaling (Samuelsen *et al.*, 2001). Studies in the MCF7 human breast cancer cell line have illustrated that BPA is genotoxic through an estrogen-mediated mechanism at concentrations in the micromolar range (Iso *et al.*, 2006). Additionally, BPA has been shown to cause micronucleus formation in MCF7 cells. The micronucleus formation was due to alteration in the Src/Raf/ Erk signaling pathway (Kabil *et al.*, 2008). BPA exposure studies in mice have shown disruption of early oogenesis and an increase in preneoplastic mammary lesions in prenatally exposed females (Murray *et al.*, 2006; Susiarjo *et al.*, 2007). Additionally, in zebrafish, BPA is able to upregulate vitellogenin, a marker for estrogenicity, and the brain aromatase, a marker for neurotoxicants (Kishida *et al.*, 2001; Muncke *et al.*, 2007). Taken together, these studies illustrate the wide reaching effects of BPA exposure during development and demonstrate the importance of understanding the fate of BPA in the environment due to its potent endocrine disrupting potential.

1.4. The Zebrafish as a Model System

1.4.1. Overview of Zebrafish Development

The zebrafish (*Danio rerio*) is a small freshwater cyprinoid telost, originating in streams in India and has become increasingly popular as a model organism in

toxicological research (Briggs, 2002). The zebrafish was traditionally used as a model for development because much is known about its morphological, biochemical, and physiological development during the embryonic, larval, juvenile and adult periods (Hill *et al.*, 2005). Recently, however, it has been recognized that the zebrafish is a useful model in studying infectious disease, immunology, human congenital and genetic disease, regulatory physiology, and cancer (Briggs, 2002; Dooley and Zon, 2000; Feitsma and Cuppen, 2008; Sullivan and Kim, 2008). Some systems are not ideal for study in the zebrafish model because they do not possess the correct tissues, for example the lung, prostate and breast, but this model lends itself to study and extrapolation to mammalian systems because the majority of the genes and pathways are homologous (Spitsbergen and Kent, 2003).

There are many advantages to using the zebrafish as a model organism to study the toxicity of different chemicals in addition to disease and development. The development of the zebrafish is rapid, from embryo to reproduction age in three months with fully characterized morphological development at each stage (Kimmel *et al.*, 1995). This development is similar to rodents, but there are many additional advantages to this system. Unlike the rodent model, embryonic development is also *ex utero*, which means that fertilization and development occur outside and can be easily visualized (Briggs, 2002). Chemicals can easily diffuse through the chorion during development allowing for longer-term exposure analyses (Kari *et al.*, 2007; Yang *et al.*, 2009a). Additionally, the embryo is completely transparent which allows for easy analysis of the resulting effects after exposure to different chemical compounds, which can be related to developmental stage (Hill *et al.*, 2005; Kimmel *et al.*, 1995). Zebrafish are small fish, about 1-1.5

inches, which helps in decreasing costs for housing and decreasing overall space needs. Zebrafish reproduction results in high fecundity, approximately 300 embryos per mating on a weekly basis, which allows for experimental replication as well as increased sample size (Hill *et al.*, 2005). Most importantly however, is the ability to extrapolate the effects seen in the zebrafish after exposure to a toxicant to human health and environmental health (Dooley and Zon, 2000; Kari *et al.*, 2007; Law, 2003).

The zebrafish genome has been fully sequenced and is approximately 1.6 Gb distributed across 25 chromosomes (Freeman *et al.*, 2007). Zebrafish proteins display approximately <70% homology to human equivalents but the homology in the conserved functional domains is considerably greater, thus supporting the use of zebrafish as a model for human disease because most toxicants are known to act with the functional domains (Langheinrich, 2003; Shin and Fishman, 2002). Additionally, there are many tools available to study the response of exposure to xenobiotics in zebrafish at the molecular level including *in situ* hybridization probes to detect the localization of RNA expression, or morpholinos to transiently knock down gene expression (Hill *et al.*, 2005).

The developmental stages of the zebrafish are well characterized (Kimmel *et al.*, 1995), facilitating developmental toxicity studies and the characterization of the response to xenobiotic exposure. This is demonstrated by a study in which 36 of 41 known mammalian teratogens caused teratogenicity in the developing zebrafish (Nagel, 2002), allowing extrapolation from zebrafish to mammals. The stage descriptions are taken from the work of Kimmel et al. and have been summarized below. The periods of zebrafish development are, in chronological order; zygote, cleavage, blastula, gastrula, segmentation, pharyngula, hatching, larval, juvenile, adult (Table 1.4, Fig. 1.5)(Kimmel

et al., 1995). Staging of embryos is easily performed, only requiring a dissecting microscope, and is described for embryos incubated at 28.5 °C. Other temperatures will alter the rate of embryonic development (Kimmel *et al.*, 1995). The zygote period is characterized by a number of cell changes and cytoplasmic streaming and occurs until the first cellular cleavage. The cleavage period encompasses the 2-cell to the 64-cell stages, in which the blastomeres divide at regular orientations in 15-minute intervals. The blastula period is time from the 128-cell to 30% epiboly stages. This period is characterized by the appearance of the blastodisc, the main processes during this time are the midblastula transition, formation of the yolk syncitial layer (YSL) and the onset of epiboly, the spreading of YSL and blastodisc over the yolk cell. Gastrulation is the period in which the primary germ layers, the antero-posterior axis, and the dorso-ventral axes are formed, which is from 50% epiboly to the bud stage. These formations occur by the morphogenic cell movements including involution, convergence and extension. Segmentation is the period in which somites form the anterior/posterior wave, and organogenesis begins. Organogenesis results in formation of rudimentary primary organs, a recognizable body plan and the development of the optic lobes and tail bud. The pharyngula period is marked by the elongation of the embryo, the continuation of cellular differentiation and the pigmentation of the retina and embryo body. The organs of the embryo are now functional, and the pectoral fin begins to develop, and the primordial of the pharyngeal arches begin to migrate. The hatching period finds the pectoral fins developed, the jaw and gills begin developing and the embryo hatches from the chorion.

1.4.2. Caudal Development in the Zebrafish

The development of the posterior region of the zebrafish is an essential process contributing to hatching, as well as overall survival in the environment. If the caudal region is not properly formed, the swimming ability and transportation of the larval/adult zebrafish will be impaired, likely impacting the health and survival of the organism (Kanki and Ho, 1997). There were initially two different modes of thought as to the development of the caudal region, the first being that two separate processes formed the head and the tail, while the second considered tail formation to be a continuation of gastrulation with posterior development not differing qualitatively from head development (Kanki and Ho, 1997). There is evidence to support both of these hypotheses, however, the presence of zebrafish with mutated caudal regions but properly formed head regions suggest that there is a complex gene expression pattern in the caudal region. The tailbud in zebrafish is the aggregation of cells at the posterior end of the fish, that gives rise to tail cells, but also to posterior tissues anterior to the anus of the zebrafish, and is detected during the gastrula stage at approximately 10 hpf (Kanki and Ho, 1997; Kimmel *et al.*, 1995). The posterior development of the zebrafish is classified by the bilateral distribution of tailbud cell progeny, and the different types of ingression within specific areas of the tailbud (Kanki and Ho, 1997). Additionally, the tailbud cells are heterogeneous, containing distinct tissue-restricted domains with respect to cell fate. The tailbud is more prominently featured during the elongation of the embryo during the segmentation period, and is one of the main developmental processes from 10-24 hpf (Kimmel *et al.*, 1995). During the segmentation period, approximately 19 hpf, the tailbud begins to move away from the body of the embryo and Kupffer's vesicle is prominent at its base. After this

stage, the lengthening tailbud contributes greatly to the overall length of the developing embryo (Kimmel *et al.*, 1995).

In addition to the visual changes seen in caudal development, it is important to understand the processes at the molecular level. It is known that the Wnt pathway is important in posterior formation of zebrafish (Shimizu *et al.*, 2005; Verkade and Heath, 2008). Morpholino knockdowns of Wnt8 and Wnt3a, important in the canonical Wnt pathway, result in embryos with shortened posterior development. Wnt8 activates the canonical pathway and is expressed in the ventro-lateral mesoderm during gastrulation (Shimizu *et al.*, 2005). Wnt3a is important in body pattering during embryonic development, and deficiencies results in reduction or loss of somite structure and posterior body structure (Shimizu *et al.*, 2005). Overexpression of these genes is not desirable as it results in hyperactivation thus leading to dorsalization and posteriorization of the embryo (Shimizu *et al.*, 2005). In addition to the Wnts, the caudal related genes (cdxs), proteins that bind to DNA and activate hox genes important in caudal development play a role in the proper posterior development of zebrafish (Shimizu *et al.*, 2005). Interestingly, the Wnt pathway genes and the cdxs genes are interconnected, specifically the Wnt pathway has been shown to regulate the expression of some cdx genes, and the cdx genes mediate Wnt signaling in zebrafish to allow for proper caudal development (Shimizu *et al.*, 2005).

1.5 Extracellular Matrix and its Role in Caudal Development

The extracellular matrix (ECM) is involved in cell adherence and is involved in many cellular functions such as migration, proliferation, differentiation and morphogenesis (Zagris 2001). The ECM is under a constant state of flux, whether it be

dynamic homeostasis or matrix remodeling. The remodeling of the ECM requires the synthesis and deposition of ECM components but also the breakdown of these components in an organized fashion to allow for cellular morphogenesis (Stamenkovic, 2003; Zagris, 2001). In the developing embryo, the ECM provides attachment sites that guide migrating cells, keeping them in specific pathways and influencing the direction and extent of their movement (Zagris, 2001). This regulation helps to maintain the proper tissue architecture in the developing embryo.

The ECM is composed of glycoproteins, proteoglycans, glycosoaminoglycans, collagens, and gelatins assembled into two main domains, the basement membrane and the interstitial matrix (Adams and Watt, 1993; Bosman and Stamenkovic, 2003). The basement membrane and the interstitial matrix are both composed of collagen scaffolds that maintain the structural integrity of the ECM (Bosman 2003). Different proteoglycans and adhesive glycoproteins adhere to this scaffold, add stability and interact with cells that are within or adjacent to the ECM (Zagris 2001). Additionally, these scaffolds provide support for a network of signaling molecules, including growth factors and cytokines that trigger many cellular remodeling processes.

The integrin family of transmembrane proteins mediates cell-matrix interactions. Integrins are the primary receptors mediating remodeling of the ECM (Larsen *et al.*, 2006), but also play a role in cell survival, proliferation, gene transcription and the structure and function of the cytoskeleton (Bosman and Stamenkovic, 2003). Integrins are capable of binding to collagens, fibronectin and laminins and can cluster into microstructures within the plasma membrane called focal complexes (Svineng *et al.*, 2008). Integrins are responsible for the assembly of fibronectin into fibrils that attach to

the surface of a cell, which provides a framework for additional matrix protein binding (Kung *et al.*, 2009). Fibronectin has been identified as an important component in the ECM during zebrafish gastrulation, with morpholino knockdowns of fibronectin resulting in shortened tails in the developing embryo (Latimer and Jessen, 2009). Reactive oxygen species can also alter integrin signaling, specifically altering expression of one group of enzymes, the matrix metalloproteinases, which are crucial to ECM remodeling (Svineng *et al.*, 2008).

In addition to the molecules comprising the ECM, there are additional cell adhesion molecules that play a vital role in development, the cadherins. Cadherins are cell-cell adhesion molecules that form Ca^{2+} dependent homotypic interactions with each other, and are vital for cell recognition, sorting, coordinated cell movement and cell/ tissue polarity (Kung *et al.*, 2009). There are several types of classical cadherins present in vertebrates, specifically N, E, and C-cadherin (Harrington *et al.*, 2007). The cadherins have been shown to be involved in cellular rearrangements called convergent extension (CE) that regulate the anterior-posterior body axis (Harrington *et al.*, 2007). Dorsal migration is one type of CE movement and results in four stages of tailbud formation; tailbud formation, extension, protrusion and eversion (Kanki and Ho, 1997). Recently, it has been demonstrated that upon morpholino knock-down of N-cadherin expression, embryos exhibit shortened tails (Harrington *et al.*, 2007). Additionally, this study demonstrated interaction between N-cadherin and Vagl2, a gene involved in non-canonical Wnt signaling, indicating a connection to this signaling pathway for caudal development in the developing zebrafish embryo (Fig. 1.3) (Harrington *et al.*, 2007). This

indicates that there is likely crosstalk occurring between the Wnt and the N-cadherins, which can influence proper development.

1.6. Matrix Metalloproteinases

1.6.1. General Overview

ECM remodeling involves different types of proteolytic enzymes, serine proteases, cysteine proteases, aspartate proteases and the matrix metalloproteinases (MMPs). MMPs are Ca^{2+} and Zn^{2+} dependent endopeptidases that are responsible for the cleavage of different components of the extracellular matrix, such as collagen and gelatin (Visse and Nagase, 2003). Zn^{2+} is required for the three-dimensional structure, while Ca^{2+} is needed for stability and catalytic function (Bode *et al*., 1999; Nagase *et al*., 2006). Due to their ability to degrade components of the ECM and aid in its remodeling, MMPs are involved in regulating angiogenesis, cell adhesion, migration, proliferation, differentiation and morphogenesis (Chakraborti *et al*., 2003; Zagris, 2001). MMPs are able to release matrix bound growth factors, cleave cell-cell adhesions, generate pro-migratory ECM component fragments and expose cryptic integrin binding sites, all of which contribute to these processes (Raffetto and Khalil, 2008). Dysregulation of MMPs contributes to the pathophysiology of several diseases including arthritis, cancer, and those of the cardiac and nervous systems due to a dysregulation of proper ECM remodeling (Ganea *et al*., 2007).

Classification of MMPs is based on their substrate specificity and domain structure, but all must have a catalytic domain homologous to that of MMP-1 (collagenase 1) (Murphy and Nagase, 2008). Despite individual MMP substrate

specificity most function by the cleavage of the peptide bond before residues with a hydrophobic side chain, such as, leucine, isoleucine, phenylalanine, methionine and tyrosine (Visse and Nagase, 2003). There are 23 human MMPs with homologues in many species, including fruit fly, nematode, mice and zebrafish (Murphy and Nagase, 2008; Stamenkovic, 2003). The different classes of MMPs are the gelatinases (MMP-2 and –9), collagenases (MMP-1, -8, -13, and -18), stromelysins (MMP-3 and -10), matrilysins (MMP-7, and -26), membrane type (MT-MMP, MMP-14, -15, -16, 17, -24, and -25) and others (MMP-12, -19, -20, -22, -23, -28) (Visse and Nagase, 2003) (Table 1.5). The gelatinases, MMP-2 and -9, are named because they can easily degrade denatured collagens (gelatins). Additionally, the gelatinases can degrade collagen types I, III, IV, V, VII, X, and XI, fibronectin, elastin, laminin, aggrecan and vitronectin (Table 1.5) (Chakraborti *et al*., 2003; Fanjul-Fernandez *et al*., 2009). The collagens cleave interstitial collagens type I, II, and III at a specific site near the N-terminus (Table 1.5) (Visse and Nagase, 2003). Stromelysins can digest gelatin and collagens III, IV, V, and IX, as well as some pro-MMPs including MMP-1 (Table 1.5) (Chakraborti *et al*., 2003; Visse and Nagase, 2003). Matrilysins can degrade collagens IV and X, gelatin, and can process cell surface molecules including E-cadherin, Fas-ligand, and pro-tumor necrosis factor (TNF)-α (Table 1.5) (Chakraborti *et al*., 2003; Visse and Nagase, 2003). The MT-MMPs digest collagens I, II and III, gelatin, and most can activate MMP-2 (Table 1.5) (Visse and Nagase, 2003). These divisions are a bit arbitrary since the substrate overlap with MMPs is extensive and MMPs can degrade a large number of non-ECM components and additional ECM components not listed here (McCawley and Matrisian, 2001).

1.6.2 Regulation of Matrix Metalloproteinase Expression and Function

MMPs are regulated at three levels, transcription, proteolytic activation and inhibition of the active enzyme by inhibitors (Nagase *et al*., 2006). MMPs are translated as zymogens, and the signal peptide is proteolytically cleaved to create the activated pro-MMP. The propeptide consists of a consensus sequence with a Zn^{2+} binding motif HEXXHXXGXXH, and a conserved methionine, which creates a downstream "Met-turn" supporting the cleft structure around the catalytic Zn^{2+} molecule (Stamenkovic 2003). Additionally, the propeptides have a cysteine switch motif, PRCGXPD in which the cysteine residue coordinates with the Zn^{2+} in the catalytic site to keep the proMMPs inactive (Murphy and Nagase, 2008). Furthermore, many MMPs contain a haemopexin-like domain which is likely a contributor to substrate specificity (Stamenkovic, 2003).

MMP expression is regulated primarily at the level of transcription by a variety of cis-acting promoter elements, and can also be coordinately regulated (Fanjul-Fernandez *et al*., 2009). Most MMPs are regulated by an AP-1 response element, located in the proximal promoter near the TATA box (Fanjul-Fernandez *et al*., 2009). AP-1 regulation is known to regulate proteins such as Fos/Jun an AP-1 (Fanjul-Fernandez *et al*., 2009). PEA3 repsonse elements also play a role in MMP activations, which bind to the Ets family of oncoproteins (Fanjul-Fernandez *et al*., 2009; Yan and Boyd, 2007). The AP-1 and PEA3 sites can act cooperatively to promote cancer formation by allowing migration and invasiveness of cancer cells (Fanjul-Fernandez *et al*., 2009). The NF-κB pathway is responsible for regulating several MMPs, such as MMP-9, upon activation of different cytokines and growth factors (Yan and Boyd, 2007).

Proteolytic activation of MMPs is another method of regulation, in which the propetide is cleaved (Nagase *et al*., 2006). There are several methods of proteolytic activity to activate MMPs. The first involves a "bait" region, which is a proteinase susceptible region in the propeptide, which allows tissue or plasma proteinases to remove a portion of the propeptide. Full activation occurs by other active MMPs (Nagase *et al*., 2006). Several MMPs have a furin-like proprotein convertase region in the propeptide, which allows them to be intracellularly activated and secreted as active MMPs (Nagase *et al*., 2006). MMPs can also be activated by oxidants which react with the cysteine switch causing activation under inflammatory conditions (Nagase *et al*., 2006).

Endogenous inhibitors of MMPs are the tissue inhibitors of metalloproteinases (TIMP), plasma a2-macroglobulins, and tissue factor pathway inhibitor-2 (Murphy and Nagase, 2008; Raffetto and Khalil, 2008). The endogenous inhibitors bind MMPs in a 1:1 stoichiometry, typically into the active cleft similar to substrate binding (Raffetto and Khalil, 2008; Visse and Nagase, 2003). MMP regulation by endogenous inhibitors is vital for normal cellular processes because they control the amount and timing of degradation (Murphy and Nagase, 2008; Raffetto and Khalil, 2008; Visse and Nagase, 2003).

In addition to transcriptional regulation, MMPs are regulated by epigenetic regulation, posttranscriptional events, and several signaling pathways. The Focal Adhesion Kinase (FAK)/ MAPK, Ras/Raf and the Wnt pathways are involved in this regulation (Karow *et al*., 2008; Yan and Boyd, 2007). Additionally, MMPs can also be activated by reactive oxygen species, specifically by inactivating Ras and MAPK signaling, which are pathways involved in the regulation of MMP expression

(Chakraborti *et al*., 2003; Svineng *et al*., 2008). Methylation of CpG islands in the promoter region has been identified as a means for regulation of MMP-2 and -9 (Fanjul-Fernandez *et al*., 2009). Posttranscriptional regulation involves proteins capable of stabilizing or destabilizing the 5' or 3' untranslated regions of MMP transcripts, which plays an important role in mRNA turnover (Fanjul-Fernandez *et al*., 2009).

1.6.3 MMPs and Development

The MMPs, specifically MMP-2, -9 and -13, have similar functions during development but are differentially regulated. This type of regulation expresses each MMP at a specific time during development, with each playing a different role, with different substrate specificities relying on the timing and order of MMP expression for proper ECM remodeling (Stamenkovic, 2003). However, dysregulation of MMPs can occur, resulting in defects during development or disease. Overexpression of MMP-1 in mice can result in heart defects, and MMP-2 and -9 play a role in cardiac rupture (Murphy and Nagase, 2008). The key role of MMPs in the regulation of angiogenesis has been demonstrated, but the mechanisms are not entirely clear (Mott and Werb, 2004). MMP-2 is involved in tumor angiogenesis whereas MMP-9 correlates with retinal angiogenesis (Mott and Werb, 2004). MMP-9 knockout mice show abnormal development in the growth plates of their long bones, specifically the metatarsals. The knockouts develop almost normally, which is likely due to compensation by other MMPs during development (Vu *et al*., 1998). Further, membrane type-MMPs are involved in follicle rupture during ovulation in medaka (Ogiwara *et al*., 2005).

MMP-2, -9 and -13 were selected for study in our experiments because they have been shown to be critical in mammalian and zebrafish development (Crawford and Pilgrim, 2005; Murphy and Nagase, 2008). MMP-2 and -9 are the gelatinases, capable of degrading gelatin, elastin, fibronectin, aggrecan, vitronectin, laminin and collagen types I, III, IV, V, VII, X, XI (MMP-2) and IV, V, XI, XIV (MMP-9) (Fanjul-Fernandez *et al*., 2009). MMP-2 is expressed in fibroblasts, keratinocytes, endothelial cells, chondrocytes, and monocytes. Alternatively, MMP-9 is expressed in alveolar macrophages, polymorphonuclear leukocytes and osteoclasts (Fanjul-Fernandez *et al*., 2009). The gelatinases have been implicated in a number of diseases; including, cancer, bone diseases, inflammatory disorders and several vascular diseases (Fanjul-Fernandez *et al*., 2009). Additionally, MMP-2 and -9 promote angiogenesis, tumor or retinal, respectively, by exposing cryptic epitopes within collagen IV (Mott and Werb, 2004). Further, MMP-9 mobilizes VEGF (vascular endothelial growth factor) from the ECM and can cleave type IV collagen (Murphy and Nagase, 2008). In addition to angiogenesis, MMP-2 and -9 play an important role in tumor cells, with MMP-9 responsible for intravasation, allowing the tumor cells to penetrate the vascular endothelial wall (Stamenkovic, 2003). MMP-2 and -9 are regulated mainly at the level of transcription. The promoter region of MMP-9 contains an AP-1, NF-κB, PEA3, and other binding sites (Fanjul-Fernandez *et al*., 2009). The promoter of MMP-2 is quite different, lacking AP-1 and NF-κB binding sites, but possessing the PEA3 sites (Fanjul-Fernandez *et al*., 2009). In addition to transcriptional activation, MT1-MMP (MMP-14) can activate MMP-2 at the cell surface through interactions at the haemopexin domain (Mott and Werb, 2004; Nagase *et al*., 2006). Proteolytic regulation?

MMP-13, collagenase 3, is capable of degrading the interstitial collagens, I, II and III (Visse and Nagase, 2003) as well as aggrecan, fibronectin, and osteonectin (Chakraborti *et al*., 2003). Synovial fibroblasts, macrophages and chondrocytes are known to express MMP-13 (Murphy and Nagase, 2008). MMP-13 plays an important role in rheumatoid and osteoarthritis, and ablation in mice results in abnormal growth plate and an increased trabecular bone (Murphy and Nagase, 2008). Transcription of MMP-13 is regulated by several different cis-factors in the promoter including AP-1, PEA3 and Runx2 sites and TATA box (Fanjul-Fernandez *et al*., 2009).

In addition to the mammalian studies, there has been increasing interest in the role of MMPs in the developing zebrafish, and much work has been conducted to characterize MMP expression. Several MMPs, specifically MMP-2, -9, -13, and -14 are critical for proper development of the zebrafish embryo (Crawford and Pilgrim, 2005; Hillegass *et al*., 2007, 2008; Latimer and Jessen, 2009; Yoong *et al*., 2007; Zhang *et al*., 2003a, b). MMP-14 (e.g. MT-MMP1) is important during zebrafish gastrulation in determining cell polarity and motility. Additionally, MMP-14 regulation suggests an involvement of the non-canonical Wnt pathway (Coyle *et al*., 2008). MMP-2, gelatinase A, has been shown to play a minor role in mouse embryogenesis, but this role is likely due to compensation by other MMPs in the mouse model. In contrast, in the zebrafish, MMP-2 knockouts exhibit truncated axis and altered development demonstrating a vital role of MMP-2 in the developing embryo (Zhang *et al*., 2003a). MMP-9, gelatinase B, expression at 12 hpf suggests a low level of maternal transmission (Yoong *et al*., 2007). Between 12 and 24 hpf, MMP-9 is localized to the notochord, but after 24 hpf, MMP-9 is detected in circulating white blood cells (Yoong *et al*., 2007). MMP-13, a collagenase, is expressed

in zebrafish beginning at 12 hpf, peaking at 48 hpf, and can be detected at 72 and 96 hpf (Hillegass *et al*., 2007). MMP-13 knockouts demonstrate alteration in proper craniofacial formation, altered somatogenesis, blood pooling, edema body axis curvature, and shortened tails (Hillegass *et al*., 2007). Glucocorticoid exposure can also affect MMP-13 expression and activity, resulting in similar phenotypes as the knockout (Hillegass *et al*., 2007, 2008). Endogenous inhibitors are also present in the developing embryo, illustrating the complex regulation of these enzymes during development (Crawford and Pilgrim, 2005). These studies illustrate the importance of proper MMP function during embryogenesis and also that they play a role after exposure to toxicants.

1.7. Hypothesis and Objectives

In the studies contained herein, we focused our attention on microbially mediated O-methylation of TBBPA producing TBBPA DME, and BPA O-methylation forming BPA MME and BPA DME. My hypotheses are that the microorganisms capable of O-methylation are ubiquitous in the environment and transform TBBPA and BPA to metabolites recalcitrant to further degradation. Additionally, we hypothesize that the O-methylated metabolites result in decreased toxicity, as measured by mortality and lesion occurrence, to the developing zebrafish embryo than the parent compound. The goal of this project was to elucidate the gene responsible for O-methylation and also to understand the effect of the O-methylated metabolites on zebrafish development. Additionally, the role of MMPs in response to TBBPA or TBBPA DME exposure was examined.

The aims of this project were to:

1. Determine if the microorganisms capable of O-methylation are ubiquitous in the environment, and what proportion of the population they represent

2. Determine if the TBBPA DME metbolite is recalcitrant, or can be transformed by microbes in the environment.

3. Characterize O-methylation reaction, using *Mycobacterium vanbaalenii* PYR-1, and other *mycobacterial* strains to determine which are capable of O-methylation, and which compounds can be O-methylated.

4. Examine the toxicity of TBBPA, BPA and their O-methylated metabolites using the developing zebrafish embryo as a model system.

5. Examine the role of the matrix metalloproteinases after exposure to TBBPA and TBBPA DME and if they play a role in the mechanism of toxicity of these compounds.

1.8. Figures

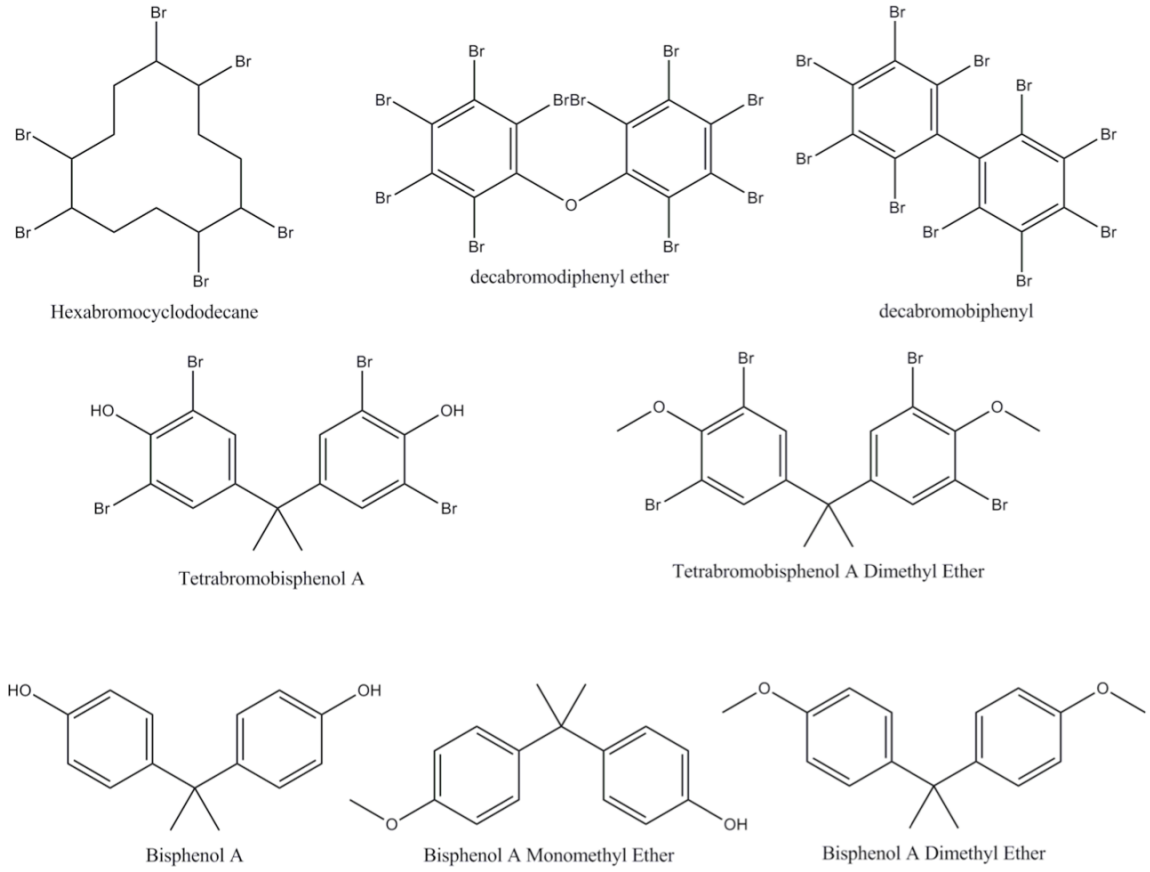

Figure. 1.1. Representative Structures of Brominated Flame Retardants, and Microbial Transformation Products. Structures include a representative from each of the major classes of BFRs, as well as the O-methyated derivative of TBBPA, TBBPA DME, as well as the reductive dehalogenation metabolite, BPA. Also shown are the structures for the O-methylated derivatives of BPA.

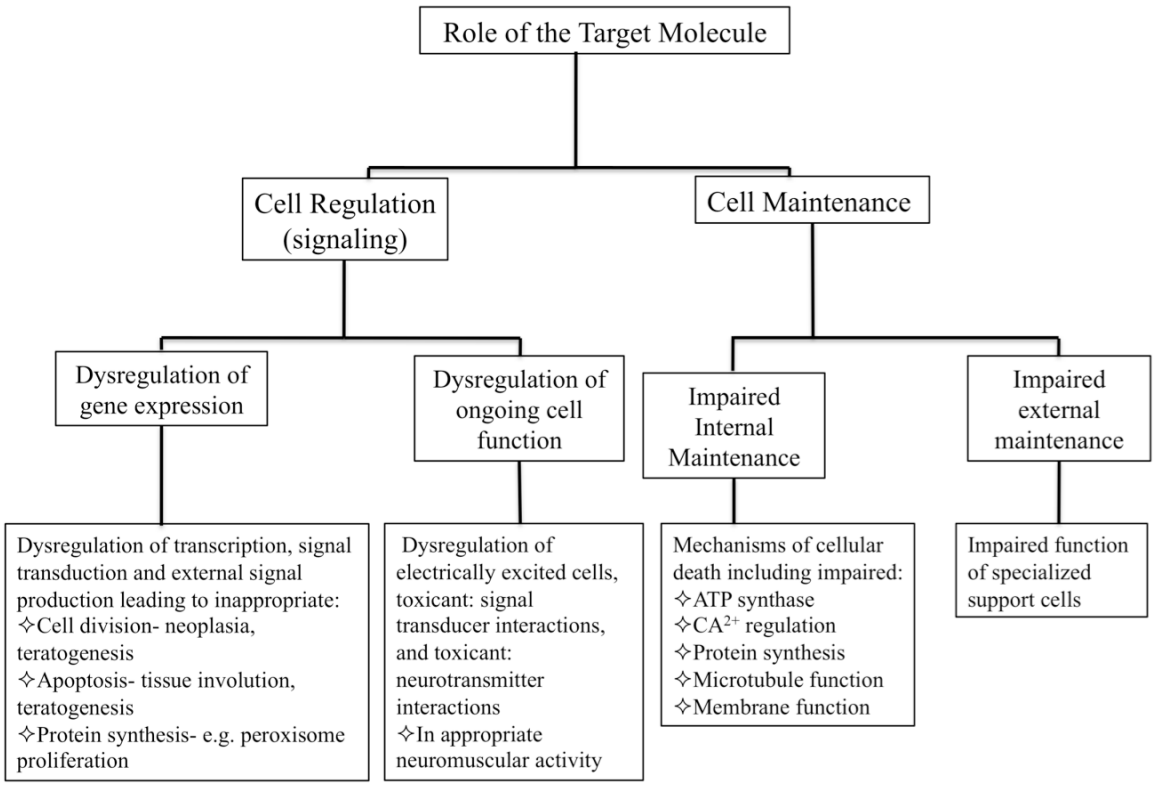

Figure 1.2. Mechanisms of Toxicity. Exposure to different chemicals can cause a variety of responses in the eukaryotic cell. This flow chart illustrates the different roles a target molecule can play, and also the resulting effects to these cells.

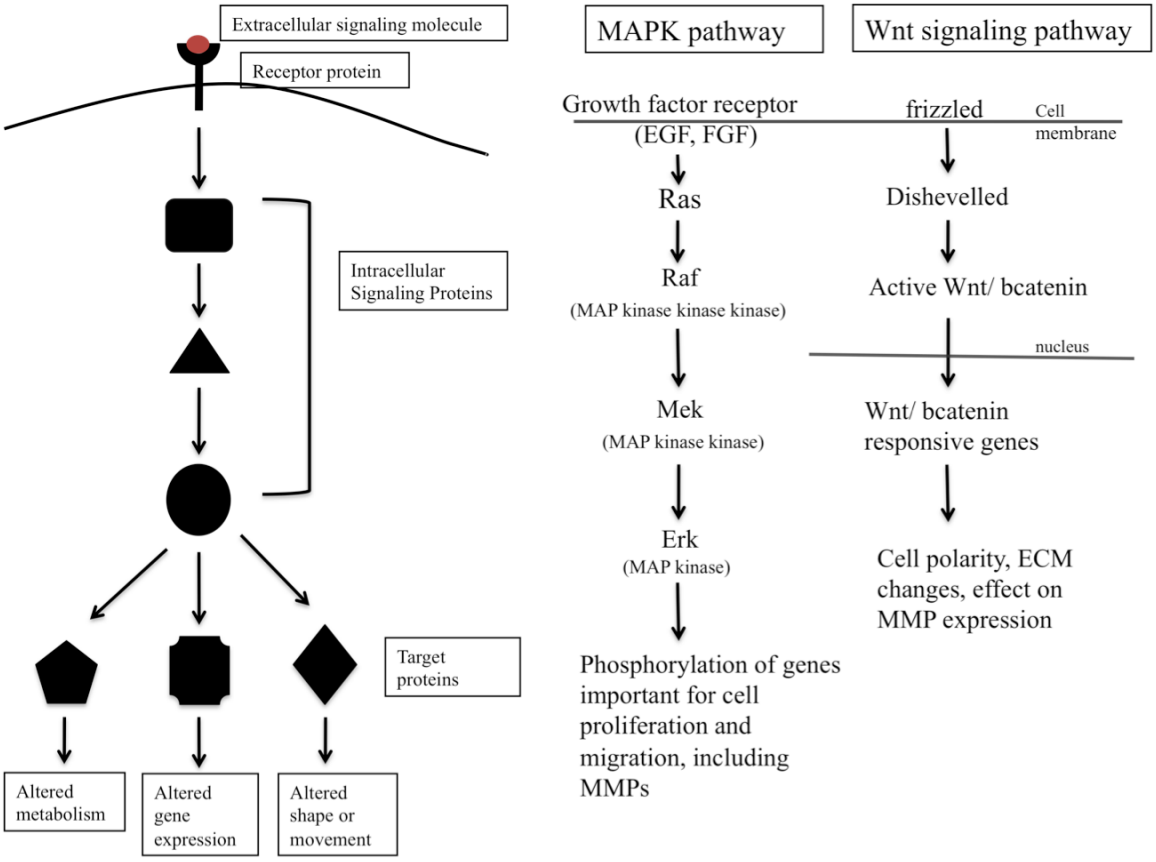

Fig. 1.3. Cellular Signaling Schematic. Cellular signaling in eukaryotic organisms is quite complex, and many pathways interconnect, but generally there are only several outcomes. Panel A illustrates a general signaling cascade with a cell membrane receptor binding a signaling molecule, which can be a growth factor, cytokine, or other protein. This binding will activate a signaling cascade, many times by phosphorylation, results in in the activation of target proteins leading to altered metabolism, gene expression or shape and movement. Panels B and C are simple schematics of the mitogen activated protein kinase and Wnt signaling pathways, and illustrate the results of activation of these pathways.

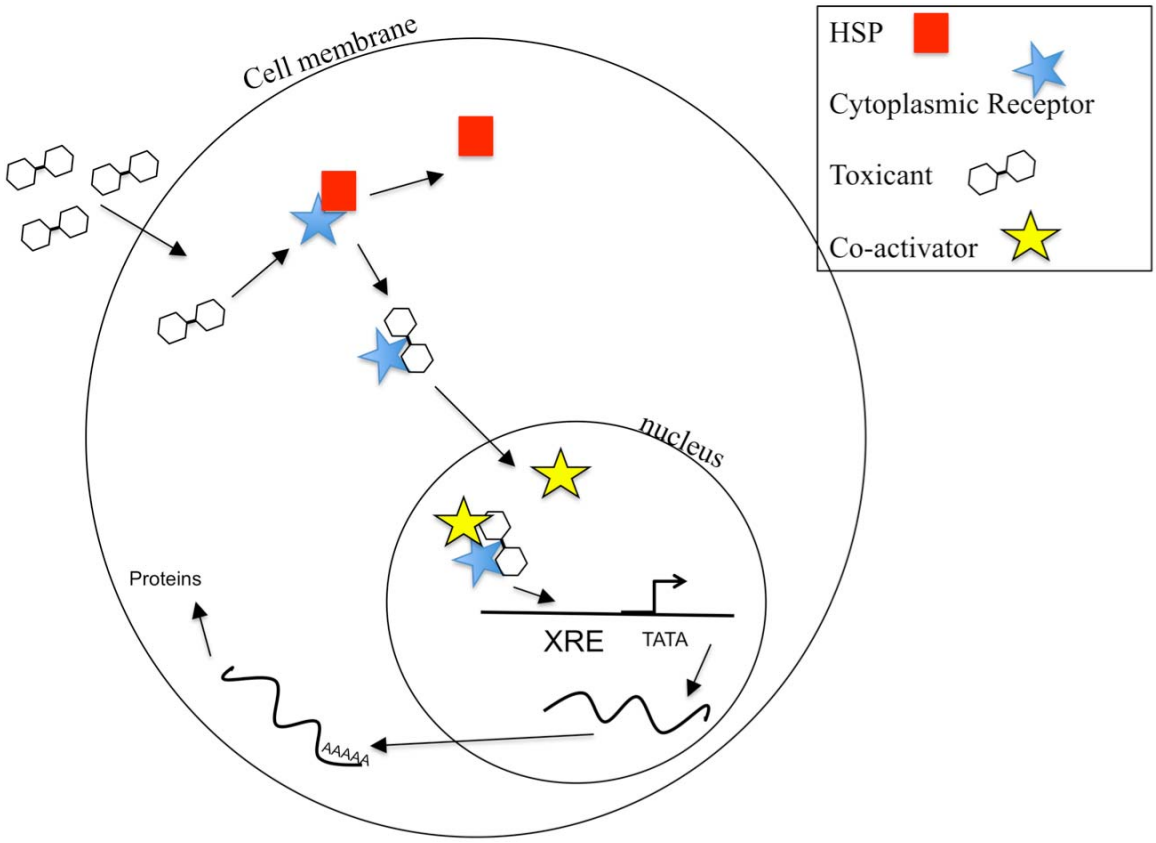

Figure 1.4. Mechanism of Nuclear Receptor Gene Expression Activation After Exposure to a Toxicant. The toxicant crosses the cell membrane and is bound by the nuclear receptor (see Table 1.2.) in the cytoplasm. The nuclear receptor is typically bound by other factors in the cytoplasm, but they break off from the complex once a toxicant is bound. The receptor then either forms a homodimer or a heterodimer with another nuclear receptor. The Receptor: Toxicant complex translocates to the nucleus, where there are co-activator molecules. The co-activator molecule binds to the receptor: toxicant complex and allows for binding to upstream promoter elements referred to as xenobiotic response elements, and either activate or repress transcription of responsive genes.

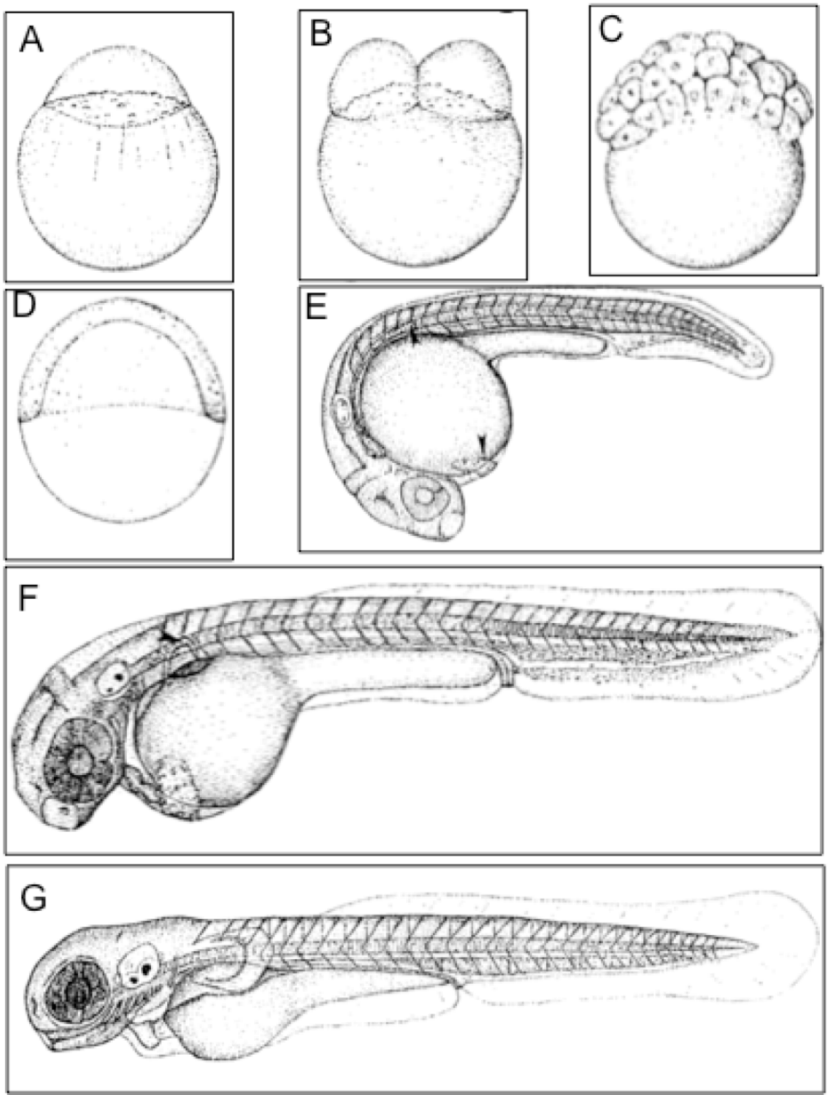

Figure 1.5. Select stages of zebrafish embryonic development. Camera lucida sketches correspond to the outset of the (A) zygote period, (B) cleavage period, (C) blastula period, (D) gastrula period, (E) pharyngula period (arrowheads indicate the posterior lateral line primordium on the dorsal side and the hatching gland on the yolk ball), (G) hatching period. Specific developmental processes occuring during each of these periods can be found in Table 1.4. Modified from Kimmel, et al. 1995.

1.9. Tables

Table 1.1. BFR Exposure and Environmental Detection

Species	Exposure: Type	Exposure: Levels (mean levels)	Exposure: Country	Exposure: Location	Compounds	Reference
Exposure						
Human	Air	Air: 15 pg m-3, 11, pg m-3, 27 pg/m-3, and 1 pg m-3 Dust: 62, 36, 2, 230 ng m-3	United Kingdom	dust from cars, indoor home and offices and outdoor air	HBCD, TBBPA	(Abdallah *et al.*, 2008)
Human	Air, Dust and Filter release	Dust house:39-49000 ng/g, Dust garage: 4-210000 ng/g	Michigan-United States	dust and air emissions from houses and garages	PBDEs, TBBPA	(Batterman *et al.*, 2009)
Human	Air	10 ng/g-domestic, 75 ng/g office	Belgium	indoor dust, office and domestic settings	BPA, Triclosan and TBBPA	(Geens *et al.*, 2009)
Human	Dust, residue from electronic shredder, environmental exposure- soils	shredder: 45500 ng/g dry wt, dust: 30700 ng/g dry wt, soil: 1910 ng/g dry wt	China	electronig waste recycling facility and soil near facility	PBDD/Fs, PBDE	(Ma *et al.*, 2009)
Human	Air: personal and stationary Skin: handwash and patch samples from clothing	air: 10-1050 ng/m3, 3-180 ng/m3. Skin: 0.09-6.7 ng/cm2, 2 ng/hands	Finland	circuit board factory, furniture workshop, electronics dismantling facility, computer classroom, offices at factories	organophosphorus flame retardants and TBBPA	(Mäkinen *et al.*, 2009)
Human	Air	55 pmol/ m3, 0.11-0.37 ng/ m3, 0.031ng/m3, 0.15 ng/m3,	Sweden	electronic recycling plant, circuit board assembly plant, computer repair factory, computer teaching center, outdoors	Deca-PBDE, triphenyl phosphate, TBBPA	(Sjödin *et al.*, 2001)
Human	Dust and furniture foam	dust: 570-7360 ng/g	United States	foam couches, chairs, mattress pads, pillows and dust samples	PBDE and organophosphate flame retardants	(Stapleton *et al.*, 2009)

Species	Exposure: Type	Exposure:Levels (mean levels)	Exposure: Country	Exposure: Location	Compounds	Reference
Human	Dust	19,000 ug/g	Japan	dust collected from cabinets at the rear of televisions and examined for TBBPA leaching from the TV to the dust	PBDEs, PBDF, TBBPA	(Takigami *et al.*, 2008)
Human	Air and Precipitation	air: 77-372 pg/m3 and rain: 1.98-15.5 ng/L	Pearl River Delta, South China	air and precipitation samples over a one year period from urban and rural areas	PBDE	(Zhang *et al.*, 2009)
Detection in Humans						
Human, neonatal	maternal and umbilical serum, adipose tissue, breast milk	breast milk: 34-9400 pg/g lw, serum: 2-783 pg/g f.w.	France	Perinatal	PBDE, HBCD, TBBPA	(Antignac *et al.*, 2008)
Human, neonatal	serum, breast milk	maternal serum:19.87 ng/g, breast milk:4.11 ng/g, cord blood: 103.52 ng/g wet weight	France	maternal adipose tissue, serum, breast milk, cord serum	tri-decaPBDEs, TBBPA	(Cariou *et al.*, 2008)
Human	Serum	1-3.4 pmol/ g lipid weight	Sweden	computer technicians with different exposure to computers	PBDEs, TBBPA	(Jakobsson *et al.*, 2002)
Human; Perinatal	maternal blood , milk, cord blood, umbilical cords	26 pg/g wet weight-maternal blood, 16 ng/g wet weight cord blood	Japan	perinatal exposure	PBDEs, hydroxylated PBDEs, PCBs, TBBPA	(Kawashiro *et al.*, 2008)
Human, perinatal	breast milk	rural: 0.97 ng/g lipid, urban: 1.4 ng/g lipid	China-Beijing	breast milk exposure	PBDEs, TBBPA	(Li *et al.*, 2008a)
Human	extraplacental gestational membranes	17.4 ± 3.9 pg/g tissue	Michigan, United States	uniquitous exposure, detection in membranes	PBDE	(Miller *et al.*, 2009)
Human; neonatal	Milk	1000 pg/g lipid weight	China	Food exposure	HBCD, TBBPA	(Shi *et al.*, 2009b)

Species	Exposure: Type	Exposure:Levels (mean levels)	Exposure: Country	Exposure: Location	Compounds	Reference
Human	Serum	less than 1 ng/g lipid weight	Norway	Age and gender body burden	PBDE, TBBPA	(Thomsen *et al.*, 2002)
Human	Breast milk		Norway	breast milk	PBDE, HBCD	(Thomsen *et al.*, 2010)
Humans, Dolphins, Sharks	Adipose tissue	human: 0.048 ng/g lipid, Bottlenose dolphin: 1.2 ng/g lipid, Bullshark: 9.5 ng/g and Atlantic Sharpnose shark: 0.872 ng/g lipid	United States	Human samples: New York City, Sharks and Dolphins off coast of Florida	HBCD, TBBPA and PBDEs	(Johnson-Restrepo *et al.*, 2008)
Humans	Serum	0.48- 1980 ng/g lipid	China	serum from office cleaners, university students, and policemen	PBDE, HBCD, hexabromobenzene, pentabromoethylbenzene, and decabromodiphenylethane	(Zhu *et al.*, 2009b)
Environmental Contamination						
n.a.	sewage sludge and sediment samples	252 ± 10 ng/g	Northwest Spain	urban sewage treatment samples, river and marine water samples	TBBPA, bromophenols	(Blanco *et al.*, 2006)
wild aquatic and terrestial bird eggs	environmental samples and biota	4.6-146 ng/g lipid wt	Yellow River Delta, North China	samples from eggs of wild aquatic and terrestial birds and 2 species of captive birds	PBDE, DDT, PCB	(Gao *et al.*, 2009)
n.s.	environmental samples and biota samples	water: 140-3200 pg/L, sediment: 330-3800 pg/g dry wt, fish: 0.29-1.7 ng/g lipid wt	England	water, sediment and fish samples frrom English Lakes	HBCD, TBBPA,	(Harrad *et al.*, 2009)
n.a.	trace environmental exposure, sediments	water: 3-160 pg/L, sediments: 5-145 pg/g	France	river water and river bed sediment	TBBPA, PBDE	(Labadie *et al.*, 2010)
Dolphins and porpoises	environmental samples, adipose tissue	4.1-51000 ng/g lw	South China	adipose tissue of dolphins and porpoises	HBCD, PBDE	(Lam *et al.*, 2009)

Species	Exposure: Type	Exposure:Levels (mean levels)	Exposure: Country	Exposure: Location	Compounds	Reference
n.a.	Sewage Sludge	12.4 ng/g	Canada	sewage sludge	nonylphenol ethoxylates, 4-nonylphenol, BPA, triclosan, pentachlorophenol, hexachlorophene, TBBPA	(Lee and Peart, 2002)
Ringed seal, polar bear	environmental exposure, primarily ingestion	from below LOD to 686 ng/g lipid weight	East Greenland	environmental samples	PBDE, MeO-PBDE, OH-PBDE, PCB, PBB, HBCD, OH-PBB	(Letcher *et al.*, 2009)
Waterbirds	environmental exposure	130-14000 ng/g lw	Pearl River Delta, South China	environmental samples near recycling plant	PBDEs, PCBs, DDT	(Luo *et al.*, 2009)
Porpoises and Seal	Food web, sediments, landfill, sewage	sediments: 1-451 ug/kg dry weight, landfill: 54 ug/kg dry weight, sewage: 7.5-95 ug/kg dry weight, animals: 0.3-136 ug/ kg lipid	North Sea, The Netherlands, UK	sewage sludge, landfill leachates, sediments and food web organisms	HBCD and TBBPA	(Morris *et al.*, 2004)
Falcons	environmental samples	0.08-53.1 ng/g lw	California, United States	falcon eggs, demonstrate metabolism in wildlife from decaBDE to nona and octaBDE	PBDE, PCB	(Park and Choi, 2009)
Farmed fish and wild birds	environmental samples and biota samples	sediment: 2-1987 ng/g dry wt, air: 1-9200 pg/m3, sewage sludge: 1-52000 ng/g dry wt, soil: 0.05-95 ng/g dry wt	Pearl River Delta and e-waste area, South China	surface sediments, sewage sludge, air, fish and birds from near an e-waste recycling facility	PBDE, TBBPA-DBPE, DBDPE	(Shi *et al.*, 2009a)
Shellfish	environmental exposure	0.1-1.4 ng/g ww, near or below LOQ in most samples	Netherlands	dietary	HBCD, me-TBBPA, PBDE, TBBPA	(van Leeuwen and de Boer, 2008)
mysid shrimp	environmental samples and biota	PBDE: 14-22 ng/g dw, 1765-2962 ng/g lipid; TBBPA: trace detection	Netherlands	water, sediment and mysid shrimp from the Scheldt estruary	PBDE, HBCD, Nonylphenol, nonylphenol carboxylates	(Verslycke *et al.*, 2005)

Species	Exposure: Type	Exposure:Levels (mean levels)	Exposure: Country	Exposure: Location	Compounds	Reference
tuna, albatross, polar bear	natural production	0.54 ± 0.38 ng/g ww	Pacific, Atlantic, Indian and Artic Oceans	environmental samples and natural metabolism	PBDE, MeO-PBDE, OH-PBDEs, bromophenols, MeO-bromophenols	(Wan *et al.*, 2009)
n.a.	Air	0.04-0.85 pg/m-3	Germany, Denmark	environmental samples	TBBPA	(Xie *et al.*, 2007)

Table 1.2. Nuclear Receptors

Ligand activated transcription factor	Endogenous Ligand	Exogenous Ligand	Effect
Estrogen Receptor (ER)	Estradiol	Bisphenol A, Ethynylestradiol, DDT, Methoxychlor	Mammary and hepatic cacinogenesis
Glucocorticoid Receptor (GR)	Cortisol	Dexamethasone	Teratogenesis, Apoptosis
Retinoid Acid Receptor (RAR, RXR)	All-trans-retinoic acid	13-cis retinoid acid	Teratogenesis
Aryl Hydrocarbon Receptor (AhR)	Unknown	TCDD, PCBs, PAHs	Enzyme Induction (CYP1A1), Teratogenesis, Hepatocarcinogenesis
Androgen Receptor (AR)	Testosterone	BPA, DDT, PAHs, PCBs	Malformations in reproductive tract development, anti-male phenotypes
Thyroid Receptor (TR)	Thyroxine	TBBPA, BPA	Altered activation of thyroid hormone dependent genes, nervous system development

Table 1. 3. Toxicological Studies Involving TBBPA

Species	Model	Endpoints	Exposure; vehicle	Exopsure time	Doses tested	Results	Other chemicals tested	Reference
Endocrine								
Quail	Japanese quail egg exposure	uptake of radioactive compounds, reproductive behavior, egg laying, oviduct morphology	injection for embryos, injected or orally exposed adults, DMSO	5 days, 9 days for adult exposure, 15 days for embryonic exposure	^{14}C labelled substrates, 250 mg/ bird, 0.8-67 mg/g egg injection	Accumulation of chemical in eggs was very low, no significant estrogen effects detected. Maternal transfer to egg was low	BPA	(Halldin *et al.*, 2001)
Human and Rat	H4IIE (rat)-DR, U-2 OS (human)-AR and PR, T47D (human)- ER	Arylhydrocarbon, androgen (AR), estrogen (ER), progesterone (PR) receptors, thyroid hormone interference	culture medium, DMSO (0.2-0.4%)	24 or 48 hours	10-12.5 μM	TBBPA potent T4 binding competitor, inhibitor of E2 sulfation	PBDe, HBCD, 2,4,6-TBP, OH-PBDEs, E2, TBBPA-DPBE	(Hamers *et al.*, 2006)
Frog *(Xenopus laevis)*	tadpoles of *Xenopus laevis*	Thyroid hormone regulated biomarkers inlcuding receptors, body length, limb length	water, ethanol (0.001%)	21 days for long term, 24, 48, 72 hours for short term	2.5-500 mg/L	500 mg/L caused developmental malformations in long term study, short term study determined antagonist activities of TBBPA to thyroid receptor	T4 as positive control	(Jagnytsch *et al.*, 2006)
Rat,	rat pituitary cell line GH3 responsive to thyroid hormone, and the rat pituitary cell line MtT/E-2 responsive to estrogen	cell proliferation of both cell types, thyroid hormone binding assays,	culture medium, DMSO	7 days	10^{-4} to 10^{-6} M	TBBPA and TCBPA caused proliferation of GH3 cells, all increased proliferation of MtT/E-2 cells, TBBPA and TCBPA inhibited T3 binding to TR	TCBPA and BPA	(Kitamura *et al.*, 2002)
Human, Rat and Mouse	human MCF-7, rat GH3, mouse NIH3T3 and rat *in vivo* model	estrogenic, androgenic and thyroidal hormone activity, uterine weight	culture medium, not indicated	24, 48 or 72 hours	10-5 to 10-8 M, 20, 100, 300, 500 mg/kg	thyroid hormone activities of TBBPA, lower estrogenic potential	BPA, TCBPA, and other derivatives	(Kitamura *et al.*, 2005b)

Species	Model	Endpoints	Exposure; vehicle	Exopsure time	Doses tested	Results	Other chemicals tested	Reference
Hamster and Frog tadpole	CHO-K1 (ovary cells) and tadpole metamorphosis	thyroid hormone activity- binding assays	culture medium, DMSO (0.1%)	24 hours, 10 minutes for binding assay, 3 days for tadpole assay	10^{-4} to 10^{-11} M	anti-thyroid effects on T3, tail shortening	TCBPA, TMBPA, DMBPA, BPA, DPP and T3	(Kitamura *et al.*, 2005a)
European Flounder	adult exposure	aromatase, vitellogenin, thyroid hormone T4, microsomal enzyme activities	water for TBBPA, food and sediment for HBCD. DMSo 0.1%	105 days for TBBPA, 78 days for HBCD	0, 0.001, 0.01, 0.1,0.2, 1.4, 0.8 μM	aromatase increased, T4 and T3 increased. No cahnge with microsomal tests	HBCD	(Kuiper *et al.*, 2007a)
Zebrafish	Embryonic exposure, and MolDarT assay	Vitellogenin expression, MolDarT assay	egg water, EtOH (0.01%)	24,48,72,96,and 120 hours post fertlization	5,10,15 μM	Vitellogenin increased with 15 mM BPA exposure	estradiol, nonylphenol, atrazine, cyproconazol	(Muncke *et al.*, 2007)
Human	H295R human adrenocortical carcinoma cell line	CYP 450 gene expression,	culture medium, DMSO (0.5% v/v)	24 and 72 hours	0.025, 0.05 0.5 μM	TBBPA only increased CYP21, OH-BDEs affect gene expression than MeO-PBDEs	PBDE, OH-PBDE, MeOPBDE	(Song *et al.*, 2008)
Human and Monkey	CV-1 (African Green Monkey kidney cells)	luciferase assay for thyroid hormone binding	culture medium, DMSO 0.2%	24 hours	1-100 μM	Cytotoxicity at 100 μM, thyroid disruption, no luciferase activated-antagonist activity	BPA, TCBPA	(Sun *et al.*, 2009)
Pacific Tree Frog (*Pseudacris regilla*)	tadpole exposure and metamorphosis	thyroid receptor expression, MMP-9 expression, tail resorption	water exposure, DMSO	24, 48, 72 96 hours	10 or 100 nM	increased tail resorption by 72 hours, altered expression of TH-receptor alpha in tail and brain, MMP-9 in brain and PCNA in tail and brain	T3	(Veldhoen *et al.*, 2006)
Neurological								
Zebrafish	Embryonic exposure	p450aromB (brain aromatase)	Embryo medium, DMSO (0.005%)	2-48 hours post fertlization	0.01, 0.1, 1.0, 10 μM	BPA= weak agonist. Less than 3x increase with 10 mM exposure	17b-estradiol, diethylstilbestrol	(Kishida *et al.*, 2001)
Rat	Brain synaptosomes and vesicles	Dopamine, glutamate and GABA uptake	culture medium, not indicated	10 or 15 minutes	2,5, 10, and 20 μM	inhibited dopamine/ glutamate uptake	PBDE, HBCD	(Mariussen and Fonnum, 2003)

Species	Model	Endpoints	Exposure; vehicle	Exopsure time	Doses tested	Results	Other chemicals tested	Reference
Mice	Behavioral	TBBPA levels in brain, behavioral activities and memory	corn oil, oral administration	3 hours	0.1, 5, 250 mg/kg body weight	increase in horizontal movements, more freezing behavior, increased spontaneous alternation	none	(Nakajima *et al.*, 2009)
Rat	Cerebellar Granule Cells	Cell death, free radical formation, calcium influx, extracellular glutamate concentrations	culture medium, DMSO 0.01%	30 min, 6, 12, 18, 24 hours, 180min	2,5,10,20 μM	ROS formation induction, increase intracellular calcium, extracellular glutamate	BPA	(Reistad *et al.*, 2007)
Human	neuroblastoma cells SK-N-MC	lipid peroxidation, cell death, ROS formation	culture medium, DMSO 0.1%	3 hours, 24 hours	1-50 μM	synergistic ROS formation, induction of cytotoxicity	BDE-47 and BDE-99	(Tagliaferri *et al.*, 2009)
Cell Signaling								
Mussels	Hemocytes	MAP kinases, lysosomal membrane stability	culture medium, DMSO	15-60 minutes	1,5,25 μM	induces hemocyte membrane destabilization, increase in JNK, MAPK and PKC, extracellular oxide production	none	(Canesi *et al.*, 2005)
Mouse	TM4 Sertoli cells	cell death, Ca2+ homeostasis	culture medium, DMSO 1%	30 min, 18 hours	0-60 μM	causes apoptosis (18 mM), increase in intracellular calcium, disrupt ER Ca2+ ATPases	none	(Ogunbayo *et al.*, 2008)
Human	Neutrophil Granulocytes	MAP Kinase pathway, Protein Kinase C	culture medium, DMSO 1%	5, 30 60 minutes	0.1,0.5,1,2,4,6,12 μM	ROS production by respiratory burst, activation of NADPH oxidase by Erk1/2 mechanism	BPA	(Reistad *et al.*, 2005)

Species	Model	Endpoints	Exposure; vehicle	Exopsure time	Doses tested	Results	Other chemicals tested	Reference
Human, Rat	Cal-62 human thyroid, NRK rat kidney, A549 human epithelial alveolar	MAP kinase pathway, cell proliferation and viability	culture medium, DMSO	24,48,72, 96 hours	10-200 μM	causes G2/M arrest, cell viability decreased in dose-dependent manner, MAPK cascades involved	BPA	(Strack *et al.*, 2007)
Miscellaneous								
zebrafish	adult exposure, proteomics and genomic (microarray) studies	Gene survey, both RNA and protein in liver	water exposure, semi-static renewal, DMSO 0.01%	semi-static every 3-4 days for 14 days	0.75 and 1.5 μM	interference of thyroid and vitamin A homeostasis. Also, oxidative stress, cellular metabolism-lipid, carbohydrate and organic acid	none	(De Wit *et al.*, 2008)
Rat	Liver cells	cytochrome P450s	oral administration in feed, corn oil	28 days	30, 100, 300 mg/kg body weight	no significant findings with TBBPA	HBCD	(Germer *et al.*, 2006)
Zebrafish	Embryo development	malformations, heat shock protein, superoxide dismutase, lipid peroxidation	water exposure daily renewal, DMSO 0.05%	96 hours	0,0.002,0.01,0.05,0.25,0.75,1.5 mg/L	Concentrations greater than 0.75 mg/L TBBPA result in mortality, lesions. TBBPA could cause oxidative stress and Hsp70 overexpression	HBCD	(Hu *et al.*, 2009)
Rat	prenatal exposure to TBBPA, postnatal exposure to PAH	body/ organ weight, histopathology	food exposure	3 weeks (TBBPA)	0, 0.01, 0.1, 1% to food	increased incidence of follicular cell hyperplasia in thyroids	perchlorate and N-bis(2-hydroxypropyl)nitrosamine, 7,12-dimethylbenz(a)	(Imai *et al.*, 2009)
Rats	ZFL Liver cells	proteomic	culture medium, DMSO 0.2%	24, 72 hours	0.05,0.5,5 and 220 μM	increased gluconeogenesis, affect proper protein folding, NADPH production, and cell-cycle control	HBCD	(Kling and Forlin, 2009)

Species	Model	Endpoints	Exposure; vehicle	Exopsure time	Doses tested	Results	Other chemicals tested	Reference
Zebrafish	embryo, adult exposure	lifecycle: clutch size, hatching	adults- water exposure daily. Juvenile- semi-static renewal (2x/ week). Embryonic- water exposure	adults- 30 days. Juveniles- 5 weeks (semi-static renewal), Embryos- 3days	0.023, 0.047, 0.094, 0.188, 0.375, 0.75, 1.5, 3 μM	decreased hatching and post hatch survival at 1.5 mM. Egg production decreased in all doses above 0.047 mM	none	(Kuiper *et al.*, 2007b)
Wheat	soil exposure	chlorophyll content, malondialdehyde, superoxide dismutase, peroxidase, catalase	TBBPA added and mixed into soils where wheat plants were planted	7 and 12 days	50-5000 mg/kg	caused oxidative stress, peroxidase and catalase good biomarkers for exposure	none	(Li *et al.*, 2008b)
Zebrafish	adult exposure	maternal transfer, analysis of TBBPA concentrations	adult exposure via food, ethanol was evaporated	every day for 42 days	10 and 100 nmol	TBBPA found in both mother and in eggs. Determined BFR can be transferred maternally due to high lipophilicity	HBCD, PBDE, TBBPA DBPE, TBBPA OHEE, 2,4,6-dibromophenol	(Nyholm *et al.*, 2008)
Rat	maternal/ gestational exposure	thyroid weight, T3 concentrations, brain development, reproduction studies	oral administration in feed, corn oil	GD 10 to day 20 post delivery	0,100,1000,10000 ppm	decrease in T3 levels in serum, no changes in body or organ weight or brain development	HBCD	(Saegusa *et al.*, 2009)
Earthworm	biomarker study	superoxide dismutase, catalase, reduced and oxized glutatione, and malondialdehyde	absorption-filter-paper contact method	48 hours	0, 0.01, 0.05, 0.1, 0.5, and 1 mg/L	generating ROS and causing oxidative damage	none	(Xue *et al.*, 2009)
Human and Rat	Human S9 and microsomes and Rat livers	biological transformations of TBBPA in cells	culture medium	2 hours	20-200 μM	TBBPA cleaved near central carbon, producing hydoxylated metabolites, which are chemically reactive	none	(Zalko *et al.*, 2006)

Table 1.4. Developmental Stages of Zebrafish Development (adapted from Kimmel, et al)

Developmental Period	Stage	Time (hpf)	Description
Zygote	1-cell	0.00	cytoplasmic streaming toward animal pole to form blastodisc
Cleavage	2-cell	0.75	Partial cleavage
	4-cell	1.00	2x2 blastomere array
	8-cell	1.25	2x4 blastomere array
	16-cell	1.50	4x4 blastomere array
	32-cell	1.75	4x8 blastomere array
	64-cell	2.00	3 blastomere tiers
Blastula	128-cell	2.25	5 blastomere tiers; irregular cleavage patterns
	256-cell	2.50	7 blastomere tiers
	512-cell	2.75	9 blastomere tiers; formation of yolk synctial layer (YSL)
	1000-cell	3.00	11 blastomere tiers; single row of YSL nuclei; asynchronous cell cycle
	High	3.33	>11 blastomere tiers;two rows of YSL nuclei; blastodisc flatterning
	Oblong	3.66	Multiple rows of YSL nuclei; blastodisc flattening
	Sphere	4.00	Blastula obtains spherical shape; flat border between blastodisc and yolk
	Dome	4.33	Yolk cell bulging toward animal pole
	30% epiboly	4.66	Blastoderm of uniform thickness forms; 30% of yolk cell covered by blastoderm
Gastrula	50% epiboly	5.25	50% of yolk cell covered by blastoderm
	Germ-ring	5.66	Germ ring visible from animal pole; 50% epiboly
	Shield	6.00	Embryonic shield visible from animal pole
	75% epiboly	8.00	Dorsal side distinctly thicker; epiblast, hypoblast,
	90% epiboly	9.00	Axis and neural plate formation; presence of brain, notochord and muscle rudiments
	Bud	10.00	Prominent tail bud; 100% epiboly
Segmentation	1-4 somites	10.33	First somite furrow
	5-9 somites	11.66	Kupffer's vesicle, optic vesicle and neural keel form
	10-13 somites	14.00	Pronephrous forms
	14-19 somites	16.00	embryo length (EL)=0.9 mm; optic placode, brain neuromeres
	20-25 somites	19.00	EL=1.4 mm; lens, optic vesicle, hindbrain neuromeres
	26+ somites	22.00	EL=1.6 mm; blood islands, otoliths, midbrain-hindbrain boundary
Pharyngula	Prim-5	24.00	EL=1.9 mm; early pigmentation, heartbeat
	Prim-15	30.00	EL=2.5 mm, early touch reflex, retina pigmented
	Prim-25	36.00	EL=2.7 mm; early motility, tail pigmentation
	High-pec	42.00	EL=2.9 mm; pectoral fin rudiments
Hatching	Long-pec	48.00	EL=3.1 mm; elongated pectoral fin buds; first cartilage and bone appear
Larval	Protruding Mouth	72.00	EL=3.5 mm; mouth opens and protrudes anteriorly behind eye

Table 1.5. Matrix Metalloproteinases and ECM Substrate Specificity

Subfamily	Name(s)	ECM Substrates
Collagenase	MMP-1/ Collagenase-1	Collagen I,II,III, VII, VIII, X, XI; gelatin, entactin/ nidogen, fibronectin, laminin, vitronecin, aggrecan
	MMp-8/ Collagenase-2	Collagen I, II, III; gelatin, entactin, aggrecan, tenascin
	MMP-13/ Collagenase-3	Collagen I, II, III, IV, VIII, IX, X, XIV; gelatin, fibronectin, vitronectin, aggrecan, osteonectin
	MMP-18/ Collagenase-4	Collagen IV
Gelatinase	MMP-2/ Gelatinase-A	Colagen I, III, IV, V, VII, X, XI; gelatin, fibronectin, fibrillin, elastin, laminin-5, vitronectin, aggrecan, osteonectin, tenascin
	MMP-9/ Gelatinase-B	Collagen IV, V, XI, XIV; geltin, decorin, fibrillin, elastin, laminins, vitronectin, aggrecan
Stromelysin	MMP-3/ Stromelysin-1	Collagen III, IV, V, VII, IX, X, XI; gelatin, decorin, elastin, entactin/ nidogen, fibronectin, fibrillin, laminin, vitronectin, aggrecan, osteonecin
	MMP-10/ Stromelysin-2	Collagen III, IV, V; gelatin, elastin, fibronectin, aggrecan
	MMP-11/ Stromelysin-3	Fibronectin, laminin, aggrecan
Matrilysin	MMP-7/ Matrilysin-1	Collagen I, IV; decorin, elastin, fibrillin, fibronectin, laminin, vitronectin, aggrecan, osteonectin
	MMP-26/ Matrilysin-2	Collagen IV, gelatin, fibronectin, fibrin/ fibrinogen
Membrane-Type	MMP-14/ MT1-MMP	Collagen I, II, III; gelatin, fibronectin, vitronectin, aggrecan
	MMP-15/MT2-MMP	Fibronectin, laminin, entactin, aggrecan, proteoglycans
	MMP-16/MT3-MMP	Collagen III, fibronectin
	MMP-17/MT4-MMP	Gelatin, fibrin/ fibrinogen
	MMP-24/MT5-MMP	Fibronectin, gelatin, proteoglycans
	MMP25-MT6-MMP	Collagen IV, gelatin, laminin-1, fibronectin, proteoglycans, fibrin/ fibrinogens
Other MMPs	MMP-12/Macrophage elastase	Fibronectin, elastin, laminin, proteoglycans, fibrin/ fibrinogen
	MMP-19/ RAS-1	Collagen IV, gelatin, laminin, fibronectin, fibrin/ fibrinogen
	MMP-20/ Enamelysin	Amelogenin, aggrecan
	MMP-21/ XMMP	Gelatin
	MMP-23/ CA-MMP	Gelatin
	MMP-27/ CMMP	Gelatin
	MMP-28/ Epilysin	Not determined

2.0. Embryonic Exposure to Tetrabromobisphenol A and its metabolites, Bisphenol A and Tetrabromobisphenol A dimethyl ether disrupts normal zebrafish (*Danio rerio*) development and matrix metalloproteinase expression

2.1. Abstract

Tetrabromobisphenol A (TBBPA) is a widely used brominated flame retardant that is persistent in the environment and detected in human serum and breast milk. TBBPA is microbiologically transformed in anaerobic environments to bisphenol A (BPA) and in aerobic environments to TBBPA dimethyl ether (TBBPA DME). Despite the detection of TBBPA DME in the environment, the resulting toxicity is not known. The relative toxicity of TBBPA, BPA and TBBPA DME was determined using embryonic exposure of zebrafish, with BPA and TBBPA DME exhibiting lower potency than TBBPA. TBBPA exposure resulted in 100% mortality at 3 (1.6 mg/L) and 1.5 μM (0.8 mg/L), whereas BPA and TBBPA DME did not result in significant embryonic mortality in comparison to controls. While all three caused edema and hemorrhage, only TBBPA specifically caused decreased heart rate, edema of the trunk, and tail malformations. Matrix metalloproteinase (MMP) expression was measured due to the role of these enzymes in the remodeling of the extracellular matrix during tissue morphogenesis, wound healing and cell migration. MMP-2, -9 and -13 expression increased (2-8 fold) after TBBPA exposure followed by an increase in the degradation of collagen I and gelatin. TBBPA DME exposure resulted in only a slight increase (less than 2 fold) in MMP expression and did not significantly increase enzymatic activity. These data suggest that TBBPA is more potent than BPA or TBBPA DME and indicate that the

trunk and tail phenotypes seen after TBBPA exposure could be due to alteration of proper MMP expression/ activity.

2.2. Introduction

Tetrabromobisphenol A (TBBPA) is a widely used brominated flame retardant that is a persistent and prevalent contaminant in the aquatic environment (Fernandes *et al.*, 2008; Kuiper *et al.*, 2007a; Kuiper *et al.*, 2007b; Labadie *et al.*, 2010; Nyholm *et al.*, 2008; Verslycke *et al.*, 2005; Zhang *et al.*, 2009). The widespread production and use of brominated flame-retardants has raised concerns regarding their fate and effects on aquatic species. Tetrabromobisphenol A [TBBPA, 4,4'-isopropylidenebis(2,6-dibromophenol)] is the most commonly used brominated flame retardant produced globally at 120,000 metric tons in 2001 (Hakk and Letcher, 2003). TBBPA is used primarily in the production of epoxy and polycarbonate resins. The environmental persistence of TBBPA is due to its high lipophilicity (log Kow=5.9), low volatility (7.0 x 10^{-11} atm-m^3/mol), low water solubility (4.16 mg/l at 25°C in H_2O) and recalcitrance (Hakk and Letcher, 2003; Kuramochi *et al.*, 2008). The widespread use of TBBPA, and its environmental persistence in dust, sediments and accumulation in biota has led to increased concerns regarding its effects on wildlife and humans (Johnson-Restrepo *et al.*, 2008).

TBBPA undergoes two different types of transformations (Fig. 2.1) mediated by microorganisms in the environment, reductive debromination to bisphenol A (BPA, 4,4'-isopropylidenediphenol) or O-methylation to TBBPA monomethyl ether and TBBPA dimethyl ether [(TBBPA DME), 4,4'-isopropylidenebis(2,6-dibromo-1-

methoxybenzene)] (Arbeli *et al.*, 2006; George and Häggblom, 2008; Voordeckers *et al.*, 2002). Notably, TBBPA DME has been detected in aquatic sediments near a plastic manufacturing plant in Sweden at concentrations of 36 and 2400 ng/g as well as in mussels in Osaka Bay, Japan (Sellström and Jansson, 1995; Watanabe *et al.*, 1983). Toxicological data on this new metabolite of TBBPA are lacking, but are crucial to understanding the impact of accumulation of TBBPA DME in the environment.

In contaminated sites, TBBPA is typically detected at parts per million (ppm) concentrations in sediments and sewage sludge near BFR production facilities, and parts per billion (ppb) at other sites (Hakk and Letcher, 2003; Hale *et al.*, 2006; Kang *et al.*, 2007). TBBPA DME has been detected in sediments at a higher level than TBBPA, but the levels are still in the ppb range (Hakk and Letcher, 2003). BPA levels in sediments and surface waters are also typically in the ppb range, but can be higher near the manufacturing plants and decrease with distance from the plant (Kang *et al.*, 2007).

It is known that TBBPA and BPA are estrogen mimics and endocrine disruptors that could interfere with normal reproduction and embryonic development (Hamers *et al.*, 2006; Jakobsson *et al.*, 2002; Kitamura *et al.*, 2005b; Law *et al.*, 2006; Meerts *et al.*, 2000; Thomsen *et al.*, 2001). Additionally, TBBPA has been shown to cause cytotoxicity in human cell culture, and neurological and reproductive effects in the mouse, rat and zebrafish animal models (Kuiper *et al.*, 2007a; Mariussen and Fonnum, 2003; Nakajima *et al.*, 2009; Reistad *et al.*, 2007; Strack *et al.*, 2007). In addition to the mammalian studies demonstrating the role of TBBPA and BPA as endocrine disruptors and estrogen mimics recent studies have illustrated similar effects in aquatic organisms. For example, TBBPA ($\geq$ 0.047 μM) exposure effects include a reduction of egg production, survival

and overall reproductive success in zebrafish (Kuiper *et al.*, 2007a). Furthermore, it has been demonstrated that maternal transfer of brominated flame-retardants from adult to embryos occurs in zebrafish (Nyholm *et al.*, 2008). Therefore, the effects of TBBPA exposure during development in aquatic species warrant further investigation.

The goal of this study was to determine the effect of developmental exposure to TBBPA and determine whether the microbial transformation products of TBBPA (BPA and TBBPA DME) differ in their developmental toxicity. Developmental toxicity data in fish exist for TBBPA and BPA, but there are no reports for TBBPA DME. The zebrafish model was used for these studies because of the ability to monitor early development and the ability to compare with previous studies and the potential to extrapolate to other aquatic species (Hill *et al.*, 2005). In our studies, TBBPA is more toxic than its metabolites, BPA and TBBPA DME. Further, embryonic exposure to TBBPA resulted in truncated bodies/tails suggesting impairment in the remodeling of tissues during development of the caudal region of the zebrafish embryo. The truncated tail phenotype suggests the hypothesis that matrix metalloproteinase (MMP) expression and activity would be altered in response to TBBPA exposure in the developing zebrafish embryo. MMPs are Zn^{2+} dependent endopeptidases that are responsible for the cleavage of different components of the extracellular matrix, such as collagen and gelatin. MMPs are involved in regulating cell adhesion, migration, proliferation, differentiation and morphogenesis (Chakraborti *et al.*, 2003; Zagris, 2001). Our study focused on the gelatinases, MMP-2 and -9, and the collagenase MMP-13. These particular MMPs were selected because they have been shown to be critical in mammalian and zebrafish development (Crawford and Pilgrim, 2005; Murphy and Nagase, 2008).

Our data show that the parent compound, TBBPA, was more toxic than BPA or TBBPA DME based on embryonic mortality and lesion occurrence. Alterations in MMP expression and activity correlated with the severity of the chemical's effects. All three compounds following embryonic exposure resulted in decreased juvenile survival (28 dph). This is the first study to characterize and compare the developmental effects of TBBPA, BPA and TBBPA DME in the developing zebrafish embryo.

2.3. Materials and Methods

2.3.1. Chemicals. Tetrabromobisphenol A (TBBPA) and bisphenol A (BPA) were obtained from Sigma Aldrich (>98% purity). Tetrabromobisphenol A dimethyl ether (TBBPA DME, purity >99% by GC-MS) was synthesized from TBBPA as previously described (George and Häggblom, 2008). Stock solutions were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich), which was also used as the vehicle control.

2.3.2. Zebrafish strains and husbandry. The AB strain of zebrafish (*Danio rerio*) was used for all experiments, and was obtained from the Zebrafish International Resource Center (ZIRC). Zebrafish were bred and maintained in a recirculating Aquatic Habitat System utilizing a light: dark cycle of 14:10 hours, respectively. All experiments in this study were conducted according to a protocol approved by the Rutgers University Animal Care and Facilities Committee.

2.3.3. Collection and Treatment of Zebrafish Embryos. Zebrafish embryos were collected at approximately 3 hours post fertilization (hpf) and were exposed in 4 ml glass

vials to TBBPA (0.75, 1.5, or 3 μM)/(0.4, 0.8, 1.6 mg/L), BPA (5, 10, or 15 μM)/(1.14, 2.3, 3.42 mg/L), DME (1, 5, or 10 μM)/ (0.57, 2.9, 5.7 mg/L) or dimethyl sulfoxide (0.15%) in sterile water for seven days. A static, non-renewal approach was used. The concentrations of TBBPA and BPA used in this study were similar to those used in previous zebrafish studies (Kuiper *et al.*, 2007b; Muncke *et al.*, 2007). The TBBPA DME doses were selected because they are in the same range as the TBBPA and BPA concentrations. One embryo was placed into each vial and observed every 24 hours for 7 days. Studies were conducted with 10-25 embryos per dose and all studies were repeated at least 3 times. Developmental lesions, including edema, hemorrhage, death and date of hatching, were recorded.

To examine juvenile survival, larvae were removed from vials after 7 days and placed into a beaker containing system water and boiled wheat seeds. They were fed paramecium culture and AP100 for the next 21 days. At 28 days post fertilization (dpf) all remaining live larvae were counted and euthanized using an overdose of MS-222 (Tricaine methanesulfonate).

2.3.4. Quantitation of Heart Beat in Exposed Zebrafish Embryos. Zebrafish embryos were collected at approximately 3 hpf and exposed to various levels of TBBPA (0.75, 1.5, or 3 μ M) or vehicle control, DMSO (0.05%) and were observed at 48 hpf. Observations were made starting at 48 hpf, because the heart is not yet fully formed at 24 hpf (Kimmel *et al.*, 1995). The embryos were observed through a dissecting microscope and mounted with a Scion video recording device to determine heart beat rate. Video was recorded at 7 frames per second for a total of 7 seconds. The videos were observed and

heart beats were counted. The number of heart beats per specified time interval were converted to heart beats per minute.

2.3.5. Alcian Blue Staining and Visualization. Zebrafish embryos were collected at approximately 3 hpf and exposed in glass plates to either TBBPA (0.75 μM), BPA (15 or 10 μM), TBBPA DME (10 or 5 μM) or DMSO (vehicle) containing 0.003% phenylthiourea. Embryos were euthanized at 7 dpf with an overdose of MS-222, and fixed with 4% paraformaldehyde. The staining and visualization procedure was performed as previously described (Hillegass *et al.*, 2008).

2.3.6. RNA Isolation from Zebrafish Embryos. Zebrafish embryos were collected at approximately 3 hpf and exposed in glass plates to either TBBPA at 0.75 μM or TBBPA DME at 5 μM or vehicle (DMSO, 0.1 %) in sterile zebrafish system water. These concentrations were selected in order to examine expression at sublethal doses. Embryos were snap frozen in liquid nitrogen at 24, 48 and 72 hpf, and stored at -80°C. RNA was isolated using Trizol (Invitrogen Carlsbad, CA). RNA was treated with Dnase (DNA-free kit Ambion, Austin, TX) and complimentary DNA (cDNA) was generated using iScript cDNA synthesis kit (BioRad, Hercules, CA). Quantitative Real Time RT-PCR was performed on a BioRad iCycler equipped with an iCycler iQ Detection System using the BioRad iQ SYBR Green Supermix (BioRad Hercules, CA). Samples were analyzed in triplicate, and normalized to the zebrafish 28S rRNA gene. Primers previously designed for zebrafish MMP-2, -9 and -13 were used (Hillegass *et al.*, 2007, 2008). Standard

curves for the MMP-2, -9 and -13 primers were prepared and used for quantitation (Hillegass *et al.*, 2007, 2008). All experiments were repeated three times.

2.3.7. *In Vitro* Zymography. Zebrafish embryos were collected and exposed to TBBPA, TBBPA DME, or vehicle as described in the RNA isolation section. Embryos were harvested in Lysis buffer containing, 150 mM NaCl, 10 mM HEPES, 2 mM DTT, and 0.1% Triton X-100, at 48 and 72 hpf. Protein concentration was determined and normalized to ensure equal protein loading. The embryos were analyzed according to the *in vitro* zymography assay as previously described (Crawford and Pilgrim, 2005; Hillegass *et al.*, 2007). Fluoresceinated gelatin and collagen I were used in these assays to analyze MMP-2 and -9 or MMP-13 activity, respectively. All data were normalized to a no lysate control to take into account background fluorescence.

2.3.8. Statistical Analyses. Sigma Stat Version 1.01 was used to determine the normality of the data, the power and the appropriate statistical test. The Mann-Whitney Rank Sum test was used to examine the hatching data. Chi square analysis was used to compare the lesion and mortality data. The Students T-test was used to analyze the data from the qRT-Real Time PCR and *In Vitro* Zymography. All data referred to as significant are $p \leq 0.05$.

2.4. Results

2.4.1. Mortality Following Exposure to TBBPA, BPA or TBBPA DME. Zebrafish exposed to TBBPA, BPA or TBBPA DME were examined for dose related embryonic mortality and gross developmental lesions. TBBPA was more acutely toxic than either

BPA or TBBPA DME (Fig. 2.2) causing 100% mortality by 3 and 5 dpf at 3 (1.6 mg/L) and 1.5 μM (0.8 mg/L) respectively. TBBPA DME exposure did not increase mortality in comparison to control at any concentration tested. BPA exposure (5-15 μM) resulted in 4-8% death, however this was not statistically different from mortality in controls (Fig. 2.2).

In order to assess whether exposure to TBBPA, BPA or TBBPA DME alters juvenile survival we assessed the number of animals surviving to 28 dpf. The mortality of exposed embryos at all doses of TBBPA (0.75 μM-47%), BPA (5 μM -47%, 10 μM -22%, and 15 μM-47%) and TBBPA DME (1 μM-16%, 5 μM -22% and 10 μM-34%) was increased as compared to the control embryos (Fig. 2.2). The differences in mortality seen in the embryonic, larval and juvenile stages after exposure to TBBPA, BPA or TBBPA DME demonstrate that TBBPA exposure (0.75 μM) was lethal at doses a magnitude lower (10 or 15 μM) than its metabolites (Table 2.1).

2.4.2. Hatching Success Following Exposure to TBBPA, BPA and TBBPA DME. The time to hatch was measured after zebrafish embryos exposure to TBBPA, BPA or TBBPA DME. TBBPA exposure (0.75 μM) displayed a significant delay in time to hatch as compared to control embryos (Table 2.1). No difference in the time to hatch was observed with TBBPA DME exposed embryos at 10, 5, and 1 μM as compared to control embryos (Table 2.1). Hatching was significantly delayed at all concentrations of BPA exposure (Table 2.1). Therefore, both TBBPA and BPA exposure delayed hatching of embryos, whereas TBBPA DME at the tested doses had no effect.

2.4.3. Lesion Occurrence in Exposed Zebrafish Embryos. Pericardial (PC) edema, yolk sac (YS) edema, and hemorrhage were observed at all concentrations of TBBPA, BPA and TBBPA DME, as shown in Table 2.1. Representative photographs of the developing embryo and lesions resulting from exposure are shown in Fig. 2.3.

Rank sum analysis was performed on data comparing control to individual chemical doses for each lesion. TBBPA exposure (0.75, 1.5 and 3 μM) resulted in a significant occurrence of PC edema compared to control (Table 2.1). A significant increase in YS edema and hemorrhage was observed at 0.75 and 1.5 μM TBBPA (Table 2.1). For BPA exposed embryos, the incidence of PC and YS edema was significant at the 15 mM dose, whereas the hemorrhaging observed was significant at all doses of BPA and TBBPA DME (Table 2.1). TBBPA DME resulted in significant YS edema at all concentrations and PC edema was observed at 5 and 10 μM (Table 2.1). These data indicate that the potency of TBBPA as compared to BPA or TBBPA DME is greater due to the numbers of lesions seen at doses of TBBPA approximately 10 fold lower than doses used for BPA or TBBPA DME exposure. However, TBBPA DME did result in significantly more vascular lesions than BPA at comparable molar concentrations.

One of the most common developmental lesions following chemical exposure to embryos is disruption of craniofacial development. To determine if there were effects of TBBPA, BPA, or TBBPA DME exposure on the craniofacial features in developing zebrafish embryos, embryos were exposed to TBBPA (0.75 μM), BPA (10, and 15 μM) and TBBPA DME (5 and 10 μM) for 7 days, and then stained with alcian blue. Intraocular distance (ID), lower jaw length (LJL) and ceratohyal cartilage length (CCL) were measured (Fig. 2.4). Embryos exposed to BPA at 15 μM exhibited a significant

increase in intraocular distance (Fig. 2.4), whereas embryos exposed to TBBPA, BPA at 10 μM (data not shown) or TBBPA DME at 10 or 5 μM showed no significant difference in ID compared to control. There were no significant craniofacial differences compared to control for any of the compounds and doses tested in LJL and CCL (Fig. 2.4).

Exposure of zebrafish embryos to TBBPA resulted in trunk edema, tail malformation, and slow heart beat that were not observed following exposure to BPA or TBBPA DME (Table 2.1 and Fig. 2.3). These lesions were most severe following exposure to TBBPA at 3 μM, with embryos demonstrating a lack of tail formation, and edema in the caudal region of the animal (Fig. 2.3). Exposure to 1.5 μM TBBPA resulted in similar lesions but with reduced severity, with the tail partially formed but was shorter and with a bent tip (Fig. 2.3). At 0.75 μM TBBPA exposure, the tail was fully formed, but was curved (Fig.2.3). The heart beat was significantly slower following TBBPA (1.5 and 3 μM) exposure at 48 hpf, and 72 hpf as compared to control embryos (Fig. 2.5). However, embryos exposed to TBBPA at 0.75 μM did not show any significant difference in heart beats compared to the control embryos (Fig. 2.5). Normal sinuous rhythm was observed in all exposed embryos, even those with reduced heart rate. Thus, TBBPA exposure at the two highest doses resulted in decreased heart rate, and stunted growth of the tail that were not seen with exposure to BPA or TBBPA DME.

2.4.4. TBBPA or TBBPA DME Exposure Leads to Alteration in MMP Gene Expression. The TBBPA induced lesions seen in the caudal regions of these embryos suggest a role for MMPs in the deformity. MMPs are known to be involved in tissue morphogenesis, wound repair and are crucial for proper embryo development (Murphy

and Nagase, 2008; Zagris, 2001). To ascertain whether MMPs play a role in the appearance of TBBPA induced developmental lesions, expression of the gelatinases MMP-2 and -9, and the collagenase MMP-13, after embryonic exposure to either TBBPA (0.75 μM) or TBBPA DME (5 μM) was examined. Exposure of developing zebrafish embryos to TBBPA resulted in a significant increase in MMP-2 by 1.25 fold at 24 hpf and slightly more than 2 fold at 48 hpf as compared to control (Fig. 2.6A). TBBPA exposure induced MMP-9 expression at 24 hpf by 3 fold at 24 hpf and by 3.5 fold at 48 hpf as compared to control levels (Fig. 2.6B). MMP-13 expression in response to TBBPA exposure increased at 24 hpf by 5.5 fold but reduced to slightly below normal at 48 hpf (Fig. 2.6C). Conversely, TBBPA DME exposure at 24 hpf and 48 hpf did not affect the expression of MMP-2 as compared to control (Fig 6A). MMP-9 expression was unchanged at 24 hpf after TBBPA DME exposure but was increased at 48 hpf by 1.5 fold relative to control (Fig. 2.6B). MMP-13 expression increased at 24 hpf by 1.5 fold with TBBPA DME exposure but was unchanged compared to that in control embryos at 48 hpf (Fig 2.6C). TBBPA exposure results in significant alteration in MMP expression at a much lower dose as compared to control in contrast to TBBPA DME exposure which did not appear to have a large impact on MMP expression, despite the dose being over 5 fold higher.

2.4.5. TBBPA or TBBPA DME Exposure Leads to Alteration in the Activity of MMPs in the Developing Zebrafish Embryo. It is important to examine MMP activity to confirm that the changes in gene expression are leading to changes in enzymatic activity. MMP activity was examined using an *in vitro* zymography assay in which native

fluoresceinated substrates (gelatin, degraded by MMPs-2 and -9 and collagen I, degraded by MMP-13) were incubated with lysates from zebrafish embryos exposed to vehicle, TBBPA or TBBPA DME. Extracts from 48 hpf TBBPA-exposed embryos demonstrated a significant increase (1.5 fold), in gelatin degradation (Fig. 2.7), a substrate for MMP-2 and -9. The 3 fold increase in degradation of gelatin correlates with the increase seen in MMP-2 and -9 mRNA expression after TBBPA exposure as does the expression of MMP-13 and the collagenase activity. Collagen I degradation was also increased (3.5 fold) in 48 hpf embryos in response to TBBPA exposure. TBBPA DME exposure resulted in significant increase in degradation of collagen I (2.5 fold) and gelatin (1.5 fold) as compared to control at 48 hpf (Fig. 2.7).

2.5. Discussion

TBBPA is transformed in the environment (Fig. 2.1) by indigenous microbes, through anaerobic reductive dehalogenation leading to BPA (Arbeli and Ronen, 2003; Arbeli *et al.*, 2006; Ronen and Abeliovich, 2000; Voordeckers *et al.*, 2002) or by aerobic O-methylation pathways forming TBBPA MME and TBBPA DME (George and Häggblom, 2008). These compounds persist and accumulate in the environment with the potential to cause toxicity to wildlife and humans. Although TBBPA DME has been detected in the environment (Sellström and Jansson, 1995; Watanabe *et al.*, 1983), it is a newly recognized metabolite of TBBPA for which little toxicological data is available. This study presents the first investigation comparing the toxicological effects of TBBPA and its metabolites in the developing zebrafish embryo and suggests that the microbially mediated transformations result in compounds with lower acute toxicity. In this study,

TBBPA concentrations used for exposure were close to environmentally detected levels (as high as mg/kg in sediments near production plants (Hale *et al.*, 2006). The doses used for BPA and TBBPA DME exposure are approximately tenfold greater than that of TBBPA. Our data show an increase in embryo or larval mortality following developmental exposure to TBBPA or BPA. TBBPA DME exposure, however, did not result in death as compared to control embryos after one-week post fertilization. TBBPA proved to be 10 times more potent than BPA or TBBPA DME exposure, resulting in 100% mortality at 1.5 and 3 μM. This potency at low doses is important considering the levels of TBBPA detected in the environment.

In contrast to the embryonic and larval mortality measured in this study, all compounds elicited mortality during the juvenile stage. In this study, long-term survival post-hatch in exposed embryos supported the findings from previous work examining post-hatch survival of embryos whose parents were exposed to 1.5 μM TBBPA (Kuiper *et al.*, 2007b). After 28 dpf, survival of TBBPA DME exposed embryos, as well as those exposed to TBBPA and BPA, was significantly reduced (Fig. 2.2). Our data show that TBBPA DME and BPA was less acutely toxic than TBBPA. TBBPA DME exposure does not cause embryonic mortality, but the sublethal lesions described below may explain the delayed mortality. The lowered juvenile survival is important as TBBPA DME has a higher log K_{ow} than TBBPA and therefore the potential to accumulate in fatty tissues and to be a persistent compound in the environment.

TBBPA, BPA and TBBPA DME exposure resulted in a variety of developmental lesions in the embryos, such as a delay in time to hatch, and vascular lesions (edema and hemorrhage). A delay in the time to hatch is an indication of the chemicals effects on

critical biochemical and developmental pathways necessary for the embryo's ability to free itself from the chorion (Nechaev and Pavlov, 2004; Sano *et al.*, 2008), and is a crucial developmental benchmark to overall larval survival of aquatic species in the environment. Our data show that exposure to TBBPA and BPA resulted in a delay in time to hatch (Table 2.1), however this was not observed in embryos exposed to TBBPA DME. Exposure to TBBPA or its metabolites caused dose dependent lesions, including PC edema, YS edema and hemorrhage in zebrafish embryos (Table 2.1). TBBPA exposure exhibited the highest potency with the highest presence of significant lesions at concentrations an order of magnitude lower than that of its metabolites. A number of common chemically induced lesions were observed (Fig. 2.3), and included edema, hemorrhage and curved tail. These lesions were previously reported for embryos exposed to similar concentrations of TBBPA and BPA (Hu *et al.*, 2009; Kishida *et al.*, 2001). These data are in keeping with studies from other laboratories showing malformed tails in zebrafish in response to exposure to BPA (68 μM) (Duan *et al.*, 2008). Environmental concentrations of BPA are typically in the nanomolar range, so it is unlikely that aquatic species would typically be exposed to such a high concentration. However, the appearance of truncated tails at higher doses of BPA exposure, resembling the tail malformations seen with TBBPA in this study suggest a similar mechanism of action of BPA and TBBPA.

Alcian blue staining of cartilage was used to measure the craniofacial features of the exposed larvae after 7 days of exposure. Our data show that there are no significant alterations in the lower jaw length or ceratohyal cartilage in the exposed embryos as compared to control (Fig. 2.4). For the intraocular distance, the only significant alteration

occurred in embryos exposed to BPA at 15 μM (Fig. 2.4). This increased distance could be due to the edema seen in the embryo during development and not necessarily a teratogenic effect. This finding demonstrates that exposure to TBBPA, BPA or TBBPA DME does not alter craniofacial development in zebrafish embryos.

The tail and trunk lesions seen in previous BPA exposure studies (Duan *et al.*, 2008), and in this study with TBBPA exposure, suggest an alteration in proper caudal formation that could be related to altered MMP expression. Previous studies illustrate a role of MMPs in the proper formation of the caudal axis (Zhang *et al.*, 2003a, b) and it is known that the Wnt pathway, which regulates caudal development, also regulates MMP expression (Harrington *et al.*, 2007; Karow *et al.*, 2008). Furthermore, TBBPA exposure is also known to cause the production of reactive oxygen species, which can play a role in the regulation of MMP expression (Reistad *et al.*, 2005; Reistad *et al.*, 2007; Svineng *et al.*, 2008). Knocking down MMP-13 expression in the developing zebrafish embryo results in body axis curvature, kinked tail and other malformations (Hillegass *et al.*, 2007), demonstrating a role for this enzyme in proper tail formation. In our study, expression of MMP-9 and -13 increased after TBBPA exposure to a greater degree than was seen with TBBPA DME exposure (Fig. 2.6). MMP-2 expression in TBBPA DME exposed embryos was similar to that of control embryos. MMP activity as measured by in vitro zymography using gelatin and collagen I as substrates was significantly increased at 48 hpf as compared to control (Fig. 2.7). Additionally, the mRNA expression increase in TBBPA exposed embryos was followed by an increase in the degradation of collagen I (1.5 fold) and gelatin (3.5 fold) (Fig. 2.7). One possible explanation is that the tail malformations and trunk edema seen during development are a result of increased MMP

expression and activity. Although we also observed an increase in MMP-9 following TBBPA DME exposure, no truncation in tail structure was observed. MMP-2 and -9 expression patterns may explain the difference in caudal development after TBBPA and TBBPA DME exposure since both enzymes are known for playing a role in promoting cellular migration and both may be required to cause the truncated tail (Murphy and Nagase, 2008). Altered expression and activity of MMPs could explain the lesions observed here and those reported by Kuiper et. al (2007). Additionally, studies examining TBBPA exposure on the effect of tadpole (Pacific Tree Frog, *Pseudacris regilla*) metamorphosis demonstrate an upregulation of MMP-9 mRNA and also abnormal timing of tail resorption during metamorphosis (Veldhoen *et al.*, 2006).

Our hypothesis that increased MMP expression and activity are related to the lesions observed in the TBBPA-exposed embryos is supported by the finding that glucocortocoid exposure of developing zebrafish also increase MMP-2, -9 and -13 expression and activity and result in similar lesions such as impaired vascularization and tail malformations similar to that seen with TBBPA exposure (Hillegass *et al.*, 2007, 2008). The data from our studies seemingly contradict the results from the morpholino knockdown of MMPs, with both resulting in similar phenotypes of the caudal region. However, this suggests that the dysregulation of MMPs is important in leading to a malformed tail. Morphogenesis is a complex process relying on the critical timing of cell proliferation, cell migration and ECM remodeling. It is likely that this dysregulation is sufficient to alter proper ECM remodeling during development of the zebrafish embryo. Taken together, these data support our hypothesis that the increase in MMP expression

due to TBBPA exposure is related to the tail malformations and altered heart beat detected in the developing embryo.

The data presented here demonstrate that developmental exposure to TBBPA, BPA or TBBPA DME result in a reduction in embryo survival. However, there are differences in their potency, with BPA and TBBPA DME being less acutely toxic than the parent compound. However, TBBPA DME does appear to be slightly more toxic than BPA in causing embryonic vascular lesions. Further, the alteration of the expression and activity of MMPs is likely playing a role in these developmental lesions due to their involvement in vascularization and caudal development. The tail malformations in TBBPA exposed embryos, may also be influencing the time to hatch as the movement of the embryos is limited with improper tail formation. These data also demonstrate that developmental exposure to these compounds results in reduced survival one-month post exposure. Ongoing use of TBBPA and increasing environmental contamination of both TBBPA due to production, and TBBPA DME due to microbial metabolism, warrants further study. Specifically, the mechanisms underlying the developmental lesions, notably the trunk and tail lesions in TBBPA exposed embryos, and potential longer-term chronic effects must be examined in order to fully appreciate the overall risk and toxicity of these compounds.

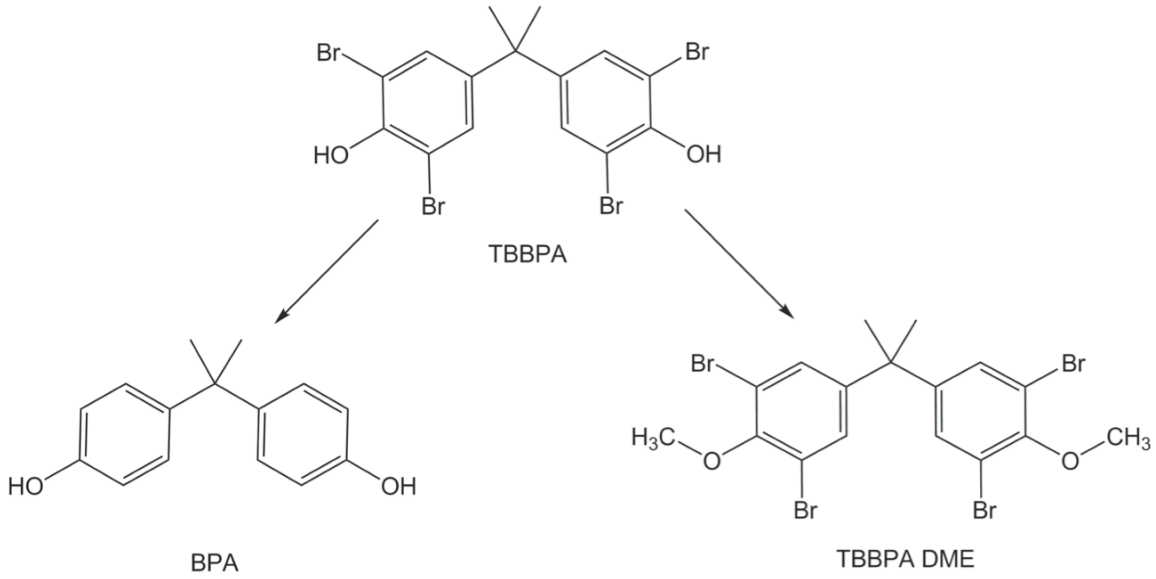

Fig. 2.1.: Microbial induced transformations of tetrabromobisphenol A. The microbial mediated transformations of TBBPA through anaerobic debromination to BPA or aerobic O-methylation to TBBPA.

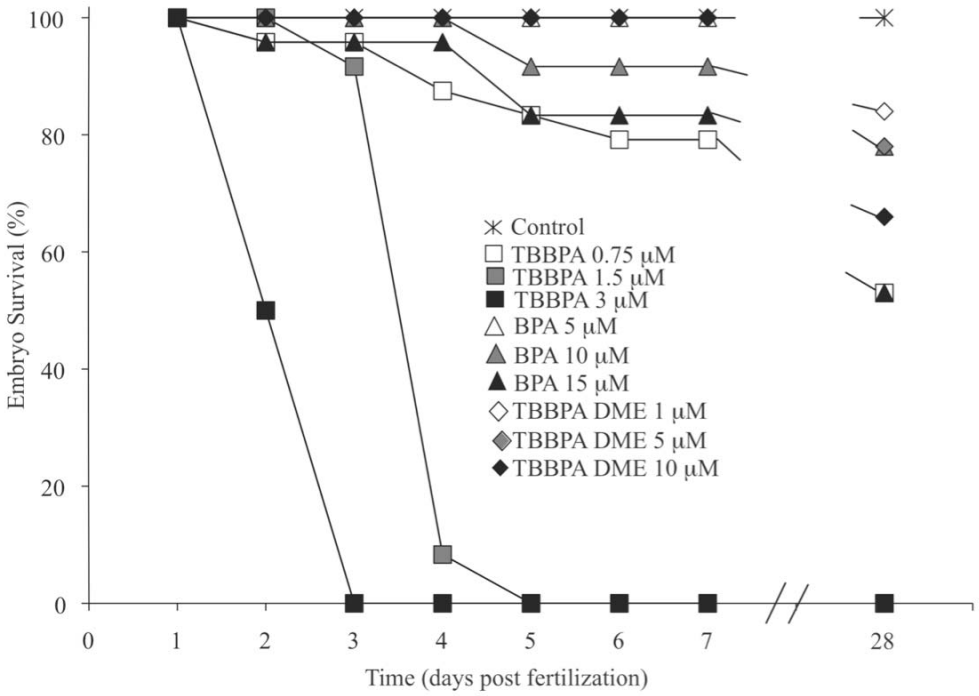

Fig 2.2.: Survival curve after exposure to TBBPA, BPA, or TBBPA DME. Zebrafish embryos were exposed to TBBPA (0.75, 1.5, or 3 mM), BPA (5, 10, 15 mM), TBBPA DME (1, 5, 10 mM) or vehicle (DMSO) and observed for 28 days. Data are representative of three different experiments. (n=25)

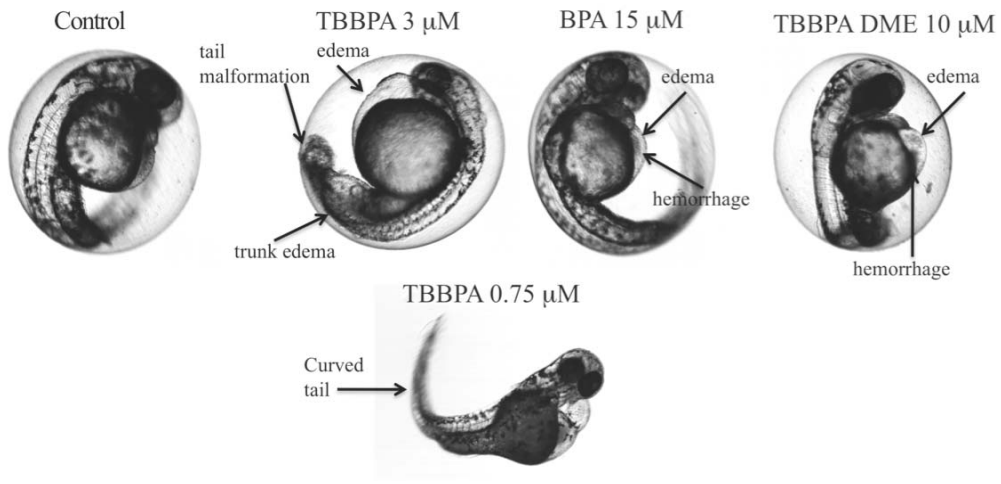

Fig. 2.3: Representative lesions due to exposure to TBBPA and its metabolites.
Representative photographs are shown illustrating lesions due to exposure to TBBPA (3 mM and 0.75 mM), BPA, TBBPA DME and vehicle (DMSO). The photographs of TBBPA at 3mM, BPA 15 mM, and TBBPA DME 10mM are of exposed embryos at 48 hours post fertilization. The photograph of the embryo exposed to TBBPA at 0.75 mM illustrates the tail malformation at a lower dose of exposure resulting in decreased severity. Arrows point to the representative lesions for each compound, including pericardial (PC) edema, hemorrhage, trunk edema and tail malformation.

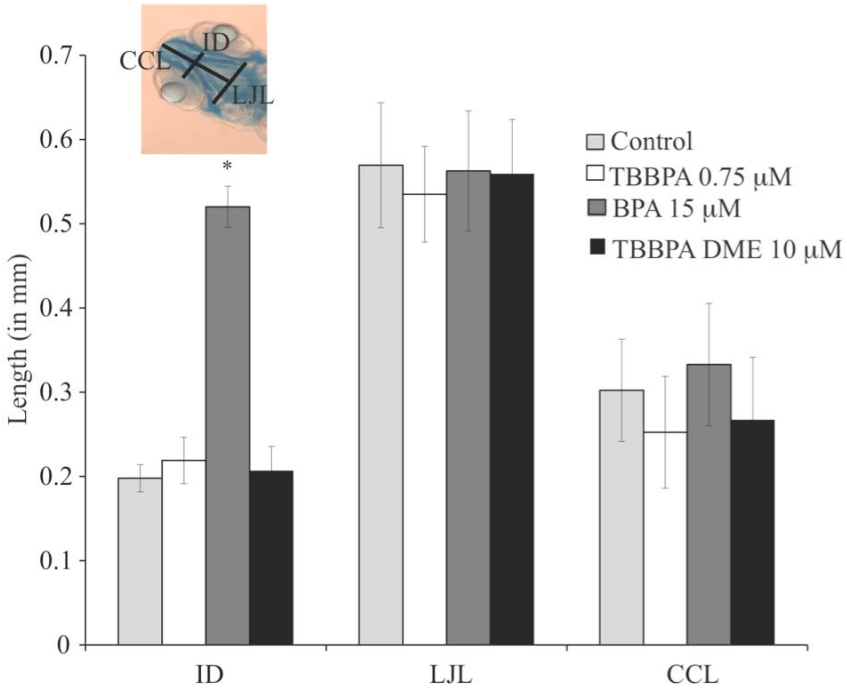

Fig. 2.4: Staining of cartliage in TBBPA, BPA, or TBBPA DME exposed embryos. Embryos were exposed in TBBPA (0.75 mM), BPA (10, and 15 mM), TBBPA DME (5 and 10 mM) or vehicle (DMSO) for 7 days, when they were euthanized and stained accordingly. Intraocular distance (ID), lower jaw length (LJL) and certohyal cartilage length (CCL) were measured. A representative photograph is shown depicting the location of each measurement. Error bars represent standard deviation and the asterisk denotes significance relative to control with a $p \leq 0.05$.

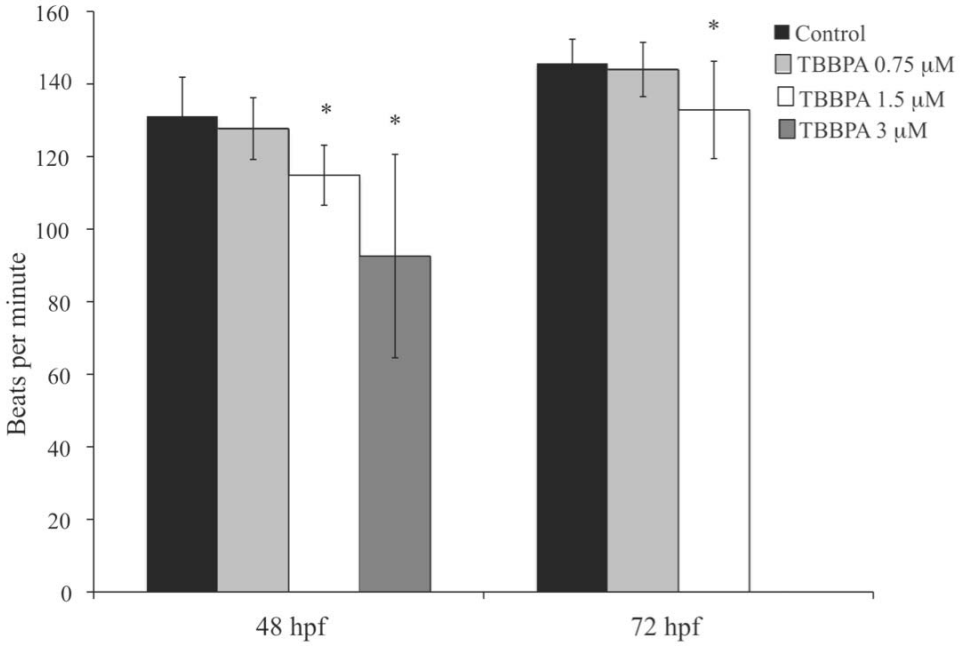

Fig. 2.5: Effect of TBBPA exposure on zebrafish heart rate. Embryos were exposed to TBBPA at 0.75, 1.5 and 3 mM. Data are the mean heart rate at 48 and 72 hpf. Error bars denote standard deviation and asterisks denote significance with a $p \leq 0.05$ as compared to control.

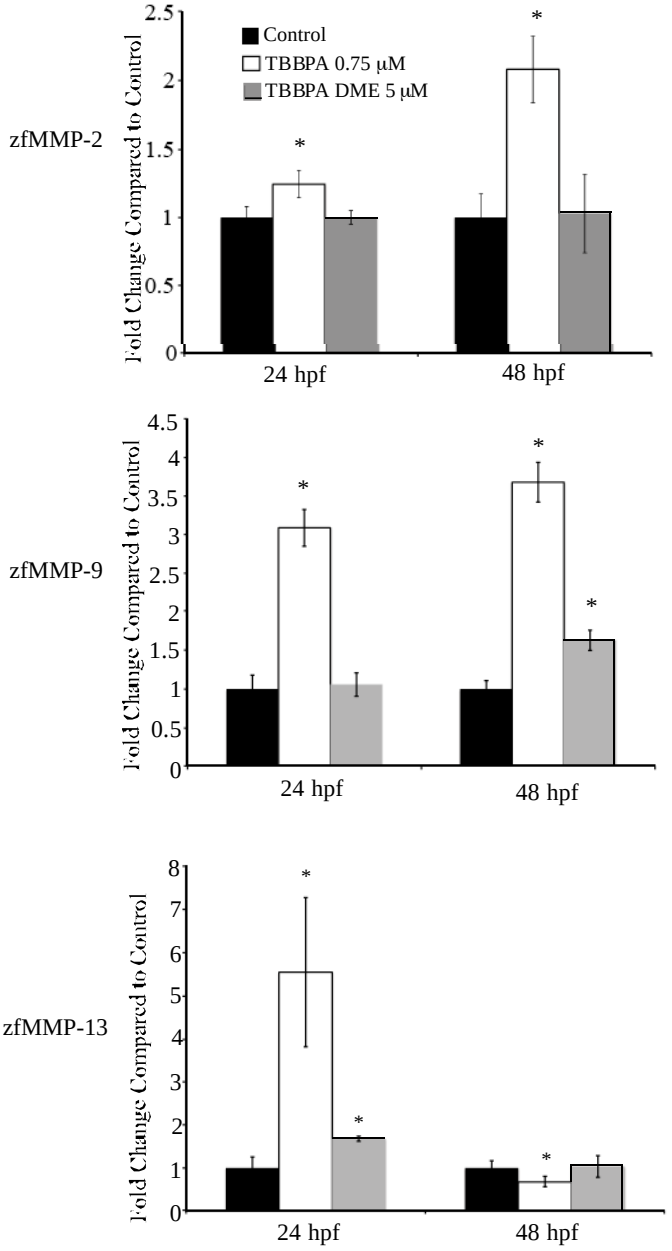

Fig. 2.6: Expression of MMP-2, -9, and -13 after exposure to TBBPA or TBBPA DME. Expression levels were measured by Quantitative Real Time PCR on total RNA isolated from embryos exposed to either vehicle, TBBPA at 0.75 mM or TBBPA DME at 5mM at 24 and 48 hpf. Data are represented as fold change relative to control. The top panel is MMP-2 expression, middle panel is MMP-9 expression and the lower panel is MMP-13 expression. Data were normalized to the 28S rRNA gene and were performed in triplicate. Data shown are representative of three separate experiments. Error bars denote standard deviation and asterisks denote significant with a $p \leq 0.05$ as compared to control.

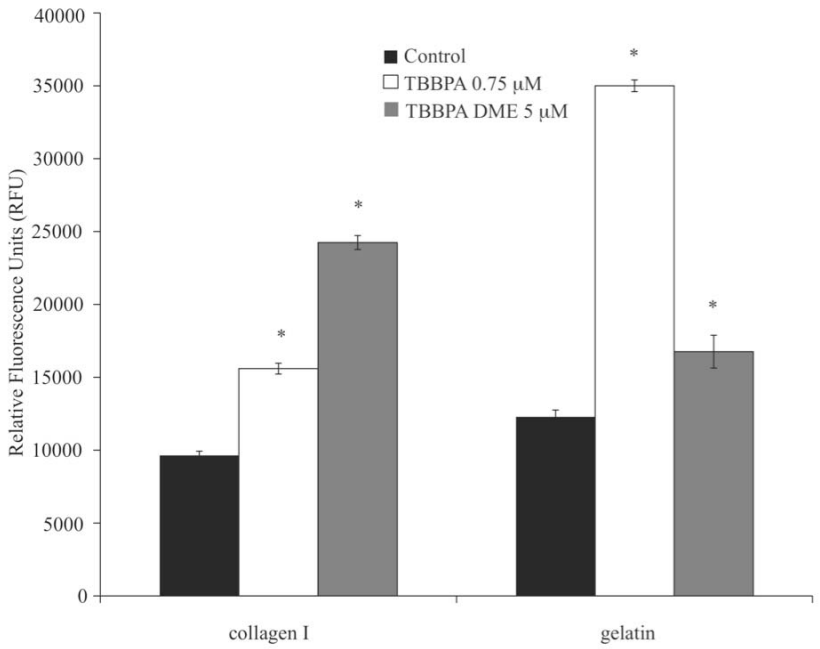

Fig. 2.7: Activity of gelatinases and collagenase after TBBPA or TBBPA DME exposure. Lysates from zebrafish embryos were obtained at 48 hours post fertilization. *In vitro* zymography was used to examine the degradation of gelatin and collagen I after TBBPA at 0.75 mM or TBBPA DME at 5 mM embryonic exposure. Data are normalized to a no enzyme control. Data are representative of three separate experiments. Error bars denote standard deviation and asterisks denote significance with a $p \leq 0.05$ relative to control.

2.7. Tables

Table 2.1:

Developmental lesions (%) observed in zebrafish (N=24 per treatment dose) exposed to TBBPA, BPA or TBBPA DME

Lesion	Control	TBBPA 0.75 μM	TBBPA 1.5 μM	TBBPA 3 μM	BPA 5 μM	BPA 10 μM	BPA 15 μM	DME 1 μM	DME 5 μM	DME 10 μM
Death	0	17	100*	100*	8	8	4	0	0	0
PC edema	8	63*	100*	50*#	33	38	46*	33	50*	46*
YS edema	4	67*	100*	29	29	17	38*	50*	46*	46*
Trunk edema	0	0	54*	0	0	0	0	0	0	0
Hemorrhage	4	75*	63*	0	38*	54*	50*	50*	58*	79*
Tail malformation	0	21	100*	50*#	0	4	0	0	0	0
Curved tail	0	17	46*	0	0	4	25	21	29	21
Time to hatch	3±0	4±0.4*	------	-----	4±0.4*	4±0*	4±0*	3±0.5	4±0.5	3±0.5

[a] Zebrafish embryos were exposed to TBBPA, BPA, TBBPA DME or vehicle (DMSO) in 4 mL vials for 7 days. Lesions were recorded and presented as lesion percentage occurring at any point during the seven day experiment. All data are shown as percentages and data are representative of three replicate experiments.

[b]* denote significance as compared to control with a $p \leq 0.05$. # denotes complete mortality in three days.

3.0. Microbially mediated O-methylation of bisphenol A results in mono- and dimethyl ether metabolites with increased toxicity to the developing zebrafish (*Danio rerio*) embryo

3.1. Abstract

O-methylation of xenobiotics, specifically phenolics and other aromatic compounds is mediated by a variety of microorganisms and is considered an alternative mechanism to biodegradation. Bisphenol A (BPA) is used in the manufacture of plastics, and has been identified in various environmental matrices, biota and human serum and breast milk. Here I demonstrate that the bacterium, *Mycobacterium vanbaalenii* strain PYR-1, in addition to strains of other *Mycobacterium* species, are able to O-methylate BPA to its mono- and dimethyl ether derivatives (BPA MME and BPA DME, respectively). The O-methylation of BPA results in metabolites of unknown toxicological impact. To evaluate the relative toxicity of BPA and its mono- and dimethyl ether derivatives, developing zebrafish embryos were exposed to BPA, BPA MME and BPA DME and examined for differences in survival and developmental lesions. BPA MME and BPA DME were more toxic than BPA, resulting in increased mortality at 5 and 28 days post fertilization and lower LC50 values. These data illustrate a new mechanism for microbial transformation of BPA, producing metabolites warranting further study to understand their prevalence in the environment.

3.2. Introduction

Bisphenol A (BPA, 2,2-bis (4-hydroxyphenol) propane) is widely used in polycarbonate plastics, dental sealants, water supply pipes and epoxy resins and is known to leach from baby bottles, food containers and reusable water bottles (Vandenberg *et al.*, 2009; Wetherill *et al.*, 2007). BPA is also used in the epoxy lining in metallic cans and has the potential to leach into the foods. Production of BPA is approximated at greater than 2.3 billion pounds per year in the United States.

The majority of exposures to BPA are through food/ water ingestion, but BPA has also been detected in dust samples and atmospheric samples due to production release (N.T.P, 2008; Vandenberg *et al.*, 2009). BPA release into the environment occurs through wastewater discharge in manufacturing plants, since BPA is not completely degraded during wastewater treatment (Kang *et al.*, 2006a). Also, BPA leaching from drainage, water and sprinkling systems has resulted in levels from 4 to 1730 mg/L (Kang *et al.*, 2007). BPA has been detected in landfills, fresh and marine waters range from 1.3 to 17,200 ng/ml, in river waters and 1 to 213 ng/g (Kang *et al.*, 2007; Kang *et al.*, 2006a). BPA can leach from the epoxy linings inside metallic cans, and from plastics used in baby bottles, infant formulas and other disposable food containers (Ackerman *et al.;* Kubwabo *et al.*, 2009; Vandenberg *et al.*, 2007; Vandenberg *et al.*, 2009). BPA has recently been detected in human colostrum at concentrations in the ng/mL range (Kuruto-Niwa *et al.*, 2007). Additionally, BPA has been detected in serum samples and urine samples of college students, correlating with the usage of polycarbonate beverage containers (Carwile *et al.*, 2009; Dirtu *et al.*, 2008). These studies indicate the prevalence

of BPA exposure to humans and indicate a need to more fully understand the fate of BPA in the environment and the resulting effects on toxicity to humans and other organisms.

BPA contamination in the environment can be a result of manufacturing waste, disposal of BPA containing products, or it can be produced as a metabolite of the reductive dehalogenation of tetrabromobisphenol A (TBBPA) by microorganisms in anaerobic environments (Arbeli and Ronen, 2003; Arbeli *et al.*, 2006; Ravit *et al.*, 2005; Voordeckers *et al.*, 2002). Microorganisms in the environment play an important role in the biodegradation of BPA. BPA can be mineralized to CO_2 under aerobic conditions by a variety of bacteria including, *Pseudomonas*, *Achrobacter*, *Streptomyces*, *Novosphingobium*, and *Sphingomonas* strains (Kang *et al.*, 2004; Lobos *et al.*, 1992; Masuda *et al.*, 2007; Toyama *et al.*, 2009; Yamanaka *et al.*, 2008; Zhang *et al.*, 2007). BPA degradation has been demonstrated in sea- and river water, sediments, rhizosphere sediments, compost leachate and sewage sludge (Danzl *et al.*, 2009; Kang *et al.*, 2004; Masuda *et al.*, 2007; Toyama *et al.*, 2009; Zhang *et al.*, 2007). In contrast, under anaerobic conditions BPA is recalcitrant (Voordeckers *et al.*, 2002).

BPA is a known endocrine disruptor, exerting effects mainly through nuclear hormone receptors. BPA is an estrogen receptor antagonist and can have the potency of estradiol (Kang *et al.*, 2007; Kitamura *et al.*, 2005b; Li *et al.*, 2009; Samuelsen *et al.*, 2001; Vandenberg *et al.*, 2009; Wetherill *et al.*, 2007). BPA is also an androgen receptor antagonist, preventing androgen-dependent transcription once bound to the androgen receptor (Vandenberg *et al.*, 2009). BPA exposure *in utero* can result in increased prostate size and weight (Wetherill *et al.*, 2007). BPA exposure can also affect proper progesterone receptor signaling (Samuelsen *et al.*, 2001). Studies in the MCF7 human

breast cancer cell line have illustrated that BPA is genotoxic through an estrogen-mediated mechanism at concentrations in the micromolar range (Iso *et al.*, 2006). Additionally, BPA exposure in MCF7 cells alters the Src/Raf/ Erk signaling pathway (Kabil *et al.*, 2008). BPA exposure studies in mice have shown disruption of early oogenesis and an increase in preneoplastic mammary lesions in prenatally exposed females (Murray *et al.*, 2006; Susiarjo *et al.*, 2007). Additionally, BPA concentrations in human urine correlate with increased risk for cardiovascular disease, oxidative stress, inflammation and diabetes (Lang *et al.*, 2008; Yang *et al.*, 2009b). Taken together, these studies illustrate the wide reaching effects of BPA exposure and demonstrate the importance of understanding the fate of BPA in the environment due to its potent endocrine disrupting potential.

O-methylation is a ubiquitous reaction characterized by the transfer of a methyl group from S-andenosyl methionine (SAM) and transferring it to an oxygen atom. This transformation is an alternative to biodegradation and has been detected in animals, fungi, plants and bacteria for several different phenolic compounds including flavenoids (Kim *et al.*, 2006a; Kim *et al.*, 2006b; Kim *et al.*, 2004; Yang *et al.*, 2004). This study is the first to demonstrate microbially mediated O-methylation of BPA to both the monomethyl (BPA MME) and dimethyl ether (BPA DME) forms under aerobic conditions by several *Mycobacterium* species. In addition, we demonstrate that these novel metabolites of BPA are more toxic in the developing zebrafish embryo than the parent compound. This study indicates that this transformation of BPA could occur in the environment, and warrants further study.

3.3. Materials and Methods

3.3.1. Chemicals. Bisphenol A (BPA) was obtained from Sigma Aldrich (>98% purity). Bisphenol A dimethyl ether (BPA DME, [CAS# 1568-83-8], purity >99% by GC-MS) was obtained from Acros Organics. To synthesize BPA MME, bisphenol A was refluxed over night in an excess of methyl iodide and potassium carbonate buffer (Knapp, 1979). The resulting mixture was approximately 50% BPA MME and 50% BPA DME by gas chromatography-mass spectrometry (GC-MS), with a small remaining amount of BPA (data not shown). The reaction product was then evaporated to dryness and dissolved in benzene. BPA MME and BPA DME were purified in a silica gel column by first eluting BPA DME with 100% benzene, followed by 5-10% acetone in benzene to elute the BPA MME. Fractions were collected, evaporated to dryness and screened by thin layer chromatography for purity (>95%). Samples were analyzed by GC-MS to confirm purity (>99%).

3.3.2. Bacterial strains and medium. *Mycobacterium vanbaalenii* strain PYR-1 (Khan *et al.*, 2002) was obtained from Dr. Carl Cerniglia (National Center for Toxicological Research, US Food and Drug Administration) and was grown in Brain Heart Infusion (BHI) broth at 28 °C with shaking. *Mycobacterium chlorophenolicum* strain PCP-1 (Haggblom *et al.*, 1994) and *Mycobacterium fortuitum* CG-2 (Häggblom *et al.*, 1988) were grown in R2A broth at 28 °C with shaking. When needed, cultures were grown on R2A agar plates and incubated at 28 °C. *Mycobacterium smegmatis* strain mc^2155, obtained from Dr. Nancy Connell (University of Medicine and Dentistry of NJ- NJ

Medical School), was grown in 7H9 supplemented with albumin, dextrose and sodium chloride with shaking at 37 °C.

3.3.3. Zebrafish strains and husbandry. The AB strain of zebrafish (*Danio rerio*) was used for all experiments, and was obtained from the Zebrafish International Resource Center (ZIRC). Zebrafish were bred and maintained in a recirculating Aquatic Habitat System utilizing a light: dark cycle of 14:10 hours, respectively. All experiments in this study were conducted according to a protocol approved by the Rutgers University Animal Care and Facilities Committee.

3.3.4. O-Methylation of bisphenol A by *Mycobacterium spp.* *M. vanbaalenii* PYR-1, *M. fortuitum* CG-2, *M. chlorophenolicum* PCP-1 and *M. smegmatis* mc^2155 were inoculated into 5 mL of Brain Heart Infusion Broth and grown at 28 °C with aeration for 48 hours. Once the culture reached an OD_{680} of 0.6 they were diluted 1:10 and grown to an OD_{680} of 1.0 at 28 °C with aeration. One mL was inoculated into sterile glass vials with Teflon cap liners, and spiked with 100 μM of BPA with and without 100 mL of 1 mg/ml of chloramphenicol to inhibit protein synthesis. Cultures were incubated at room temperature with shaking and samples were taken at specific timepoints and stored at -20 °C. The samples were acidifed with hydrochloric acid and 20 μM 2,4,6-tribromophenol was added as an internal standard followed by extraction with hexane for one hour. The hexane phase was removed and analyzed by gas chromatography-mass spectrometry (GC-MS).

3.3.5. Analytical Methods. Gas chromotography- mass spectrometry was conducted using an Agilent Gas Chromatograph Series 6890 with a Mass Selective Detector Series 5973N equipped with a splitless injector, and a HP-5MS column (30 m length x 0.25 mm i.d x 0.25 μm film thickness). The injector temperature was 250 °C with an injection volume of 1 μl, the oven temperature started at 70°C, ramping up to 300 °C by 15 °C/minute, and held at 300 °C for 5 minutes. SCAN mode was used initially in the range from 32 to 600 m/z. Once the predominant ions were identified, analysis was performed using selected ion monitoring (SIM) to detect and quantify BPA (213 and 228 m/z), BPA MME (227 and 241 m/z) and BPA DME (241 and 256 m/z).

3.3.6. Collection and Treatment of Zebrafish Embryos. Zebrafish embryos were collected at approximately 3 hours post fertilization (hpf) and were exposed in 4 mL glass vials to BPA (20, 10, 5, 2.5 and 1 mg/L), BPA MME (5, 2.5, 1, 0.5 and 0.25 mg/L), BPA DME (10, 5, 2.5, 1, and 0.5 mg/L) or dimethyl sulfoxide (0.15%) as the vehicle control, in sterile water. A static, non-renewal approach was used. The concentrations of BPA used in this study was similar to those used in previous zebrafish studies (Duan *et al.*, 2008; Kuiper *et al.*, 2007b; Muncke *et al.*, 2007). All doses were determined after initial dose response studies to obtain the optimal doses for exposure. One embryo was placed into each vial and observed every 24 hours for 7 days. Studies were conducted with 25 embryos per dose and all studies were repeated at least 3 times. Developmental lesions, including edema, hemorrhage, death and date of hatching, were recorded.

After 7 days, larvae were removed from vials and placed into a beaker containing system water and boiled wheat seeds. They were fed paramecium culture and AP100 for

the next 21 days. At 28 days post fertilization all remaining live larvae were counted and euthanized using an overdose of MS-222. The LC50, which is the dose required to cause mortality in 50% of the test population, and the standard error were determined using the probit method as described (Miller and Tainter, 1944).

3.3.7. Statistical Analyses. Sigma Stat Version 10.1 was used to determine the normality of the data, the power and the appropriate statistical test. The Mann-Whitney Rank Sum test was used to examine the hatching data. Chi square analysis was used for the lesion and mortality data. All data referred to as significant are $p \leq 0.05$.

3.4. Results

3.4.1. O-methylation of Bisphenol A. To determine whether microorganisms can O-methylate BPA, we utilized *Mycobacterium* strains known to O-methylate other hydroxylated aromatic compounds, such as polyaromatic hydrocarbons, 2,4,6-tribromophenol and tetrabromobisphenol A (TBBPA). *Mycobacterium* strains PYR-1, PCP-1, CG-2 and 155 were exposed to 100 μM BPA and analyzed for the production of the monomethyl and dimethyl ether forms of BPA. All strains used were able to O-methylate BPA (Figs. 3.1, 3.2 and Table 3.1). However, differences in the rate and extent of O-methylation were noted. Specifically, *M. fortuitum* CG-2 and *M. smegmatis* 155 only produced BPA MME after a 2 week incubation, no BPA DME was detected (Figs. 3.1, 3.2 and Table 3.1) *M. vanbaalenii* PYR1 or *M. chlorophenolicum* PCP1 converted approximately 5% of BPA to BPA MME and less than 1% of BPA to BPA DME (Fig. 3.1).

To test whether O-methylation was an inducible or constitutive reaction, cultures of *M. vanbaalenii* PYR-1 were spiked with BPA, with and without chloramphenicol to inhibit protein synthesis. The rate of BPA transformation in the cultures with chloramphenicol was near identical to that without, resulting in 5-7% transformation of BPA to BPA MME and BPA DME after 9 days (Fig. 3.2). These data illustrate that the O-methyltransferase is constitutively expressed in *M. vanbaalenii* PYR-1.

3.4.2. Mortality Following Exposure to BPA, BPA MME, or BPA DME.

The LC50 is calculated in toxicological studies to determine the concentration at which half the population will die following exposure to certain compounds. The LC50 values for BPA, BPA MME and BPA DME exposure were determined after 5 and 28 days post fertilization (dpf). These timepoints were chosen because they represent the embryonic (short-term survival) and larval (long-term survival), periods. At 5 dpf, BPA MME was more potent than BPA DME or BPA, with an LC50 of 0.66 ± 0.065 (Table 3.2). BPA DME is the next toxic with LC50 of 1.2 ± 0.06 followed by BPA 4 ± 0.37. In contrast, at 28 dpf, the LC50 for BPA DME was below 1 mg/L with total death of embryos exposed at this dose above 60%. Thus it is the most potent over the juvenile period of zebrafish development. Similar to the embryonic toxicity, the BPA MME (0.38 ± 0.05) was more toxic than BPA (1.8 ± 0.23) in juvenile zebrafish (Table 3.2).

Zebrafish embryos were exposed to BPA, BPA MME, or BPA DME and examined for dose related embryonic mortality. BPA DME exposure was more acutely toxic than BPA, resulting in 100% mortality in embryos exposed to 2.5, 5 or 10 mg/L at 4 dpf (Table 3.3). BPA MME was also more toxic than BPA, resulting in 100% mortality

of 5 mg/L exposed embryos at 2 dpf, and the 2.5 mg/L dose at 5 dpf (Table 3.3). BPA exposure resulted in 100% mortality at the 20 mg/L dose by day 2 and by day 5 for the 10 mg/L dose, but those doses are higher in comparison to the doses used for BPA MME and BPA DME. Additionally, BPA exposure at 5 mg/L resulted in significant mortality (60%) as compared to control at 5 dpf (Table 3.3).

In order assess the impact of BPA, BPA MME or BPA DME exposure on juvenile mortality we examined the number of animals surviving to 28 dpf. BPA exposure resulted in an increase in juvenile mortality for the 5 and 2.5 mg/L doses as compared to control, but not at the 1mg/L dose (Table 3.3). At 28 dpf, BPA DME exposure results in increased mortality at both the 1 and 0.5 mg/L doses with survival at 12 and 24%, respectively, as compared to survival of control embryos (Table 3.3). BPA MME exposure also resulted in significant increase in mortality in the 1, 0.5 and 0.25 mg/L doses as compared to control, resulting in embryo survival of 24, 52 and 60%, respectively (Table 3.3). These data show that BPA MME and BPA DME are more toxic than BPA to the developing zebrafish embryo with regards to embryonic and juvenile mortality.

3.4.3. Lesion Occurrence in Embryos Exposed to BPA, BPA MME or BPA DME.

Exposure to either BPA, BPA MME or BPA DME resulted in developmental lesions in the zebrafish embryo. BPA exposure resulted in significant PC and YS edema, hemorrhage and curved tail at 10 mg/L, and PC edema at 5 mg/L (Table 3.3). Lesions detected at other doses of BPA exposure were not different from control. BPA MME exposure (0.5, 1, and 2.5 mg/L) resulted in significant PC edema, with the 1 and 2.5 mg/L

doses also exhibiting significant YS edema and hemorrhage (Table 3.3). BPA MME (1 mg/L) exposed embryos had an increase in curved tails as compared to control embryos. BPA DME (0.5, 1, 2.5, 5, and 10 mg/L) exposed embryos exhibited significant PC and YS edema as compared to control embryos. Additionally, BPA DME at 0.5, 2.5, 5 and 10 mg/L exposed embryos exhibited an increase in hemorrhage as compared to controls (Table 3.3). The presence of curved tails was significant in BPA DME exposed embryos at 1 and 2.5 mg/L (Table 3.3). These data show that the O-methylated metabolites result in developmental lesions at a lower exposure dose than BPA exposure.

The lowest observable adverse effect level (LOAEL) indicates the doses needed to detect significant developmental malformations as compared to control. These levels assist in the ranking of the compounds as well as indicating levels of exposure at which development will be affected. The LOAEL was lower in BPA MME and BPA DME than in BPA exposed animals (Table 3.2). These data illustrate that the O-methylated metabolites of BPA are more toxic than BPA.

3.4.4. Hatching Success Following Exposure to BPA, BPA MME, or BPA DME. The time to hatch was measured in embryos exposed to BPA, BPA MME or BPA DME and compared to control embryos. BPA exposed embryos at 1, 2.5 and 5 mg/L exhibited a significant delay in their time to hatch (Table 3.3). BPA MME exposed embryos also exhibited a delay in time to hatch at the 1 mg/L dose, but there was no difference in the time to hatch for the 0.25 and 0.5 mg/L doses (Table 3.3). There was no difference in time to hatch for any dose of exposure to BPA DME (Table 3.3). Hatching of embryos exposed to BPA at 10 and 20 mg/L and BPA MME at 2.5 and 5 mg/L was not observed

due to mortality. Exposure to BPA or BPA MME result in differences in the time to hatch, but BPA DME exposure did not appear to affect this developmental process.

3.5. Discussion

O-methylation is a ubiquitous reaction, not limited to bacteria, but also catalyzed by plants, fungi and animals (Cho *et al.*, 2008; Do *et al.*, 2007; Huang *et al.*, 2008; Kim *et al.*, 2006a; Kim *et al.*, 2004; Kostrzewa-Susłow *et al.*, 2007; Shafiee *et al.*, 1994; Vidgren *et al.*, 1994; Wein *et al.*, 2002; Zhu *et al.*, 2009a). This type of transformation has been demonstrated for phenolic compounds such as, flavenoids, phenylpropanoids and benzyl isoquinoline alkaloids (Cho *et al.*, 2008). The O-methyltransferases catalyze the transfer a methyl group from S-adenosylmethionine (SAM) to a hydroxyl group, but the enzymes in bacteria have been poorly characterized to date. Several different bacterial and fungal species have been identified as O-methylators of xenobiotic compounds (Häggblom *et al.*, 1989a)REFS. *Mycobacterium vanbaalenii* strain PYR-1 used in this study has several putative O-methyltransferases and is capable of O-methylating high molecular weight polyaromatic hydrocarbons (Kim *et al.*, 2008; Kim *et al.*, 2004), so it was hypothesized that this organism could also O-methylate BPA. These studies illustrated that *M. vanbaalenii* PYR1 could O-methylate BPA and other phenolic compounds.

Earlier studies on O-methylation conducted using chloro- and bromophenols demonstrated a preference for the O-methylation of free hydroxyl groups that are flanked by a bulky substituent, such as a chlorine or bromine atom or methyl group (Allard *et al.*, 1987; George and Häggblom, 2008; Häggblom *et al.*, 1986; Häggblom *et al.*, 1989a;

Häggblom *et al.*, 1988). Recently, O-methylation of tetrabromobisphenol A (TBBPA) has been demonstrated with *M. chlorophenolicum* PCP1 (George and Häggblom, 2008), *M. vanbaalenii* PYR1 and other strains used in this study (unpublished data). The microbially mediated O-methylation of compounds such as TBBPA, with bromine substituents flanking the free hydroxyl group, occur at a faster rate than that of BPA, but O-methylation of BPA was observed with all strains of mycobacteria tested. The differences in O-methylation included slower rate thus only producing BPA MME.

To date, the function and purpose of O-methylation is not known, and thus additional study needs to focus on the differences amongst the strains. It was postulated that O-methylation is a detoxifying process (Neilson *et al.*, 1988), but as we show with our data in the developing zebrafish that this transformation can result in more toxic metabolites. The O-methylated metabolites of BPA are more toxic in the developing embryo than the parent compound BPA. Our data show that the BPA MME metabolite exhibited the greatest toxicity at 5 dpf compared to both BPA and BPA DME, but at 28 dpf BPA DME was the most potent with exposure resulting in less than 50% survival in all doses (Table 3.1). These data suggest that O-methylation of BPA contribute greatly to the toxicity of the compound during embryonic and juvenile development. In all, these data demonstrate that the O-methylated metabolites are of increased potency compared to BPA, resulting in decreased embryonic and long-term survival. The mechanism(s) for the difference in toxicity of BPA and its O-methylated derivatives is not yet known.

Exposure to BPA, BPA MME or BPA DME resulted in lesions, such as pericardial and yolk sac edema, and hemorrhage during embryonic development, that are commonly observed malformations after exposure to xenobiotics (Carney *et al.*, 2006;

Hill *et al.*, 2005) The lesions occurred less frequently with BPA, predominantly at the higher doses, with the lowest observable effect (LOAEL) for BPA exposure was calculated to be 5 mg/L. In contrast, the LOAEL for BPA MME and BPA DME was 0.5 mg/L (Table 3.2). Therefore, a 10 fold higher concentration required by BPA to elicit similar effects in the developing zebrafish embryo. In addition to the common lesions, a curved tail phenotype was detected in hatched larvae, which a lesion seen in previous studies from both our laboratory and others utilizing BPA exposure (Duan *et al.*, 2008) McCormick, *et al* submitted). The concentrations of BPA used in the Duan *et al* (2008) study were similar to those in our study, but at 20 mg/L only mortality was observed. The major difference in our study was an exposure period at 2 hpf as compared to 8 hpf. This may explain any contradictions between these studies, and also illustrates that the earlier the exposure, the more toxic and applicable to environmental concentrations. It is unlikely that developing organisms will be exposed to concentrations greater than 10 ppm (10 mg/L) in the environment. However, several of our doses are environmentally relevant due to detection of BPA at 1 ppm (Kang *et al.*, 2006b; Meesters and Schroder, 2002; N.T.P, 2008), which are the doses of BPA MME and BPA DME that can affect development in our studies.

Exposure to BPA (1, 2.5 and 5 mg/L) and BPA MME (1 mg/L) resulted in a delay in the time to hatch. The time to hatch is an important developmental marker for larval aquatic organisms, if altered signals a change in the biochemical and developmental pathways needed for the embryo to free itself from the chorion (Nechaev and Pavlov, 2004; Sano *et al.*, 2008). Interestingly, exposure to BPA DME caused greater mortality than BPA exposure, but did not affect the hatching ability of zebrafish embryos. These

data suggest that O-methylation is affecting biochemical pathways in the developing embryo that lead to the differences detected in the hatching potential after exposure.

This study is the first to demonstrate the ability of microbes to O-methylate BPA resulting in the production of two novel metabolites, BPA MME and BPA DME. These metabolites are of interest due to their increased lipophilicity and possible accumulation if produced in the environment. Further studies examining BPA contamination in the environment should also investigate whether these O-methyl transformation products are present. Since this transformation can be catalyzed by environmental mycobacteria, it is likely that soil/sediment samples may harbor these metabolites, or microbes capable of BPA O-methylation. Additionally, TBBPA can be O-methylated and also debrominated, so the potential for the TBBPA DME to be dehalogenated, resulting in BPA DME, needs to be elucidated. The importance in these transformations is the increased toxicity of BPA MME and BPA DME to developing zebrafish embryos.

3.6. Figures

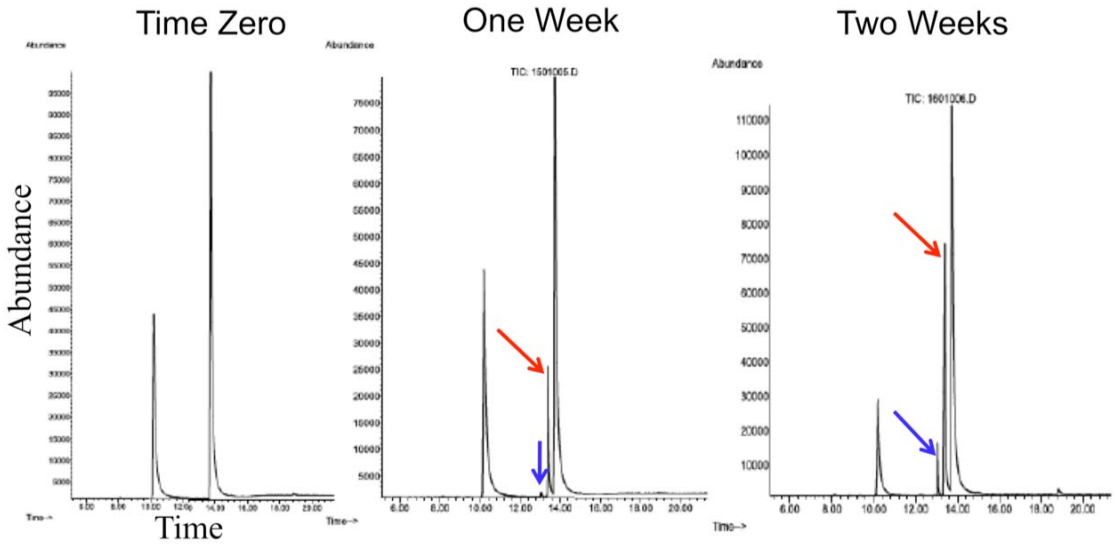

Fig. 3.1. Detection of *in vitro* O-methylation of BPA. *Mycobacterium vanbaalenii* strain PYR-1 was incubated with 100 µM BPA. Samples were taken at time zero, one and two weeks. Gas chromatograms show BPA at a retention time of 13.77min, BPA MME (red arrow) at 13.39 min, BPA DME (blue arrow) at 13.0 min and 2,4,6-tribromophenol at 10.17 minutes. These data are representative of the experiments performed with *M. chlorophenolicum, M. fortuitum and M. smegmatis.*

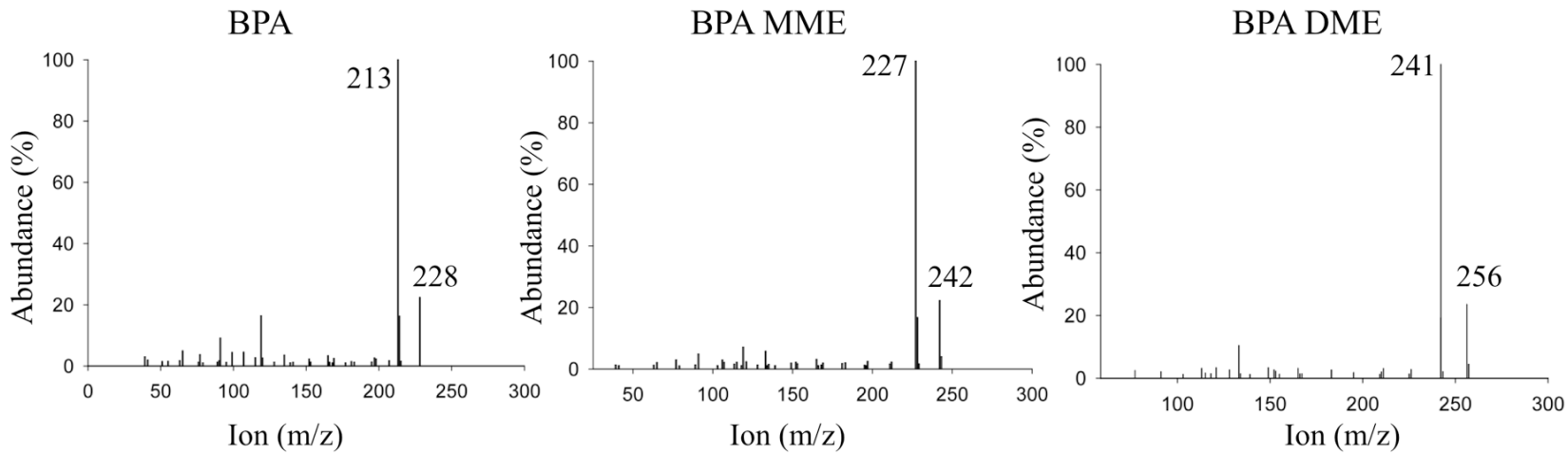

Figure 3.2. Mass Spectra of BPA, BPA MME, and BPA DME. The ions comprising at least 1% abundance are shown in the mass spectra for BPA, BPA MME, and BPA DME. The predominant ions of 213 (BPA), 227 (BPA MME) and 241 (BPA DME) were set to 100% and the abundance of all other ions was compared to these ions. The ions of 228, 242, and 256 correspond to the molecular weight of the compounds.

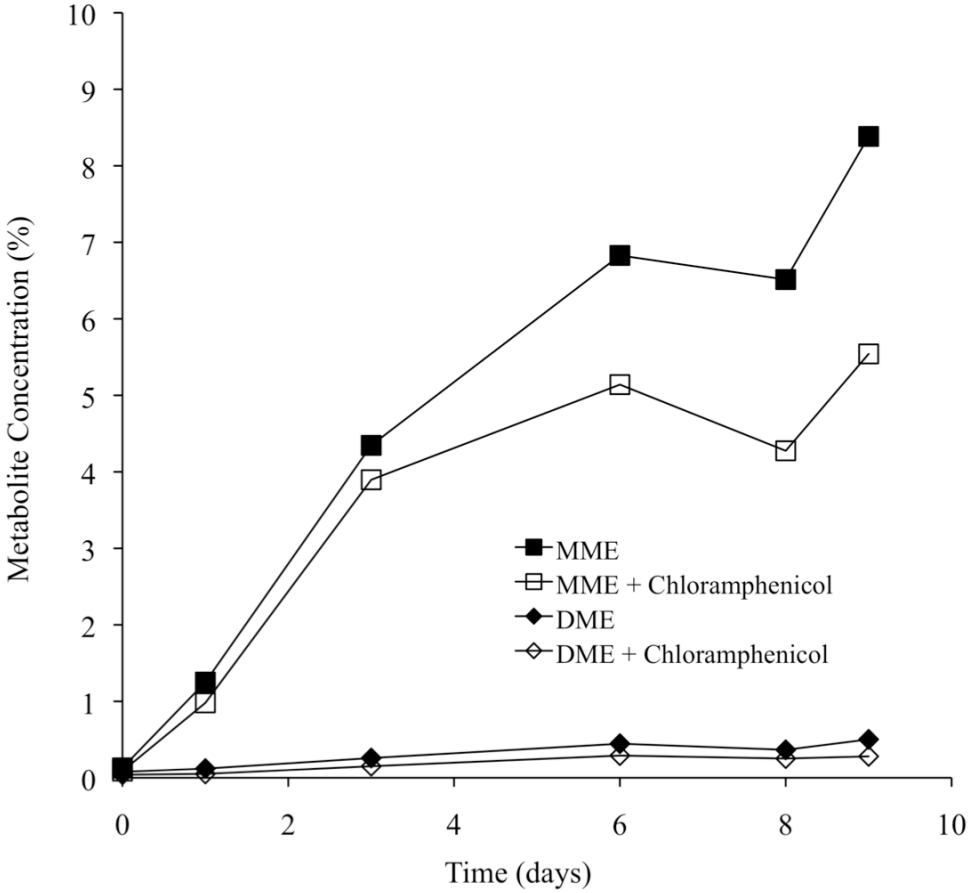

Fig. 3.3. O-methylation of BPA by M. *vanbaalenii* PYR1. *M. vanbaalenii* strain PYR-1 was incubated at 28°C with 100 μM BPA alone and with 1 mg/mL chloramphenicol to inhibit protein synthesis. Timepoints were taken over 9 nine days, extracted and analyzed by GC-MS for detection of the monomethyland dimethyl ether metabolites of BPA.

3.7. Tables

Table 3.1. O-methylation of BPA by Mycobacterium species.

O-methylation of BPA by *Mycobacterium* species

	BPA MME (%)		BPA DME (%)	
Species and Strain	1 week	2 weeks	1 week	2 weeks
M. vanbaalenii PYR1	7.5	8	0.4	0.4
M. chlorophenolicum PCP1	4.4	12	0.2	0.5
M. fortuitum CG2	0.46	0.91	0	0
M. smegmatis 155	1.1	1.2	0	0

Table 3.2. Dosage Levels for BPA, BPA MME, and BPA DME exposure to zebrafish embryos.

Short term vs. long term LC50 values and Lowest Observable Adverse Effect Levels

	5 dpf			28 dpf		
	BPA	MME	DME	BPA	MME	DME
LC50	4 mg/L	0.66 mg/L	1.2 mg/L	1.8 mg/L	0.38 mg/L	n.d
Confidence Interval	0.37	0.065	0.056	0.23	0.05	n.d
Lowest Observed Adverse Effect Level	5 mg/L	0.5 mg/L	0.5 mg/L	1 mg/L	0.25 mg/L	0.5 mg/L

Zebrafish embryos were exposed to BPA, BPA MME, BPA DME, or vehicle (DMSO) in 4 mL vials for 7 days, the surviving larvae were placed in beakers containing only system water. The LC50 was calculated based on the number of embryos dead at 5 dpf and the number of larvae dead at 28 dpf for each treatment dose. The confidence interval was determined using the methods of Miller and Tainter, 1944. The lowest observable adverse effect level was determined based on the presence of significant development lesions. These data are representative of three replicate experiments. N=25 per treatment dose, per experiment.

Lesion Occurrence (%)																
		BPA (mg/L)					BPA MME (mg/L)					BPA DME (mg/L)				
	Control	1	2.5	5	10	20	0.25	0.5	1	2.5	5	0.5	1	2.5	5	10
Death (5 days)	4	0	4	64*	100*	100*	24	12	16	100*	100	0	4	100*	100*	100*
Death (28 days)	8	4	48*	100*	-	-	40*	48*	76*	-	-	88*	76*	-	-	-
PC edema	0	20	12	56*	80*	-	20	36*	64*	64*	0	28*	40*	88*	80*	40*
YS edema	20	5	4	20	64*	-	28	40	80*	68*	0	52*	56*	88*	88*	68*
Hemorrhage	0	10	4	4	40*	-	16	20	48*	60*	0	32*	8	80*	88*	68*
Curved tail	4	15	20	8	-	-	0	12	76*	-	-	0	36*	32*	8	0
Time to Hatch	2.9 ± .4	3.2 ±0.4*	3.2 ± 0.5*	3.5 ± 0.7*	-	-	3.2 ±0.4	3.1 ± 0.5	3.7 ± 0.5*	-	-	3.1 ± 0.4	3.1 ± 0.3	2.6 ± 0.5	2.8 ±0.4	3 ±0.0

Table 3.3.: Developmental lesions seen in zebrafish exposed to BPA, BPA MME or BPA DME. Zebrafish embryos were exposed to BPA, BPA MME, BPA DME, or vehicle (DMSO) in 4 mL vials for seven days. Lesions were recorded and presented as lesion percentage occurring at any point during the seven-day experiment. All data are shown as percentages, and data are representative of three replicate experiments. * denotes significance as compared to control with a p= 0.05. N=25 per treatment dose.

4.0. Environmental Prevalence and the Characterization of the O-methylation of Tetrabromobisphenol A by *Mycobacterium* spp.

4.1. Abstract

Tetrabromobisphenol A (TBBPA) is a widely used brominated flame retardant that is persistent in the environment which can be microbiologically debrominated in anaerobic environments to bisphenol A (BPA) and in aerobic environments O-methylated to TBBPA monomethyl and dimethyl ether (TBBPA DME). The distribution of O-methylating organisms in the environment is not known, and upon examination of several sediment samples from NY/NJ, the potential for O-methylation was detected at all sites tested, although O-methylating microorganisms comprised a minor portion of the population. The fate of TBBPA DME in the environment is not well understood, but our data show that TBBPA DME was recalcitrant to degradation under both aerobic and anaerobic conditions. In this study, several *Mycobacterium* spp. were used to examine the O-methylation of TBBPA and other brominated phenols. All species tested were capable of O-methylating TBBPA to TBBPA DME, but exhibited catalyzed at different rates. *M. vanbaalenii* PYR1 O-methylated 2-bromophenol and 2,6-dibromophenol, with a faster rate for 2,6-DBP O-methylation. A gene deletion was created in the putative catechol O-methyltransferase (COMT) of *M. vanbaalenii* PYR-1 to determine if it encodes for the O-methyltransferase catalyzing the transformation of TBBPA to TBBPA DME. Overall, these data illustrate that O-methylating organisms are widely distributed in the environment, and different strains of mycobacteria are capable of O-methylation.

4.2. Introduction

Tetrabromobisphenol A (TBBPA) is the most prevalent brominated flame retardant used in the manufacture of textiles and electronics, and is utilized around the globe at over 200,000 tons per year (Hakk and Letcher, 2003). Levels of TBBPA in the environment are increasing, due to increased production and usage. There is concern about environmental contamination by TBBPA due to its high lipophilicity (log K_{ow}=5.9), low water solubility (4.16 mg/L at 25°C in H_2O) and low volatility (7.0 x 10^{-11} atm-m^3/mol) and persistence (Hakk and Letcher, 2003; Haneke, 2002).

TBBPA undergoes two different types of microbially mediated transformations by indigenous microorganisms in the environment (Fig. 2.1); debromination to bisphenol A (BPA, 4,4'-isopropylidenediphenol) and O-methylation to TBBPA monomethyl ether (TBBPA MME) and TBBPA dimethyl ether [(TBBPA DME), 4,4'-isopropylidenebis(2,6-dibromo-1-methoxybenzene)] (Arbeli *et al.*, 2006; George and Häggblom, 2008; Voordeckers *et al.*, 2002). TBBPA can be reductively dehalogenated to BPA in anaerobic sediments, but the microorganisms responsible for this degradation are unknown (Arbeli and Ronen, 2003; Arbeli *et al.*, 2006; Ravit *et al.*, 2005; Ronen and Abeliovich, 2000; Voordeckers *et al.*, 2002). Additionally, microbes in aerobic sediments have the ability to transform TBBPA by sequential O-methylation to yield the mono- and di-methyl ether forms (Allard *et al.*, 1987; George and Häggblom, 2008). The fate of TBBPA MME and TBBPA DME in the environment is currently not known. However, the increased lipophilicity and decreased water solubility of these compounds make them more likely to accumulate and persist. TBBPA DME has been detected in

sediments near a plastic manufacturing plant in Sweden at 24 and 1500 ng/g dry weight compared to TBBPA levels at 34 and 270 ng/g (Sellström and Jansson, 1995).

O-methylation is a ubiquitous reaction, characterized by the transfer of a methyl group from S-adenosyl methionine (SAM) to an oxygen atom and has been identified in several bacteria, fungi and mammals (Cho *et al.*, 2008; Coque *et al.*, 2003; Do *et al.*, 2007; Huang *et al.*, 2008; Kim *et al.*, 2006a; Lavid *et al.*, 2002; Vidgren *et al.*, 1994; Yang *et al.*, 2004). This transformation is thought to be an alternative pathway to degradation, and in many instances the function and purpose is not yet understood. Several O-methyltransferases, the enzymes that catalyze this reaction, have been characterized, but the majority of the bacterial enzymes have not been studied in detail (Cho *et al.*, 2008; Coque *et al.*, 2003; Kim *et al.*, 2004; Lavid *et al.*, 2002; Wein *et al.*, 2002; Yang *et al.*, 2004).

The O-methylation reaction in aerobic soils and sediments has been previously studied with halogen-substituted (specifically chlorinated) phenols, thiophenols and chloroguaiacols. It has also been shown that *Mycobacterium sp*. strain CG-1 and CP-2 and *Mycobacterium fortuitum* strain CG-2 and other soil bacteria catalyze the O-methylation reaction (Allard *et al.*, 1987; Häggblom *et al.*, 1986; Häggblom *et al.*, 1988; Neilson *et al.*, 1988). O-methylation of TBBPA was previously demonstrated, and occurred at a slower rate than 2,6-dibromophenol (Allard *et al.*, 1987; George and Häggblom, 2008; Valters *et al.*, 2005). Additionally, O-methylation of high molecular weight PAHs has been observed as a step in the biodegradation pathway of these compounds (Hückelhoven *et al.*, 1997; Kim *et al.*, 2008; Kim *et al.*, 2004; Kweon *et al.*, 2007; Wunder *et al.*, 1997). Interestingly, O-methylated halogenated polybrominated

diphenyl ethers (MeO-PBDE) similar in structure to industrial PBDEs, also exist naturally in the environment (Teuten *et al.*, 2006; Teuten *et al.*, 2005; Vetter *et al.*, 2007) and have been detected in fish, whales, dolphins, marine sponges, algae and acorn worms (Teuten *et al.*, 2005; Valters *et al.*, 2005). In addition to naturally formed MeO-PBDEs, derivatives originating from PBDEs used as flame-retardants have been identified (Valters *et al.*, 2005; Wan *et al.*, 2009).

Recently, an environmental *Mycobacterium* species, *M. vanbaalenii* was isolated from petroleum-contaminated estuarine sediments based on its ability to degrade high molecular weight polyaromatic hydrocarbons (PAHs) such as anthracene, fluoranthene, phenanthrene, biphenyl and benzo[*a*]pyrene (Khan *et al.*, 2002). The degradation of PAHs by this strain involves a novel O-methylation before ring cleavage early in the degradation pathway (Kim, *et al.*, 2004b; Kweon, *et al.*, 2007; Moody *et al.*, 2002; Moody *et al.*, 2004). *M. vanbaalenii* PYR-1 is most closely related to *M. austroafricanum*, another known PAH degrader (Khan, *et al.*, 2002). The genome of *M. vanbaalenii* PYR-1 has been fully sequenced at 6.5 Mb (6,491,865 bp) with 5,979 predicted protein coding regions on one circular chromosome with an average G + C content of 67% (Kim *et al.*, 2008). With regard to genes involved in O-methylation, there are nine putative O-methyltransfereases in the *M. vanbaalenii* PYR-1 genome and one probable catechol O-methyltransferase. The catechol O-methyltransferase is thought to be the enzyme responsible for the PAH O-methylation (Kim *et al.*, 2004a; Kim, *et al.*, 2004b).

O-methylation of TBBPA and polybrominated diphenyl ethers, another type of brominated flame-retardants, has been demonstrated, but much remains to be learned

about this transformation in the environment and at the molecular level. In these studies we demonstrate that *M. vanbaalenii* PYR-1 is capable of O-methylating TBBPA to TBBPA DME. Additionally, we show that O-methylating organisms are ubiquitous in the environment, but represent a small portion of the community. Furthermore, these studies demonstrate that the TBBPA DME metabolite is recalcitrant and is not degraded either aerobically or anaerobically. We have attempted to create a mutated allele of the catechol O-methyltransferase gene in *M. vanbaalenii* PYR-1 and experiments are ongoing to determine if this knockout will prevent O-methylation of TBBPA.

4.3. Materials and Methods

4.3.1. Chemicals. Tetrabromobisphenol A (TBBPA) was obtained from Sigma Aldrich (>98% purity). TBBPA was refluxed over night in an excess of methyl iodide and potassium carbonate buffer (Knapp, 1979). A portion of the sample was analyzed by gas chromatography-mass spectrometry (GC-MS), and the resulting mixture was >99% TBBPA DME (data not shown). The reaction product was then evaporated to dryness and analyzed by GC-MS. Stock solutions were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich), which was also used as the vehicle control.

4.3.2. Bacterial strains and medium. *Mycobacterium vanbaalenii* strain PYR-1 (Khan*, et al.*, 2002) was obtained from Dr. Carl Cerniglia at the Food and Drug Administration and was grown in Brain Heart Infusion (BHI) broth at 28°C with shaking. *Mycobacterium chlorophenolicum* strain PCP-1 (Apajalahti and Salkinoja-Salonen, 1986; Briglia *et al.*, 1994; Häggblom*, et al.*, 1994) and *Mycobacterium fortuitum* CG-2

(Häggblom *et al.*, 1988) were grown in R2A (Difco) broth at 28°C with shaking at 160 rpm. When needed, cultures were grown on R2A agar plates and incubated at 28°C. *Mycobacterium smegmatis* strain mc^2155, obtained from Dr. Nancy Connell at the University of Medicine and Dentistry of NJ, was grown in 7H9 supplemented with albumin, dextrose and sodium chloride with shaking at 37°C.

4.3.3. Most Probable Number Assay. The number of O-methylating organisms was quantified using a five tube MPN assay using sediment samples collected from the NJ/ NY area; Arthur Kill, Newark Bay, Jamaica Bay and Shinnecock Bay with the number of O-methylators to total heterotrophs. TBBPA was spiked into all vials sealed with Teflon caps at a final concentration of 20 μM. The sediment slurry (10%) was diluted to extinction (10^{-9}) in R2A broth. Vials were observed for turbidity over an eight-week period. Once the eight weeks were completed, samples were scored positive or negative for growth. In order to determine the number of O-methylating organisms, the cultures were acidified with HCl and extracted with hexane for GC-MS analysis. Detection of TBBPA MME or TBBPA DME scored the vial positive for O-methylation. A five tube MPN table was used to generate the number of heterotrophs and the number of O-methylating organisms in these sediments (Cochran, 1950; DeMan, 1975).

4.3.4. O-Methylation of TBBPA by *Mycobacterium vanbaalenii* PYR-1. *Mycobacterium vanbaalenii* PYR-1 was inoculated into 5 mL of BHI Broth and grown at 28 °C with aeration for 48 hours. Once the culture reached an OD_{680} of 0.6 it was diluted 1:10 and grown to an OD_{680} of 1.0 at 28 °C with aeration. One mL was inoculated into

sterile glass vials with Teflon cap liners, with 100 μM of TBBPA. Additional cultures were inoculated with 100 μL of 1 mg/ml of chloramphenicol to inhibit protein synthesis. Cultures were incubated at room temperature with shaking and at designated timepoints aliquots were taken and stored at -20 °C in glass vials. Samples were acidifed with hydrochloric acid and 20 μM 2,4,6-tribromophenol was added as an internal standard followed by extraction in toluene for one hour. The toluene phase was removed and analyzed by GC-MS.

4.3.5. O-methylation of brominated phenolic compounds by *M. vanbaalenii* PYR1. *Mycobacterium vanbaalenii* PYR-1 was inoculated into 5 mL of BHI Broth and grown at 28 °C with aeration for 48 hours. Once the culture reached an OD_{680} of 0.6 it was diluted 1:10 and grown to an OD_{680} of 1.0 at 28 °C with aeration. One mL was inoculated into sterile glass vials with Teflon cap liners, with 100 μM of 2-bromophenol (2-BP), 2,6-dibromophenol (2,6-DBP) or 2,4,6-tribromophenol (2,4,6-TBP). Additional samples were inoculated with 100 μM 2,4,6-tribromophenol and 100 μL of 1 mg/ml of chloramphenicol to inhibit protein synthesis. Samples were incubated at room temperature with shaking and at designated timepoints aliquots were taken and stored at -20 °C in glass vials . Samples were acetylated and extracted according to a previously established protocol (Häggblom *et al.*, 1986). The hexane phase was removed and analyzed by GC-MS.

4.3.6. Analytical Methods. An Agilent Gas Chromatograph Series 6890 with a Mass Selective Detector Series 5973N equipped with a splitless injector, and a HP-5MS

column (30 m length x 0.25 mm i.d x 0.25 μm film thickness) was used for analytical analysis. The injector temperature was 250 °C with an injection volume of 1 ml, the oven temperature started at 70 °C, ramping up to 300 °C by 15 °C per minute, and holding at 300 °C for 5 minutes. SCAN mode was used initially in the range from 32 to 600 m/z to identify the prominent ions for TBBPA, TBBPA MME and TBBPA DME and the bromophenols. Selected Ion Monitoring (SIM) was used for quantitation and the ions used to detect TBBPA (529 and 544 m/z), TBBPA MME (543 and 558 m/z) and TBBPA DME (557 and 572 m/z), 2,4,6-tribromophenol (330, 332), 2,4,6-tribromoanisole (344, 346), 2,6-dibromophenol (252), 2,6-dibromoanisole (266), 2-bromophenol (172) and 2-bromoanisole (186).

4.3.7. Creation of a Knockout of the Catechol O-methyltransferase Gene in *M. vanbaalenii* PYR-1.

DNA isolation: DNA was isolated from *M. vanbaalenii* PYR-1 with a modified phenol:chloroform protocol (Kerkhof and Ward, 1993). Lysozyme was added and incubated at 37 °C for one hour followed by the addition of 10% SDS and proteinase K. NaCl (5M) and CTAB was then added, mixed and incubated for 10 minutes at 65 °C. Chloroform:isoamyl alcohol (24:1) was used to extract, and DNA was precipitated with isopropanol and washed with 70% ethanol (Safi *et al.*, 2008).

Amplification of flanking regions to generate mutant allele: PCR primers were generated for the upstream and downstream regions flanking the catechol O-methyltransferase gene (Table 4.1.), generating two products of approximately 1500 bp each. The conditions for the amplification were (per reaction); 2.5 μL Pfx buffer, 0.5 μL

$MgSO_4$, 0.25 μL dNTPs, 1.25 μL primer mix (forward and reverse at 10 nM each), 2μL PYR1 genomic DNA, 0.25 μL Pfx polymerase (Invitrogen), and Pfx Enhancer at 3X concentration for the right fragment and 0x concentration for the left fragment. The PCR cycling program was as follows; 95 °C for 5 min, then 30-40 cycles of 94 °C for 50 sec, 55 °C for 30 sec (right) or 60 °C for 30 sec (left) and 68 °C for 1 min, 72 °C for 5 min and a hold at 4 °C. PCR products were screened on a 1% agarose gel and then digested with XhoI/Acc65I for the right fragment and XhoI and HindIII (Promega) for the left fragment.

Generation of Suicide Vector: The fragments were ligated into a p2nil vector digested with HindIII/Acc65I (Parish and Stoker, 2000; Safi*, et al.*, 2008). Ligation products were transformed into *E.coli* TOP10 cells, grown on LB plus kanamycin (50 μg/mL) for selection. Colonies were picked, grown in 4 mL cultures (LB plus kanamycin 50 μg/ml), and plasmid DNA isolated the following day by a traditional isopropanol precipitation protocol (Safi*, et al.*, 2008). Plasmid DNA was digested with SacI (Promega), cutting only in the two flanking DNA fragments to screen for positive colonies containing the mutant allele in the p2nil backbone.

Construction of the Suicide Vector Used for Homologous Recombination. Positive clones, containing the mutant COMT gene in the p2nil backbone, were digested with PacI (New England Biolabs) for 3 hours and dephosphorylated for four hours with alkaline phosphatase (Roche). The Pac cassette was cut from the pGOAL vector, and ligated with the p2nil ΔCOMT construct at 18 C overnight, and transformed into *E.coli* TOP10 cells, with XGAL (50 μg/mL) and Kanaycin (50 μg/mL) selection. XGAL selection resulted in blue colonies, which were inoculated into broth and plasmid DNA

isolated. Colonies were screened by restriction enzyme digestion with BamH1 and HindIII (Parish and Stoker, 2000; Safi*, et al.*, 2008).

Generation of PYR-1 Catechol O-methyltransferase Knockout: The final suicide vector construct was transformed into competent *M. vanbaalenii* PYR1 cells. *M. vanbaalenii* competent cells (20 ml) were prepared the same day as used by washing in 10% glycerol three times at 3000 rpm for 10 minutes, and resuspending in final volume of 400 μL 10% glycerol and used the same day. *M. vanbaalenii* PYR1 competent cells, 200 μL, and plasmid DNA, final construct 7.5 mL of 760 ng/μl stock) were combined and electroporated at 2500V and 25 μFin a 2 mm cuvette.

PCR primers were designed to screen for potential gene deletion mutants (Table 4.1.). Amplification conditions were as follows: 95 for 5 minutes, followed by 30 cycles of 95 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 50 sec, concluded with a final extension at 72 °C for 5 min. These primers were designed at the edges of the deletion region in order to achieve maximal difference in band size to compare to the wild type strain.

4.4. Results

4.4.1. Environmental Distribution of O-methylating Microorganisms. O-methylation of TBBPA has been previously demonstrated in the environment and laboratory (ref), but the prevalence of O-methylating organisms in different environments was not known. In order to determine the environmental distribution of O-methylating organisms, a most probable number assay (MPN) was utilized on sediments from the NY/NJ region. These sites were chosen because the Arthur Kill, Newark Bay and Jamaica Bay have high levels

of xenobiotic contamination. The Shinnecock Bay site was chosen as a clean site. Sediments from the Arthur Kill had the highest ratio of O-methylating microorganisms, and those organisms were able to transform TBBPA to both TBBPA MME and DME (Table 4.2.). The microorganisms in Newark Bay sediments were able to O-methylate TBBPA to both TBBPA MME and DME, but the number of O-methylating organisms was a magnitude less as compared to the Arthur Kill population (Table 4.2.). The Shinnecock Bay and Jamaica Bay samples had low levels of O-methylators, and the only metabolite detected was TBBPA MME (Table 4.2.).

4.4.2. O-methylation of TBBPA and 2,4,6-tribromophenol. *M. vanbaalenii* PYR-1 is capable of O-methylating TBBPA to TBBPA DME within two weeks (Fig. 4.1.). TBBPA MME was detected at low levels on day 2 and 3 but was further converted to TBBPA DME. It has been previously hypothesized that the O-methylation of halogenated phenols is constitutive (Neilson *et al.*, 1988). We therefore tested this with a culture of *M. vanbaalenii* PYR-1 spiked with 2,4,6,-TBP in the presence of chloramphenicol, a protein synthesis inhibitor. Transformation of 2,4,6-TBP occurred at a similar rate with or without chloramphenicol, indicating that this transformation is indeed constitutive (Fig. 4.2.). TBBPA O-methylation in the presence of chloramphenicol is also constitutive (data not shown).

Previous studies have found that bulky flanking substituents, such as a halogen or methyl group, facilitate O-methylation (Allard *et al.*, 1987). In order to determine if *M. vanbaalenii* PYR1 had a similar preference, 2-bromophenol (2-BP) and 2,6-dibromophenol (2,6-DBP) were spiked into *M. vanbaalenii* PYR1 cultures (Fig. 4.3). The

rate of 2,6-DBP O-methylation was faster than that for 2-BP, resulting in accumulation of 100% of 2,6-tribromoanisole after 2 weeks as compared to 10% of 2-bromoanisole (Fig. 4.3).

To determine the similarities and differences in the O-methylation reaction by different *Mycobacterium* species, *M. vanbaalenii, M. fortuitum, M. chlorophenolicum* and *M. smegmatis* cultures were spiked with TBBPA and could O-methylate TBBPA (Table. 4.3.). There were differences noted in the strains, specifically regarding the rates of O-methylation and production of metabolites. *M. smegmatis* was the slowest in catalyzing TBBPA O-methylation, with no TBBPA DME after 2 weeks (Table 4.3.). *M. chlorophenolicum* catalyzed O-methylation was also slower than that of *M. fortuitum* or *M. vanbaalenii* resulting in only TBBPA MME, whereas *M. vanbaalenii* and *M. fortuitum* converted almost 100% of TBBPA within 2 weeks to TBBPA DME.

4.4.3. Phylogenetic Analysis of Catechol O-methyltransferase in *M. vanbaalenii* PYR1. The genome of *M. vanbaalenii* PYR-1 has nine open reading frames annotated as putative O-methyltransferases. One of these, the catechol O-methyltransferase (COMT) was shown to O-methylate PAHs during degradation (Kim *et al.*, 2004). A neighbor-joining phylogenetic tree was constructed with amino acid sequences from the nine O-methyltransfereases of PYR-1, homologous COMTs and general O-methyltransferases from other bacterial species in order to determine the relationship amongst these genes. The COMT from *M. vanbaalenii* clustered with the other COMT genes, which upon homology search, were all from different *Mycobacterium* spp. Additionally, the COMT genes from *M. vanbaalenii* and *M. gilvum* cluster together, which is expected as they are

both from PAH degrading mycobacteria (Fig. 4.4.) (Gauthier *et al.*, 2003; Willumsen *et al.*, 2001). Five O-methyltransferases from *M. vanbaalenii* PYR-1 clustered together, and they share a putative role in polyketide synthesis, but cluster away from the COMT. One O-methyltransferase of PYR-1 clusters with *Saccharopolyspora erythraea*, which is a rhamnosyl 4'-O-methyltransferase encoding for insecticidal macrolide biosynthesis (Fig. 4.4.) (Huang *et al.*, 2008). The remaining two O-methyltransferases cluster with the O-methyltransferase from *Bacillus cereus*, which is known to O-methylate flavenoids (Fig. 4.4.). These data illustrate that the COMT O-methyltransferase (accession number YP_954084) is a most likely catalyzing the O-methylation of TBBPA.

4.4.5. Construction of a COMT mutant strain of *M. vanbaalenii* PYR-1. O-methyation of TBBPA is a constitutive reaction, and thus, the only way to determine which gene is responsible for producing the protein catalyzing this reaction is to generate a mutant strain with a deleted copy of that gene (Fig. 4.5.). Using a method developed by Parish and Stoker, a vector construct was created, with a gene deletion of the probable catechol O-methyltransferase. This construct was transformed into the *M. vanbaalenii* PYR1 strain (Fig. 4.5.) in an attempt to create a homologous recombination event resulting in a gene deletion mutant. These experiments are ongoing.

4.5. Discussion

TBBPA is the most prevalently used brominated flame retardant in the world, and is increasingly detected in the environment and in different organisms (Cariou*, et al.*, 2008; Choi *et al.*, 2009; Dirtu*, et al.*, 2008; Geens*, et al.*, 2009; Halldin*, et al.*, 2001;

Jakobsson, *et al.*, 2002; Johnson-Restrepo, *et al.*, 2008). The lipophilicity of TBBPA contributes to its environmental accumulation as well as recalcitrance to biodegradation. O-methylated metabolites have been detected in the environment, (Hakk and Letcher, 2003), but the function and prevalence of this transformation is not understood. These studies examined the potential for O-methylation at different sites in NY/NJ. Finally, *M. vanbaalenii* and other species of mycobacteria were determined to O-methylate TBBPA and a mutation of the COMT was created in *M. vanbaalenii* PYR1.

TBBPA O-methylation has been demonstrated with *M. fortuitum* and *M. chlorophenolicum* (George and Häggblom, 2008), but the overall distribution of this transformation in the environment was not previously known. An MPN assay was conducted to determine whether there were organisms in local sediments capable of O-methylation. The Arthur Kill had the highest percentage of O-methylating organisms as compared to the total heterotrophic population at 0.7%, followed by Shinnecock Bay, Newark Bay and Jamaica Bay (Table 4.2.). Surprisingly, no O-methylators were found in landfill leachate from Hackensack, but overall, the MPN data demonstrate that O-methylation occurs at most sites tested. Contrary to previous data from our laboratory, the percentage of O-methylating organisms (< 1%) (Table 4.2.) was lower than previously detected (10%) (George and Häggblom, 2008). This difference could be due to the use of different sampling sites, different times of year, different medium used, and finally the sediments were in cold storage for an extended period of time and could contribute to a reduction in O-methylating microorganisms.

M. vanbaalenii PYR1 is a known high molecular weight PAH degrader, with a novel O-methylation during the degradation process (Khan, *et al.*, 2002; Kim, *et al.*,

2004b; Stingley *et al.*, 2004). Additionally, the genome of this bacterium has been sequenced, making it a useful tool in analyzing the O-methylation transformation on a molecular level. *M. vanbaalenii* PYR1 was able to O-methylate TBBPA to TBBPA DME completely within two weeks of incubation (Fig. 1.1). Previous studies have suggested that O-methylation is a constitutive process, which our data confirms (Fig. 4.2.). When compared to other species of mycobacteria, *M. vanbaalenii* PYR1 had one of the faster rates similar to that of *M. fortuitum*. The O-methylation rates of *M. chlorophenolicum* PCP1 and *M. smegmatis* mc^2155 are slower than that of the other species (Table. 4.3.).

Phylogenetic analysis of the amino acid sequence of the O-methyltransferase genes in *M. vanbaalenii* PYR1, and other bacteria illustrate that these genes cluster based on substrate specificity (Fig. 4.4.). The COMT genes cluster together, and interestingly, the two genes from environmental isolates cluster away from those of the mycobacterial species known to cause disease (Fig. 4.4.) These data support the choice to target the COMT gene as the one likely for encoding the O-methyltransferase catalyzing the transformation of TBBPA to TBBPA DME. Additionally, the endogenous substrate for other known COMTs is dopamine, which is an aromatic compound similar in structure to TBBPA, suggesting that TBBPA would be a likely substrate for this enzyme. Due to the structural similarities, it is likely this protein is the O-methyltransferase responsible for bromophenol, bisphenol A and even possibly chlorophenol O-methylation, but this would need to be examined in more detail with the mutated strain. Experiments transforming the ΔCOMT construct into *M. vanbaalenii* PYR1 are ongoing, and if a successful gene deletion mutant is obtained, the *M. vanbaalenii* PYR1 ΔCOMT strain (Fig. 4.5.) would be used analyze for its ability to O-methylate TBBPA.

The function of O-methylation of TBBPA remains to be elucidated, but these studies have contributed to the knowledge surrounding this transformation. TBBPA O-methylating organisms are widely distributed, detected in most samples tested, and O-methylation is a constitutive reaction in mycobacteria. Additional examination of other sediment samples would be useful to understand the distribution of O-methylating organisms outside the NY/NJ area. Additionally, completion of the gene deletion mutant in *M. vanbaalenii* PYR1 is necessary to understand whether the COMT is the gene responsible for the O-methylation of TBBPA and the brominated phenols. Overall, these data provide additional insight into the O-methylation transformation, further characterizing another *Mycobacterium* sp. capable of this transformation.

4.6. Figures.

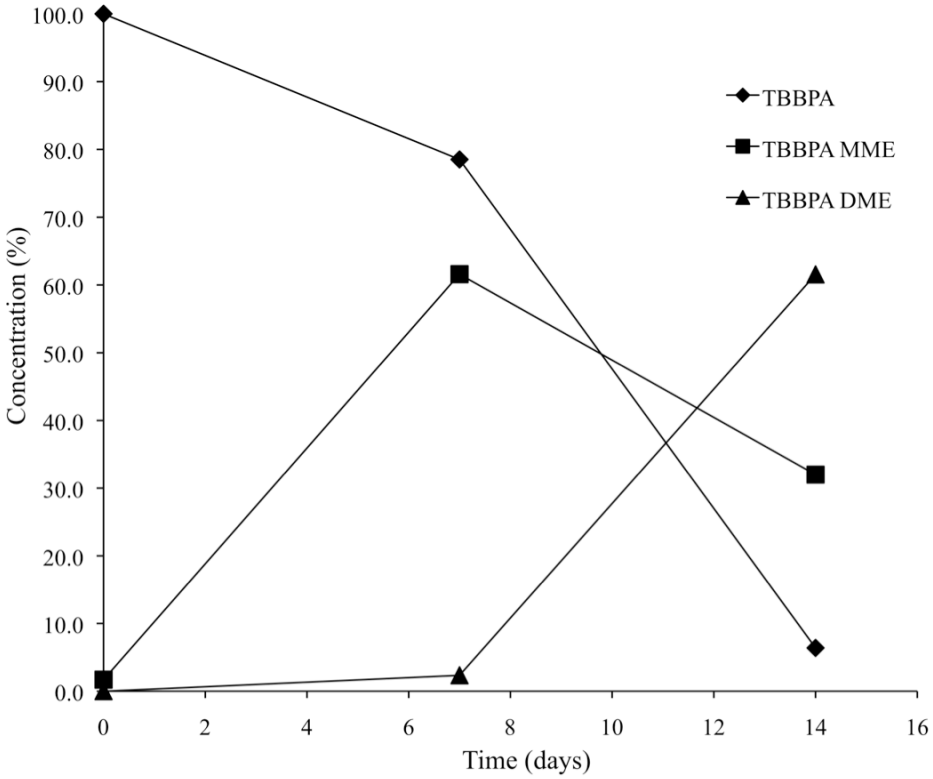

Fig. 4.1. O-methylation of TBBPA by *M. vanbaalenii* PYR1. *M. vanbaalenii* PYR-1 was spiked with 100 mM of TBBPA and incubated for two weeks. Samples were taken, analyzed by GC-MS for the presence of the mono- and dimethyl ether metabolites.These data are representative of three replicate experiments.

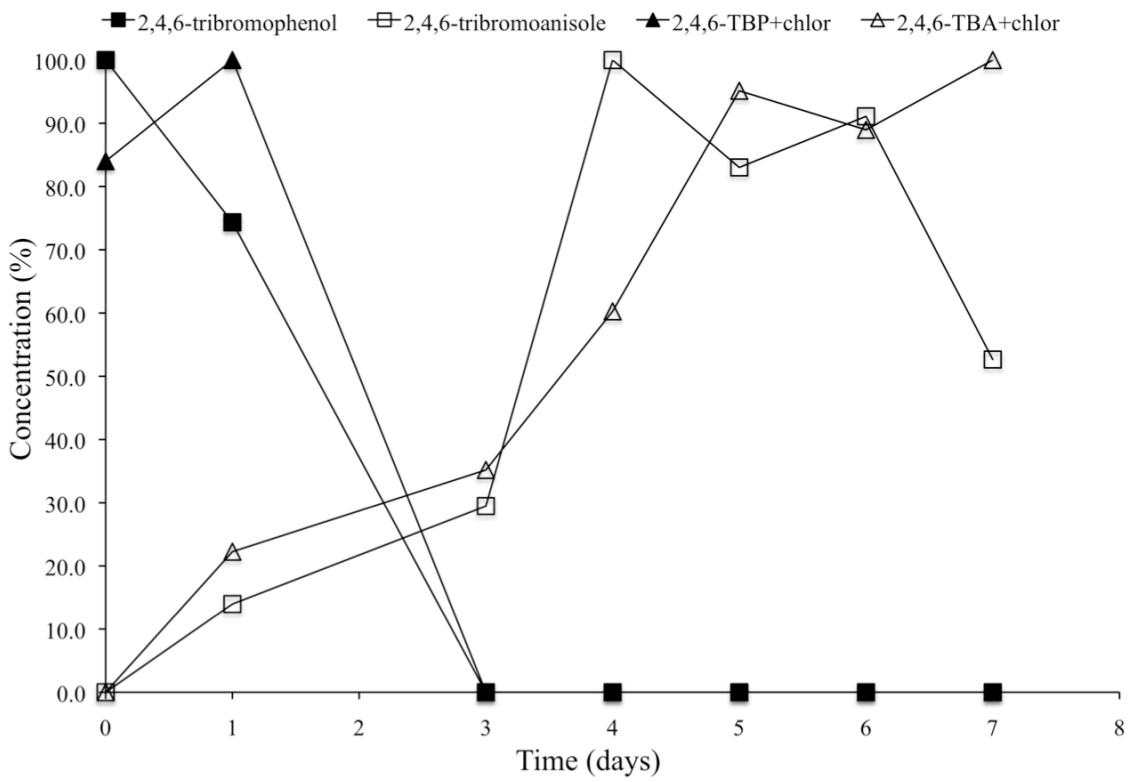

Fig. 4.2. O-methylation of 2,4,6,-tribromophenol with and without chloramphenicol. *M. vanbaalenii* PYR1 was spiked with 100 μM 2,4,6-tribromophenol with and without chloramphenicol. Samples were taken over a period of seven days and analyzed by GC-MS for the presence of 2,4,6-tribromoanisole.

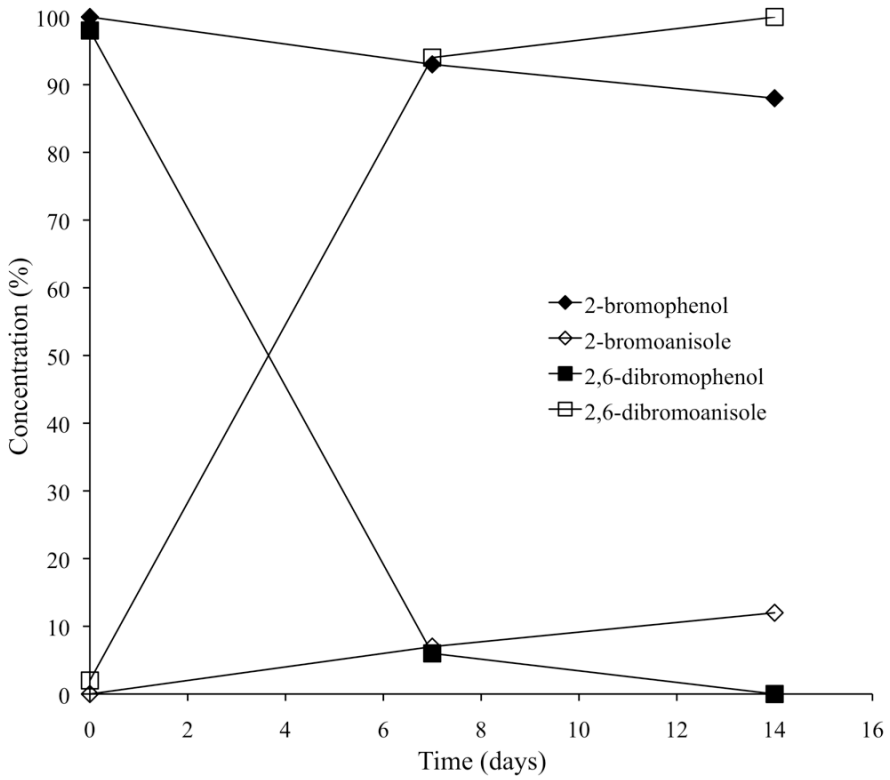

Fig. 4.3. O-methylation of bromophenols by *M. vanbaalenii* PYR-1. *M. vanbaalenii* PYR1 was spiked with either 2-bromophenol or 2,6-dibromophenol and samples were analyzed by GC-MS for the presence of the brominated anisole derivative after seven and fourteen days of incubation.

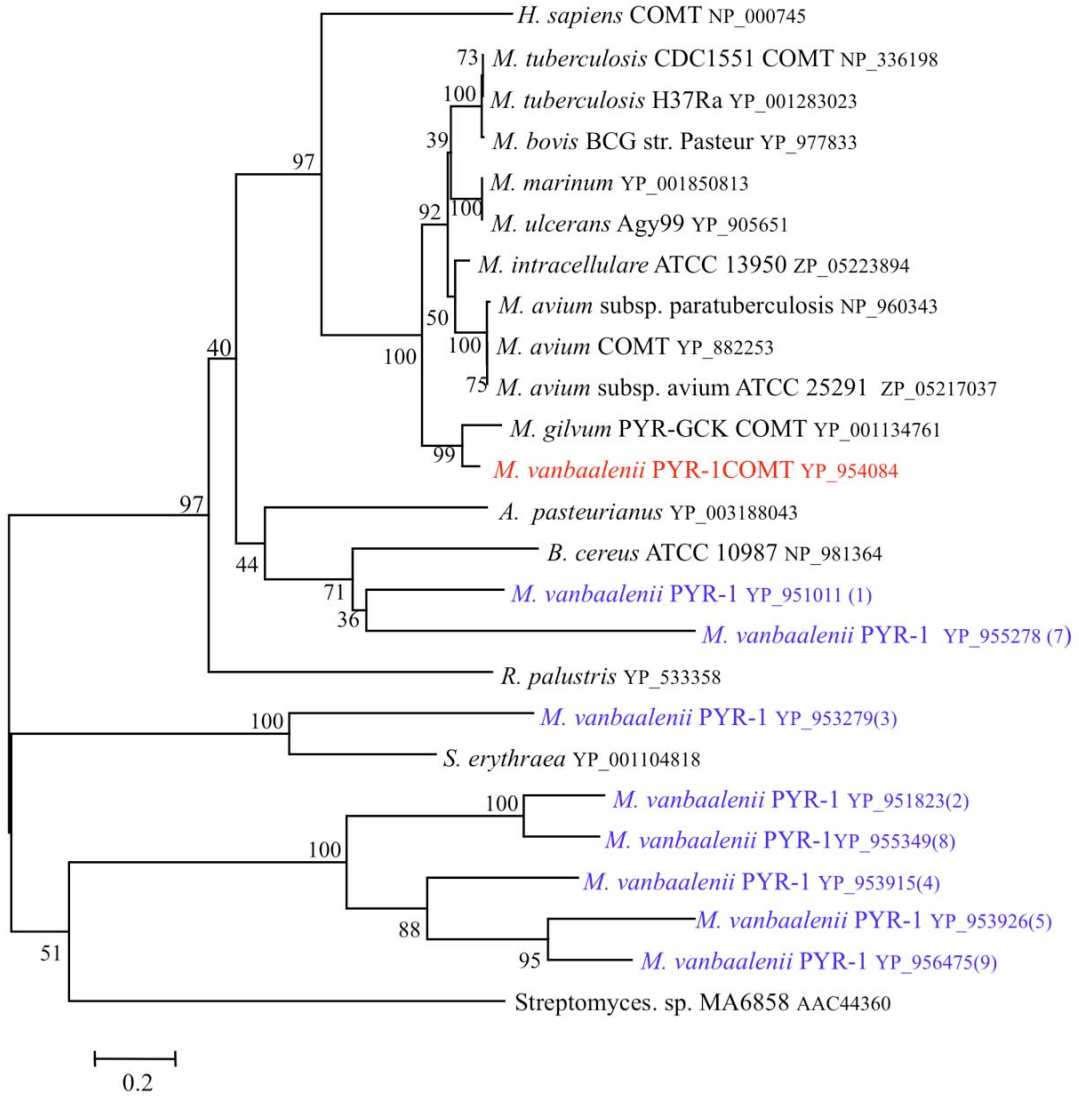

Fig. 4.4. Phylogenetic analysis of O-methyltransferase amino acid sequences. Amino acid sequences of the nine putative O-methyltransferase genes from *M. vanbaalenii* PYR1, other known O-methyltransferases and homologous COMT sequences were compared using neighbor joining phylogenetic analysis. The COMT from *M. vanbaalenii* PYR1 is shown in red, and the other putative O-methyltransferases are shown in blue.

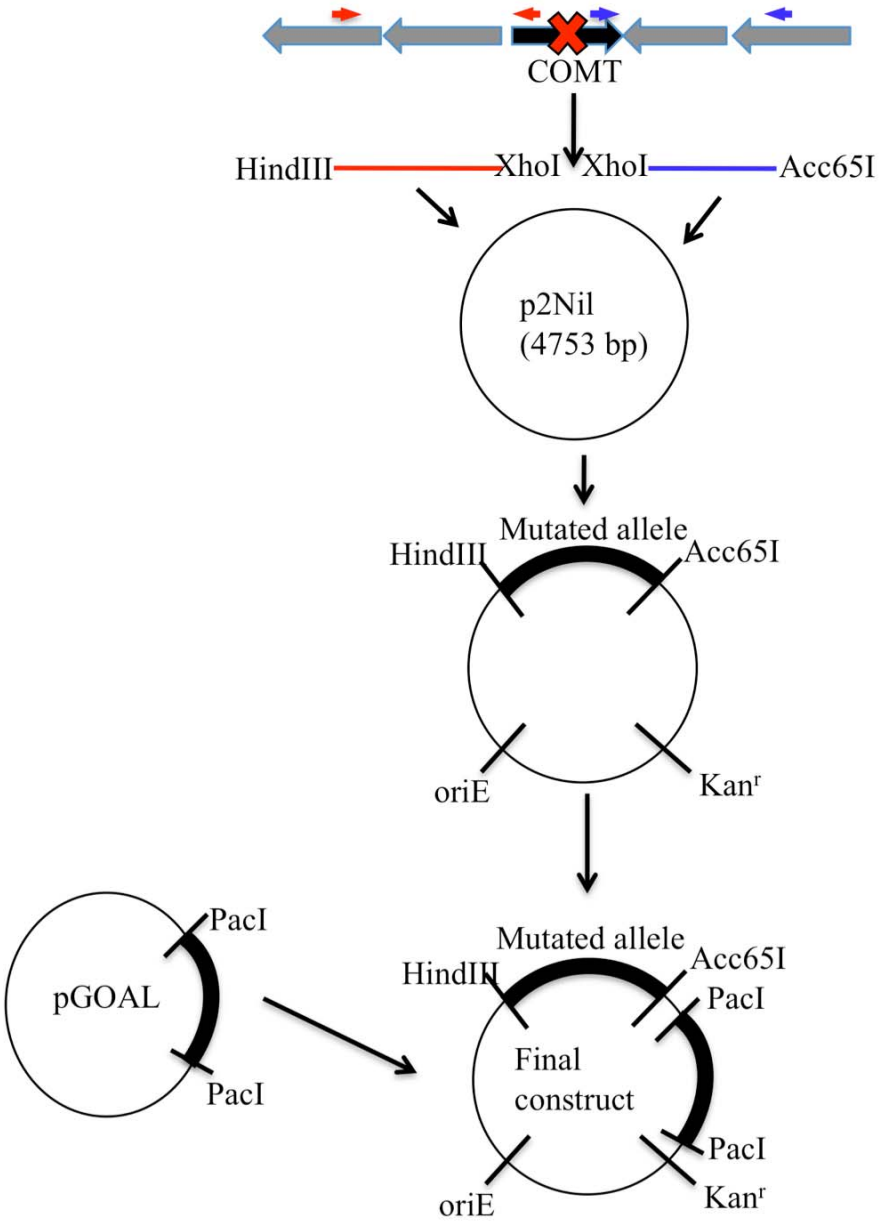

Fig. 4.5. Construction of Suicide Vector for Deletion of the COMT gene in *M. vanbaalenii* PYR1. This schematic illustrates the design of the constructs to generate a COMT gene deletion mutant as demonstrated in Parish and Stoker (2000). The primer sequences for the flanking regions of the COMT are shown in red (left flanking) and blue (right flanking). The restriction sites utilized for cloning are also shown above.

4.7. Tables

Table 4.1. Primer sequences used to create mutant allele for catechol O-methyltransferase gene.

Primers to Knock Out Catechol O-methyltransferase gene	
	Sequence (5' to 3')
Left Flanking Region	
Forward	TTTAAGCTTAACGACGTCGTCTCGTCGAG
Reverse	CCGAGCTCGAGCACCAGG
Right Flanking Region	
Forward	TTTCTCGAGGCTGGAGTCCGAGTATCT
Reverse	TTTGGTACCATGCCCGATGTTTCGACGC
Screening Primers	
Forward	GCATGCCGTCGTTCGATG
Reverse	GCGTGGCAGGTACTGCAGGA

Primer sequences were chosen approximately 1500 bp upstream or downstream of the COMT gene. One primer in each pair was located in the COMT gene, but resulted in a deletion of at least 500 bp of the center of the gene. Sequences in red correspond to restriction enzyme cutting sites used to create the mutant allele cloned into the p2nil vector. The screening primers were used to screen the wild type and the knockout to determine if the gene deletion was incorporated into the *M. vanbaalenii* PYR1 genome by homologous recombination.

Table 4.2. Distribution of O-methylating microorganisms in NY/NJ samples.

Site	Presence of TBBPA-DME	Presence of TBBPA-MME	Number of Heterotrophs per gram sediment	Number of Methylators per gram sediment	Ratio of Methylators per total heterotrophs (%)
Arthur Kill	+	+	4.7×10^5	3460	0.73
Hackensack Landfill Leachate	-	-	2.3×10^5	0	0
Jamaica Bay	-	+	5×10^5	100	0.02
Newark Bay	+	+	6.5×10^5	220	0.034
Shinnecock Bay	-	+	1×10^4	470	0.047

A five tube MPN Assay was used to determine the number of heterotrophic organisms in each site, by scoring positive or negative for growth. The total number of O-methylating organisms per site was determined by using a most probable number table, and the positive tubes were those detecting either the mono- or dimethyl ether metabolites of TBBPA.

O-methylation of TBBPA by *Mycobacterium* strains

	TBBPA MME (%)		TBBPA DME (%)	
Species and Strain	1 week	2 weeks	1 week	2 weeks
M. chlorophenolicum PCP1	3	8.8	0.5	3
M. fortuitum CG-2	39.7	57.8	16.3	22.3
M. smegmatis mc2155	15.8	18.9	0	0

Table 4.3. O-methylation of TBBPA by different *Mycobacterium* spp. Mycobacterial cultures were spiked with TBBPA and samples were taken at one and two weeks and analyzed for TBBPA MME and TBBPA DME. Data are concentrations of TBBPA MME or TBBPA DME presented as in percentages.

5.0. Discussion

BFRs have been the focus of many studies around the world which have demonstrated the presence of these compounds in breast milk, serum, cord blood and other samples (Table 1.1) (Antignac*, et al.*, 2008; Cariou*, et al.*, 2008; Kawashiro*, et al.*, 2008; Thomsen*, et al.*, 2001; van Leeuwen and de Boer, 2008; Zhu*, et al.*, 2009b). In the environment, TBBPA and PBDEs can be O-methylated by microorganisms, but despite environmental detection of these metabolites, there has been little analysis of human or animal samples for these O-methylated compounds (Sellström and Jansson, 1995; Teuten*, et al.*, 2006; Vetter *et al.*, 2007b; Watanabe*, et al.*, 1983). The studies in this thesis were the first to demonstrate the toxicological effects of O-methylated metabolites of TBBPA and BPA on the developing zebrafish embryo. Additionally, this is the first report of O-methylation of BPA by several environmental mycobacteria *spp.*, including *M. vanbaalenii* PYR-1 The prevalence of O-methylating organisms in the environment (Table 4.2.) as well as targeted the gene likely responsible for this reaction in *M. vanbaalenii* PYR-1 were examined (Table 4.1 and Fig. 4.5). O-methylated metabolites are more lipophilic and more likely to accumulate in the environment, but their toxicological effects are unknown. Therefore, it is important to gain an understanding as of the impact of these compounds on organisms in the environment, which can be extrapolated to other species, including humans.

Despite the literature illustrating that BFRs are endocrine disruptors (Hamers*, et al.*, 2006; Kang*, et al.*, 2007; Kitamura*, et al.*, 2005b; Legler and Brouwer, 2003), they are still in demand and used globally. Several BFRs, specifically penta- and octaBDE have recently been banned due to recognized toxicity issues, but due to past use are detected in many environmental matrices including sediments, dust, soil and water (Chen

et al., 2009; Choi*, et al.*, 2009; Morf*, et al.*, 2005; Segev*, et al.*, 2009; Shi*, et al.*, 2009a; Takigami*, et al.*, 2008). TBBPA toxicity is not considered to be a major concern despite its acceptance as an endocrine disruptor, specifically the ability to disrupt proper thyroid hormone binding (Hamers*, et al.*, 2006; Haneke, 2002; Meerts*, et al.*, 2000). However, other studies also illustrate that TBBPA can be neurotoxic, cytotoxic and can also disrupt Ca^{2+} homeostasis. (Kitamura*, et al.*, 2005a; Kuiper*, et al.*, 2007b; Nakajima*, et al.*, 2009; Ogunbayo *et al.*, 2008; Reistad*, et al.*, 2005; Reistad*, et al.*, 2007; Saegusa *et al.*, 2009; Strack*, et al.*, 2007). Additionally, TBBPA can cause developmental malformations in zebrafish (Kuiper*, et al.*, 2007b). These studies suggest that TBBPA exposure causes toxicological effects and TBBPA should be a contaminant of concern. This is further supported by the numerous studies citing TBBPA detection in dust, sediments, aquatic organisms, in addition detection in serum, breast milk and other human samples (Cariou*, et al.*, 2008; Fernandes*, et al.*, 2008; Geens*, et al.*, 2009; Johnson-Restrepo*, et al.*, 2008; Morf*, et al.*, 2005; Shi*, et al.*, 2009b; Takigami*, et al.*, 2008; Xie*, et al.*, 2007). Additionally, it is known that TBBPA can be passed maternally from mother to embryo in zebrafish, due to the high lipophilicity of the compound (Nyholm *et al.*, 2008), which suggests that TBBPA can biomagnify through the food chain, and cause chronic effects to exposed organisms that are not currently understood. Furthermore, TBBPA and other BFRs such as PBDE can be O-methylated, and those metabolites have an increased likelihood to accumulate in the environment and in the fatty tissues of animals, thus these studies are vital in characterizing the toxicity of O-methylated metabolites.

5.1. Microbially Mediated O-methylation of Phenolic Compounds (a lot of repeat)

Our data demonstrate the O-methylation can result in metabolites either more or less toxic than the parent compound to developing zebrafish (Ch.2. and 3). These results indicate that it is important to understand more about the O-methylation reaction in general, primarily concerning the microbes capable of catalyzing this reaction, and the prevalence of these organisms in the environment. TBBPA can be O-methylated by several species of mycobacteria (George and Häggblom, 2008), but the prevalence these O-methylating microorganisms in the environment is poorly understood. An MPN assay was used to determine the percentage of O-methylators in a given microbial population by detecting TBBPA DME formation in 6 different sediment samples spiked with TBBPA from NJ/NY (Table 4.2.). These data illustrate that O-methylation appears to be ubiquitous, although the O-methylating organisms comprise a relatively minor (<1%) proportion of the total heterotrophic population. These data may or may not be reflective of the true microbial populations because a previous study from our laboratory suggested that O-methylating organisms can represent approximately 10% of total heterotrophs (George and Häggblom, 2008). Likely explanations for these discrepancies may include different sampling sites/locations, different media used in the MPN assays, and the sediments having been in cold storage for an extended time before analysis with a possible die-off of some microorganisms. Further, it is possible that the amount of TBBPA used in these assays was growth inhibitory to the microorganisms, and could then explain the low percentage of heterotrophs in these sites. In order to test this hypothesis, an experiment examining growth during exposure to different concentrations of TBBPA to a diluted *M. vanbaalenii* culture should be performed. It is possible that

TBBPA is toxic to microorganisms, due to its ability to uncouple the mitochondrial membrane, and thus depending on the concentration could decrease the number of total heterotrophs.

Even if TBBPA is toxic to some microbes, TBBPA DME and MeO-PBDEs have been detected in the environment and tissue samples at levels equal to and greater than TBBPA and the PBDE parent compounds (Sellström and Jansson, 1995; Teuten*, et al.*, 2006; Vetter*, et al.*, 2007b; Watanabe*, et al.*, 1983). A more comprehensive analysis should be conducted utilizing sediments from other sites to elucidate the prevalence of O-methylation and the specific O-methylated compounds existing in different types of environments. Our data suggest that TBBPA DME is recalcitrant in aerobic and anaerobic environments (Appendix 1). This information will be needed for a more thorough assessment of the environmental concerns surrounding TBBPA and its metabolic processes.

Previous studies indicated that phenolic compounds lacking bulky substituents, such as a halogen or methyl group, flanking the free hydroxyl group, would not be O-methylated (Allard*, et al.*, 1985; Häggblom*, et al.*, 1988; Neilson*, et al.*, 1988). Interestingly, our data indicate that BPA can be O-methylated, but that the rate of this transformation is very slow compared to TBBPA O-methylation, which would explain why previous studies did not detect O-methylation of nonhalogenated compounds (Figure 3.1) (Allard*, et al.*, 1985; Allard*, et al.*, 1987; Häggblom*, et al.*, 1988). This difference could also be due to limited nutrient availability and/or cell density (Fig. 3.2 and 4.1). Additionally, the rates of O-methylation differ between species of mycobacteria. Two species analyzed for O-methylation, *M. fortuitum* and *M. smegmatis* do not produce BPA

DME, but BPA MME is detected (Fig. 3.2). Similarly, *M. smegmatis* is slower at O-methylating TBBPA (Table 4.3.), as compared to the other three species of mycobacteria tested. This data suggests that there is a difference in the enzymes responsible for O-methylation.

In addition to the differences seen between TBBPA and BPA O-methylation, differences in the relative rate of transformation were determined when examining O-methylation of 2-bromo-, 2,6-dibromo- and 2,4,6-tribromophenol with the highest rate of O-methylation was observed for 2,4,6-tribromophenol and 2,6-bromophenol then 2-bromophenol (Fig. 4.3.). These data support previous studies indicating that the O-methylation with flanking substituents is favored, and additional study needs to determine if the enzyme catalyzing these reactions for TBBPA and BPA are different, or the same.

O-methylation is catalyzed by O-methyltransferases, which are enzymes with many different cellular functions including methylation of DNA and degradation of different compounds. O-methyltransferases, including the COMT, typically use the methyl group from S-adenosyl methionine and transfer it to the free hydroxyl group, but it is not known which of the proteins, since there are nine putative genes in *M. vanbaalenii* PYR1, catalyzes the O-methylation of TBBPA or BPA. Since these enzymes are also found in eukaryotic organisms, it is possible that eukaryotic metabolism contributes to the levels of O-methylated compounds in the environment. Phylogenetic analysis of the *M. vanbaalenii* PYR1 O-methyltransferase genes and homologs from other bacteria indicate that they cluster based on substrate specificity (Fig. 4.4.) Based on previous data indicating the COMT from *M. vanbaalenii* PYR1 is the protein responsible for the O-methylation of PAHs during degradation, and phylogenetic analysis indicating

the COMT gene clusters away from all other O-methyltransferases, would be a likely candidate for the O-methylation of TBBPA and BPA (Fig 4.4.) (Kim *et al.*, 2004) Dopamine is the endogenous substrate for COMT, having a similar aromatic ring structure, indicating if this is the gene responsible for O-methylation of TBBPA, then the possibility remains that other aromatic structures could also be substrates for O-methylation.

In order to determine if the COMT gene is responsible for encoding the protein catalyzing the O-methylation of TBBPA and BPA, a gene deletion mutant needed to be created. TBBPA and BPA O-methylation is a constitutive reaction, which makes identifying the responsible protein a challenge. A suicide plasmid construct was created in *E. coli* and transformed into *M. vanbaalenii* PYR1 competent cells (Fig. 4.5.). The results from this experiment are still pending.

5.2. Toxicological Effects of TBBPA, BPA and TBBPA DME Exposure on Developing Zebrafish Embryos

In the environment, TBBPA undergoes two different types of microbially mediated transformations. In addition to the aerobic O-methylation (Chapters 2 and 4), it can be dehalogenated to BPA in anaerobic environments (Arbeli*, et al.*, 2006; Ravit*, et al.*, 2005; Ronen and Abeliovich, 2000; Voordeckers*, et al.*, 2002). The toxic effects of BPA have been well studied, and like TBBPA, it is a known endocrine disruptor (Vandenberg*, et al.*, 2009; Wetherill*, et al.*, 2007). BPA acts primarily as an estrogen mimic, whereas TBBPA exerts its effects mainly through the thyroid pathway but the effects of these two compounds during development are not well understood (Meerts*, et*

al., 2001; Samuelsen*, et al.*, 2001; Sun*, et al.*, 2009). In contrast, the toxicity of TBBPA DME had not previously been studied, but the information is crucial in understanding the effects of exposure to the TBBPA O-methylated metabolites on different species.

In order to gain an understanding of how microbially mediated transformations of TBBPA, either anaerobic or aerobic, affect toxicity, zebrafish embryos were exposed to TBBPA, BPA, or TBBPA DME to observe developmental lesions caused by exposure to these compounds (Chapter 2.). TBBPA exposed embryos resulted in 100% mortality by 3 days at a 3 μM dose and by 5 days at a 1.5 μM dose (Fig. 2.2.). In contrast, TBBPA DME and BPA exposed embryos exhibit low levels of mortality in the embryonic period. Long-term survival experiments demonstrated that all compounds resulted in significantly decreased survival as compared to control embryos. These studies indicated that TBBPA is the most toxic followed by BPA, and then TBBPA DME as measured by mortality (Fig. 2.2 and Table 2.1.). These data illustrate that exposure to these compounds, at close to environmental concentrations, in the environment would have a significant adverse impact on developing aquatic species.

In addition to increased mortality, TBBPA, BPA and TBBPA DME exposed embryos also exhibited developmental lesions. Specifically, embryos exposed to all compounds experienced edema of both the yolk sac and pericardial region in addition to hemorrhages, (Table 2.1 and Fig. 2.3) which commonly seen in embryos exposed to xenobiotic compounds. TBBPA exposed embryos exhibited slower heart rates as compared to control embryos. Interestingly, embryos exposed to 1.5 and 3 μM TBBPA also exhibited poorly formed or truncated caudal regions. Embryos exposed to TBBPA at 0.75 μM TBBPA exhibited curved tails (Fig. 2.3). BPA exposure also resulted in a dose

response in curved tail formation, but at a low percentage, whereas TBBPA DME exposed embryos exhibited similar occurrence of this lesion at all doses tested (Table 2.1). The curved tail phenotype observed in previous studies of TBBPA and BPA exposures in zebrafish, but the BPA doses used were approximately 7 fold higher than in our studies (Duan*, et al.*, 2008; Kuiper*, et al.*, 2007b). Interestingly, the toxicity based on lesion occurrence suggests that TBBPA is the most toxic, followed by TBBPA DME and then BPA, which is different than the mortality data. These data suggest that lesions occurring in response to TBBPA, BPA and TBBPA DME exposure may be caused through different mechanisms, and that TBBPA is more potent at affecting proteins and processes involved in the proper caudal formation of zebrafish embryos.

The improper formation of the posterior region of TBBPA exposed zebrafish suggested a role of the MMPs in TBBPA toxicity. MMPs are involved in cellular migration, wound healing, ECM remodeling and cellular morphogenesis (Mott and Werb, 2004; Stamenkovic, 2003). Previous studies illustrate a role of MMPs in the proper formation of the caudal axis (Zhang*, et al.*, 2003a; Zhang*, et al.*, 2003b) and it is known that the Wnt pathway, which regulates caudal development, also regulates MMP expression (Harrington*, et al.*, 2007; Karow*, et al.*, 2008). Furthermore, TBBPA exposure is also known to cause the production of reactive oxygen species, which can play a role in the regulation of MMP expression (Reistad*, et al.*, 2005; Reistad*, et al.*, 2007; Svineng*, et al.*, 2008). Previous data has demonstrated that morpholino knockdowns of MMP-13 in zebrafish results in a similar truncated tail phenotype (Hillegass *et al.*, 2007). Additionally, studies with MMP-2 and -9 have shown them to be essential for normal development in zebrafish (Yoong*, et al.*, 2007; Zhang*, et al.*, 2003a).

Since the toxicological effects of BPA are well understood, and our interest was mainly in investigating the difference in toxicity between TBBPA and its O-methylated derivative, only these two compounds were used to examine the role of MMPs after exposure in the developing zebrafish. Expression of MMP-9 and -13 increased after TBBPA exposure to a greater degree than what was seen with TBBPA DME exposure (Fig. 2.6). MMP-2 expression in TBBPA DME exposed embryos was similar to that of control embryos. Additionally, the mRNA expression increase in TBBPA exposed embryos was followed by an increase in MMP activity (Fig. 2.7). One possible explanation is that the tail malformations and trunk edema seen during development are a result of increased MMP activity. Although we also observed an increase in MMP-9 following TBBPA DME exposure, no truncation in tail structure was observed. MMP-2 and -9 expression patterns may explain the difference in caudal development after TBBPA and TBBPA DME exposure since both enzymes are known for playing a role in promoting cellular migration (Murphy and Nagase, 2008). TBBPA exposure has been shown to upregulate MMP-9 mRNA expression and an effect on tail resorption in studies examining tadpole metamorphosis in the Pacific Tree Frog (Veldhoen*, et al.*, 2006). Altered expression and activity of MMPs could explain the lesions observed here and the aberration noted in hindgut formation reported by Kuiper et al, (2007).

Our hypothesis that the lesions observed in TBBPA exposed embryos are related to increased MMP expression and activity is supported by the finding that glucocortocoid exposure of developing zebrafish also increase MMP-2, -9 and -13 expression and activity and result in similar lesions such as impaired vascularization and tail malformations as detected after TBBPA exposure (Hillegass*, et al.*, 2007; Hillegass*, et*

al., 2008). Similar phenotypes are observed in the developing zebrafish embryos when morpholino knockdowns of MMP-13 are performed to prevent translation (Hillegass *et al.*, 2007). The data in this thesis seemingly contradict the results from the morpholino knockdown of MMPs, with both resulting in similar phenotypes of the caudal region. However, this suggests that it is the dysregulation of MMPs, which is important in leading to a malformed tail, not whether there is an increase or decrease in their expression. Morphogenesis is a complex process relying on the critical timing of cell proliferation, cell migration and ECM remodeling. It is likely that this MMP dysregulation is sufficient to alter proper ECM remodeling during development of the zebrafish embryo. Taken together, these data support our hypothesis that the tail malformations and altered heart beat detected in the developing embryo are related to the increase in MMP expression due to TBBPA exposure.

Additional experiments examining the role of MMP regulators during TBBPA exposure would be useful to understanding the molecular basis of lesion formation. Specifically, knockdowns of the MAPK pathway and analysis of MMP expression/activity would allow for an understanding in the role of MAPK and their regulation of MMPs in response to TBBPA. Additionally, analysis of the expression of proteins involved in the Wnt pathway in response to TBBPA or TBBPA DME exposure would help in understanding the differences in toxicities of these compounds to the developing embryo.

5.3. Toxicity of BPA, BPA MME and BPA DME to the developing zebrafish embryo

Experiments performed to determine the substrate range of *M. vanbaalenii* PYR-1 O-methylation capabilities, it was discovered that this microorganism can also O-methylate BPA (Fig. 3.1 and Table 3.1). In order to ascertain the toxicity of these novel compounds on the developing zebrafish embryos they were synthesized and purified. Dose response studies were performed to determine the best concentrations to use during the exposure studies. In contrast to the toxicity of TBBPA DME relative to TBBPA, the O-methylated metabolites of BPA exhibited much greater toxicity than BPA. This result was the opposite of the toxicity data showing that TBBPA DME resulted in decreased mortality and lesion occurrence in developing embryos when compared to TBBPA (Fig. 2.2, 2.3). Additionally, previous experiments with chlorinated guaiacols, a methyl benzene, illustrated equally toxic effects with the corresponding chlorinated veratrole (Neilson *et al.*, 1984). The BPA MME and BPA DME metabolites caused increased mortality to developing embryos at a lower dose than did BPA (Table 3.1). In the embryonic period, the LC50 values for these compounds ranked them from most toxic to least toxic as BPA MME, BPA DME and BPA in the embryonic period. In contrast, juvenile survival illustrated that BPA DME was the most toxic, with all doses resulting in less than 50% survival at 28 dpf (Table 3.1, 3.2). At 28 dpf, BPA MME was again more toxic than BPA. These data indicate that O-methylation does not always detoxify a compound, but can instead result in a much more toxic metabolite to developing organisms.

In addition to mortality, other lesions were identified in BPA, BPA MME, and BPA DME exposed embryos. Edema of the YS and PC region and hemorrhage in the

yolk sac were common after exposure to all three compounds, but the occurrence was greater in embryos exposed to BPA MME or BPA DME at comparable doses (Table 3.3.). Additionally, the embryos exposed to BPA DME have a higher percentage of significant lesions than the BPA MME at comparable concentrations (Table 3.3.). These data suggest that while BPA MME has an LC50 lower than BPA DME, the addition of another methyl group causes lesions that initially are sublethal, but eventually result in death of the embryo. Embryos exposed to lower levels (such as 0.25 mg/L) of BPA MME, may be able to recover from the lesions seen in the embryonic period of development (Table 3.2. and Table 3.3.). These data suggest that even if the BPA MME exposed embryos exhibit less lesions than those exposed to BPA DME, both compounds result in embryonic mortality are environmentally relevant doses.

A delay in the time to hatch is an indication of the effects due to chemical exposure on critical biochemical and developmental pathways necessary for the embryo's ability to free itself from the chorion (Nechaev and Pavlov, 2004; Sano*, et al.*, 2008), and is a crucial developmental benchmark to overall larval survival of aquatic species in the environment. Despite the high percentages of lesions, the time to hatch for the BPA DME embryos was not affected, but was delayed for BPA exposed embryos (Table 3.2). Interestingly, this same trend in the time to hatch was noted with TBBPA and TBBPA DME (Table 2.1). It is possible that the O-methyl groups are important in regulating the time to hatch, since without them, exposure to the parent compounds BPA or TBBPA results in delayed hatching. Another explanation may be that the TBBPA exposed embryos lacking a properly formed caudal region, exhibit impaired movement inside the chorion and thus hatching is delayed. TBBPA and BPA may affect the proper production

and secretion of the hatching protein, which assists the chorion in disintegrating enabling hatching of the embryo, but to date, the effects of TBBPA or BPA exposure to these protein levels have not been tested. The exact mechanism is still unknown, but despite the differences in toxicity and lesion production, this trend is similar between chemicals, and is likely a function of the O-methylation reaction.

5.4. Comparison of Toxicological Effects Observed After Exposure to TBBPA, BPA and their O-methylated metabolites

Interestingly, even though both TBBPA and BPA were O-methylated, the toxicity of the corresponding O-methylated metabolites was markedly different. This finding sparks some interesting questions regarding the mechanism of toxicity and immediately suggests the importance of the bromine substituents in influencing overall toxicity. The structures of TBBPA DME and BPA DME differ only in the bromine substitutes present, but exposure of the developing zebrafish embryo to BPA DME results in increased mortality. TBBPA exposure results in a truncated caudal region, a lesion not detected after exposure to any other compound tested. MMP expression data is not yet available for embryos exposed to BPA, BPA MME, or BPA DME, but the embryos seem to develop normally, thus it is likely that MMPs are be altered during development. However, MMPs can be regulated by both the estrogen and thyroid signaling pathways, and as such may be altered after BPA, BPA MME or BPA DME exposure (Elliot *et al.*, 2008; Glassberg *et al.*, 2008; Kitamura*, et al.*, 2002; Kitamura*, et al.*, 2005a) Specifically, estrogenicity increases as the number of bromines decreases (BPA), however increased thyroid binding corresponds to increased number of bromine

substituents (TBBPA) (Hamers, *et al.*, 2006; Jagnytsch, *et al.*, 2006; Kitamura, *et al.*, 2005a; Meerts, *et al.*, 2001). Thus, even though BPA and TBBPA are both endocrine disruptors, TBBPA acts mainly through the thyroid-signaling pathway as an agonist to the thyroid receptor while BPA is found to act through the estrogen pathway, but is also a thyroid receptor antagonist. Interestingly, TBBPA exposure causes increased resorption of tadpole tails during frog metamorphosis, but BPA blocks this developmental process (Heimeier *et al.*, 2009; Iwamuro *et al.*, 2003; Jagnytsch, *et al.*, 2006; Kitamura, *et al.*, 2005a; Veldhoen, *et al.*, 2006). It is likely that the differences in toxicity we see between the TBBPA DME and the BPA MME/BPA DME are a result of the bromine substituents and the molecular pathways being affected by exposure. Future studies should examine the role of the thyroid and estrogen signaling pathways should be conducted to determine if they are affected by exposure, and contribute to the developmental malformations seen in the developing zebrafish embryo after exposure to these chemicals.

5.5. Biodegradation Potential of O-methylated Metabolites

The O-methylation of BPA to BPA MME and BPA DME by *M. vanbaalenii* PYR1 is novel and not previously demonstrated. This transformation results in metabolites with increased toxicity as compared to BPA, but the environmental prevalence of these O-methylated metabolites is not known. Activated sludge samples were examined for the ability to O-methylate BPA, but our data suggest that BPA mineralization was a competing process. BPA can be mineralized in aerobic sediments within 5 days by indigenous microorganisms (Kang, *et al.*, 2004; Lobos, *et al.*, 1992;

Masuda*, et al.*, 2007). The O-methylation reaction is also aerobic but is much slower than the mineralization of BPA (Fig. 3.1 and 3.2.). Future studies should focus first on the detection of these metabolites in the environment and then attempt to identify this reaction *in vitro*. In addition to environmental detection, toxicity of TBBPA, BPA and their O-methylated metabolites on microorganisms needs to be determined. These metabolites have the potential to be harmful to microorganisms, which would affect the potential for further biotransformation. It is possible that under our culture conditions, O-methylation was not optimal, but could proceed more similarly to the rate of TBBPA O-methylation.

The fate of these O-methylated metabolites in the environment is not currently understood, but they are more lipophilic, and likely more recalcitrant to degradation. Microorganisms are capable of utilizing many different compounds and functional groups as carbon sources, electron donors and acceptors. TBBPA can be reductively dehalogenated to BPA in anaerobic sediments(Arbeli and Ronen, 2003; Arbeli*, et al.*, 2006; Ravit*, et al.*, 2005; Ronen and Abeliovich, 2000; Voordeckers*, et al.*, 2002). TBBPA DME anaerobic microcosms with sediments from NJ/NY were prepared and incubated under sulfidogenic and non-sulfidogenic conditions to determine whether or not TBBPA DME could be debrominated. No transformation of TBBPA DME was detected under any of these conditions. It is possible that our experimental design, specifically the medium composition or temperature of incubation was not appropriate for the bacterium possibly capable of dehalogenation. Additionally, we tested two sites, Arthur Kill and Shinnecock Bay, but it is possible that neither site sample contained dehalogenating microorganisms. Preliminary studies with *Desulfoluna spongiiphila* strain

AA1 were not successful because the culture was not growing well due to a phenol degrading contaminating organism, and therefore no dehalogenation was detected (data not shown). However, it would be advantageous in the future to use a pure culture of *D. spongiiphila* strain AA1 spiked with TBBPA DME to determine if dehalogenation is possible, with TBBPA as a control. *D. spongiiphila* AA1 is known to debrominate 2,6-TBP easily within two weeks under sulfidogenic conditions and can also dehalogenate other bromophenols and iodophenols, but not fluorinated or chlorinated phenols (Ahn *et al.*, 2009). This information is vital in further understanding the formation of BPA MME and DME in the environment. Our data do not conclusively show BPA O-methylation in environmental samples, since BPA degradation competes with the O-methylation and occurs at a faster rate. It is possible that a BPA degrading organism was present in our activated sludge samples, but the conditions were not ideal for optimal growth and metabolism. However, BPA DME formation could occur if TBBPA DME was dehalogenated. Due to the increased toxicity of the BPA MME and BPA DME metabolites in the developing zebrafish, it is crucial to understand the different mechanisms by which these metabolites can form in the environment.

In addition to dehalogenation, it is possible that TBBPA DME could be de-O-methylated by microorganisms. One such group are the acetogens, which are anaerobic bacteria, and have been shown to de-O-methylate methyl tert-butyl ether (MTBE) (Youngster, 2009). Our laboratory has several cultures capable of degrading methyl tert-butyl ether (MTBE) by a de-O-methylation reaction, and one culture was used to examine the possible O-demethylation of TBBPA DME using syringate as a co-metabolite (Chapter 4). Syringate was monitored by HPLC and use by the microorganisms was

detected initially, but the TBBPA DME spiked into the culture was not used by the culture. Repeated additions of syringate found the culture did not use it for de-O-methylation so it is possible the microbial consortia died, or because it was not a pure culture, the other organisms thrived on the by products from the medium and syringate. Future studies should employ the use of an isolated culture, of a known de-O-methylator, such as *Xanthomonas retroflexus, Pseudomonas putida,* or *Acinetobacter radioresistens* spiked with TBBPA DME to determine if it can be transformed (Goswami *et al.*, 2007). It is likely one of these organisms would be able to O-demethylate TBBPA DME, because they can O-demethylate 2,4,6-trichloroanisole. Additional cultures should also be prepared with BPA DME and BPA MME to understand the fate of BPA MME and DME in the environment. Our data thus far suggest that TBBPA DME is highly recalcitrant with no detectable degradation in anaerobic cultures in over a year, but it is possible that a pure culture will yield different information improving upon the knowledge of possible degradation of this compound. However, studies have indicated TBBPA DME has been detected at 6 fold higher concentrations in the environment than TBBPA, this suggests prevalent O-methylation, but also recalcitrance of the O-methylated metabolite (Alaee*, et al.*, 2003; Hakk and Letcher, 2003; Hale*, et al.*, 2006).

5.6. Conclusions

Collectively these studies suggest that microbial mediated O-methylation of halogenated and non-halogenated phenolics is a process occurring ubiquitously in the environment resulting in metabolites exhibiting altered toxicity. These microbially mediated transformations impact the environment, human health and also the economy.

Mainly, the economic impact results from cork taint in wine (Alvarez-Rodriguez*, et al.*, 2002; Coque*, et al.*, 2003; Miki*, et al.*, 2005), but recently, there have been reports about tainted Tylenol, and Tylenol products, including Rolaids, citing a musty odor. It was determined that these products were contaminated with 2,4,6-tribromoanisole, the O-methylated metabolite of 2,4,6-tribromophenol (McCoy, 2010). The 2,4,6-tribromoanisole was a contaminant originating from the wooden pallets used to transport these medicines (McCoy, 2010). There is a wide reaching recall for these products, and some people who have ingested them have experienced intestinal discomfort (McCoy, 2010). The presence of O-methylated metabolites in Tylenol illustrates the importance in understanding the O-methylation of phenolic compounds, and their resulting toxicity, since exposure to these compounds can occur from many different sources. There is much to be learned yet about the function of this reaction, but the studies in this thesis have contributed much information as to the toxicity of these compounds, and have demonstrated that O-methylated metabolites can have toxic effects greater than their parent products.

6.0. References

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7.0. Appendices

Appendix 1: Microbial Mediated Degradation of TBBPA DME

In order to test the biodegradation potential of TBBPA DME, several different types of cultures were prepared. All anaerobic cultures were grown in a basic anaerobic medium containing (in g/L) 1.3 KCl, 0.2 KH_2PO4, 1.17 NaCl, 0.5 NH_4Cl, 0.1 $CaCl_22H_2O$, 3.0 $MgCl_26H_2O$, 2.5 Na_2CO_3 and 2.84 Na_2SO_4 for sulfidogenic medium. Also, vitamins, trace salts, resazurin, and Na_2S-$9H_2O$ were added. The medium used to culture acetogens was the basic non-sulfidogenic medium, but contained 0.18 g/L of $MgCl_26H_2O$ instead of 3.0 g/L. All cultures were sealedwith a Teflon cap at room temperature. Sulfidogenic and non-sulfidogenic cultures were prepared and spiked with TBBPA DME, and monitored for one year. No degradation of TBBPA DME was detected in the twelve months of cultures (data not shown). Additionally, cultures were prepared with non-sulfidogenic medium, and spiked with 100 μM syringate, to stimulate growth of de-O-methylating organisms. These cultures were also monitored for one year, the organisms within used the syringate, but did not de-O-methylate TBBPA DME. Aerobic cultures were also prepared, but no degradation was noted in these cultures. These data demonstrate that TBBPA DME is recalcitrant under the conditions tested in these experiments.

Appendix 2: Expression of CYP450 genes after exposure to TBBPA or TBBPA DME

In order to investigate the role of the Aryl Hydrocarbon Receptor (AhR) pathway after exposure to TBBPA or TBBPA DME, Real Time PCR was conducted to analyze gene expression of CYP1A1 and CYP1B1. RNA was isolated from developing zebrafish embryos at 24, 48, and 72 hours post fertilization, and Real Time PCR was conducted as per methods described in Chapter 2. The 28S rRNA gene was used to normalize the PCR data. These data are representative of at least three replicates, but the data never fully repliated and so these genes were not examined further. To date, it has not been demonstrated that TBBPA is an AhR agonist, and is very weakly estrogenic, thus the CYP450s were not examined in further detail.

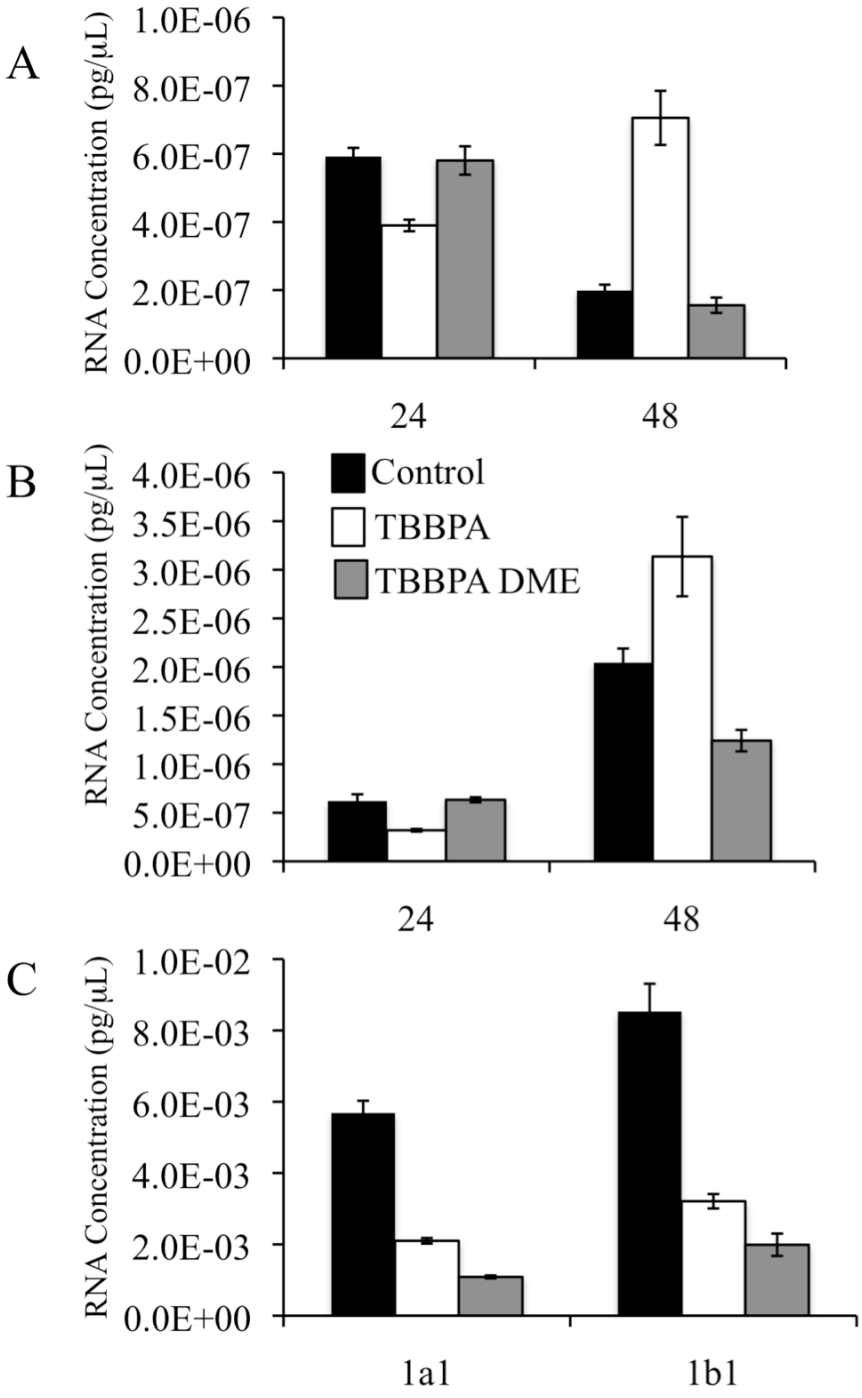

Figure A1: Expression of zebrafish CYP1A1 and CYP1B1 in response to TBBPA and TBBPA DME exposure. A. Expression of CYP1A1 after 24 and 48 hpf exposure to TBBPA and TBBPA DME, B. Expression of CYP1B1 after 24 and 48 hpf exposure to TBBPA and TBBPA DME, C. Expression of CYP1A1 and CYP1B1 after 72 hpf. Data are representative of three experiments.

Appendix 3: Activity of Matrix Metalloproteinases after TBBPA and TBBPA DME exposure in the developing zebrafish

In order to better understand the role of the matrix metalloproteinases in the developmental lesions observed in the developing zebrafish after exposure to TBBPA and TBBPA DME, *in vitro* zymography was conducted to examine MMP activity. The methods used to obtain these data are described in detail in Chapter 2.

These data were not included in the second chapter because the trends seen in these data do not follow the trends in the RNA data. Additionally, since these assays were analyzed on total protein, it is possible that there were changes due to widespread expression in the developing embryo.

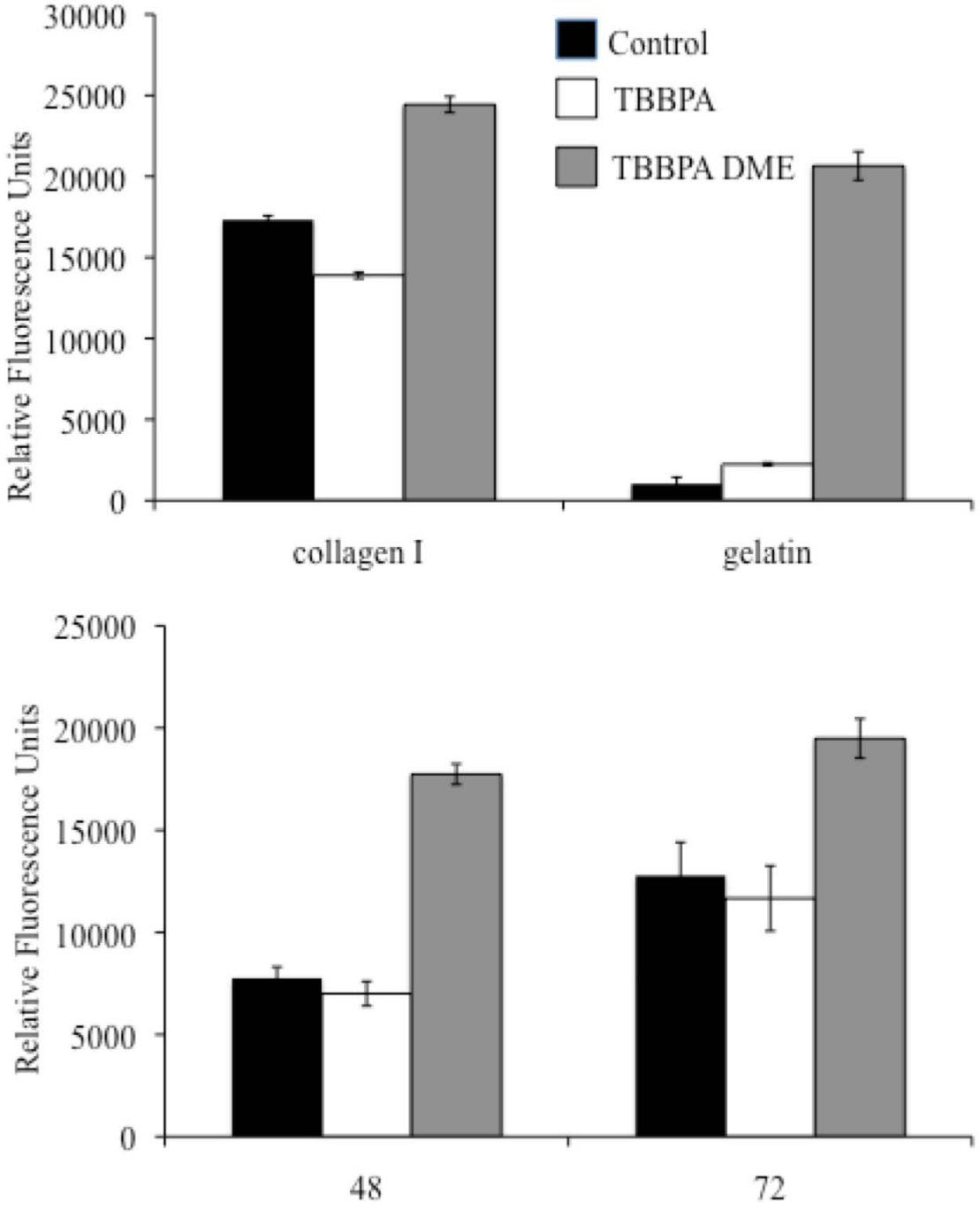

Fig. A2. *In Vitro* Zymography of TBBPA and TBBPA DME protein samples. Panel (A) shows data from 72 hpf for collagen I and gelatin. Panel (B) contains data using collagen IV as the fluoresceinated substrate.

Appendix 4: Rate of Heartbeats in the developing zebrafish in response to BPA, BPA MME or BPA DME

After exposure to BPA, BPA MME or BPA DME, initial observation demonstrated a possible effect on embryo heart rate. To investigate this further, zebrafish embryos were exposed to BPA, BPA MME, BPA DME or vehicle and their heartbeats were counted at 48 and 72 hpf. The methods for this experiment are explained in detail in Chapter 2. In the BPA exposed fish, in addition to a significant decrease in heart beat an arrhythmia was detected at 48 and 72 hpf, but could be due to high dosage and impending death of the embryo. Embryos exposed to BPA MME had a significant decrease in heartbeat at 72 hpf, but the BPA DME exposed embryos did not exhibit a decreased heartbeat. Additionally, embryos exposed to BPA MME or BPA DME did not exhibit arrhythmias. It is likely that these alterations in heart rate was due to the higher doses of treatment, since the embryos died within the embryonic period and were exhibiting other lesions such as hemorrhage and edema.

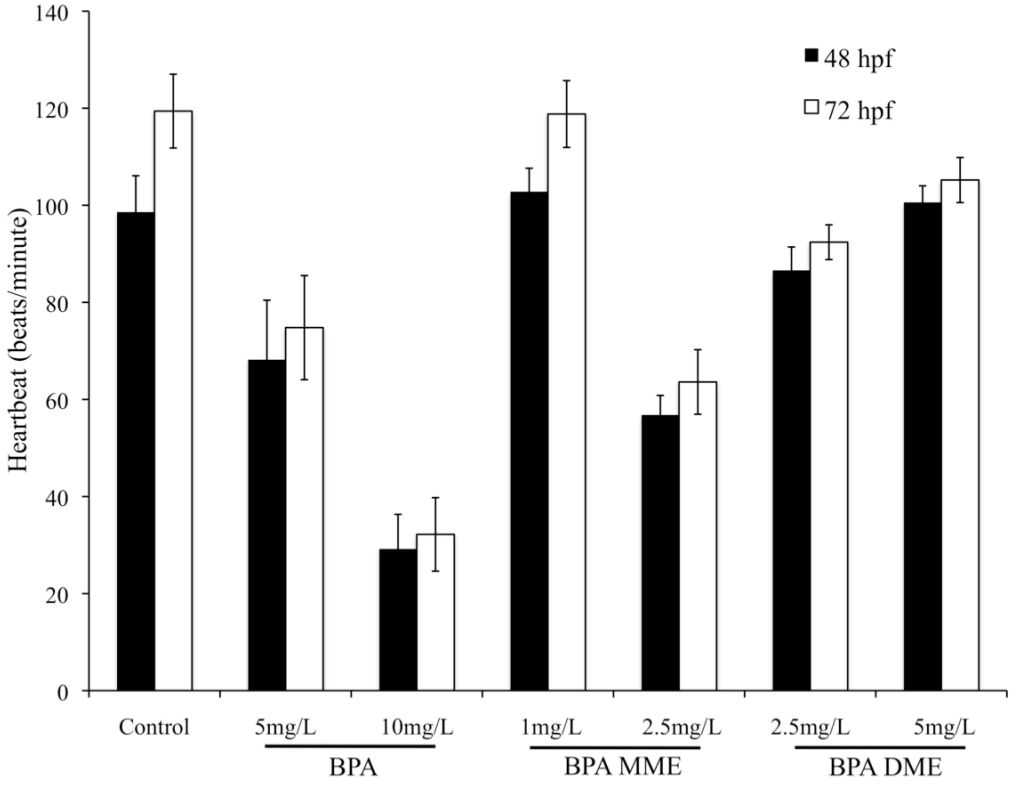

Fig A3: Zebrafish embryo heart rate after exposure to BPA, BPA MME or BPA DME.

Appendix 5: Alcian Blue Staining of Cartilage in Zebrafish Embryos Exposed to BPA, BPA MME or BPA DME

A common developmental lesion after exposure to xenobiotic compounds is an alteration in the cartilage structure. To measure any changes in cartilage structure in the developing zebrafish, alcian blue staining was performed as per the detailed methods in Chapter 2. These data show that exposure to BPA, BPA MME or BPA DME do not cause biologically significant malformation in larvae after exposure to these compounds at the doses chosen.

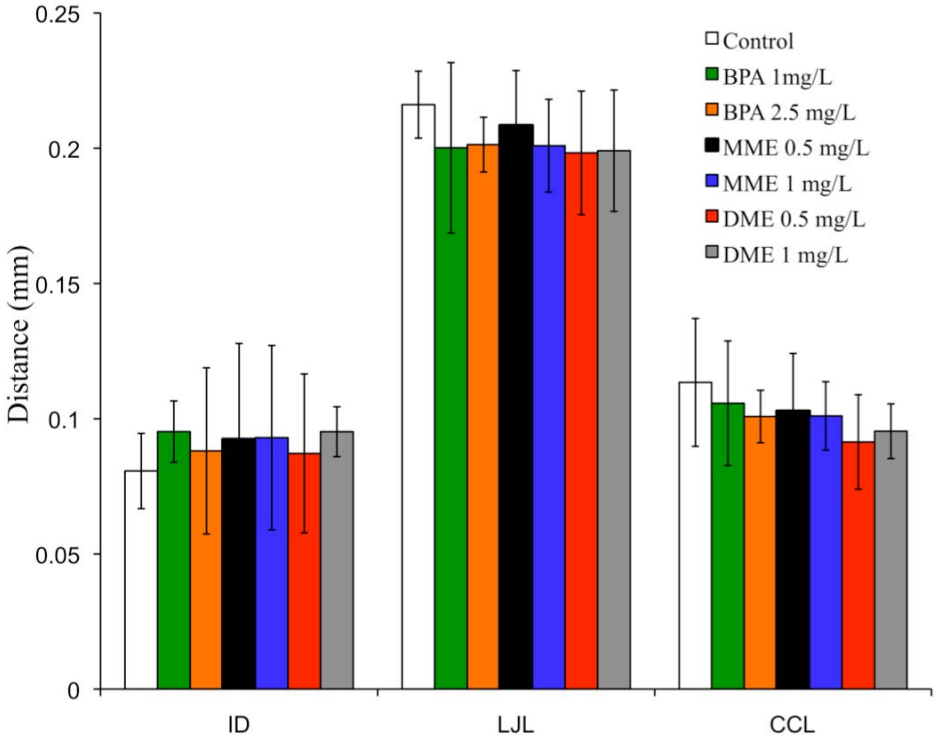

Fig. A4: Alcian Blue Staining of Zebrafish Embryos exposed to BPA, BPA MME, or BPA DME. Zebrafish embryos were exposed to BPA, BPA MME, BPA DME, or vehicle, and the cartilage was stained at 7 dpf with alcian blue. Measurements were taken of the interocular distance (ID), the lower jaw length (LJL) and cerohyal cartilage length (CCL).

Appendix 6: TBBPA O-methylation by *M. vanbaalenii* PYR-1 is a constitutive reaction

In order to determine if the O-methylation of TBBPA by M. vanbaalenii PYR1 was inducible or constitive, 100 uM TBBPA was spiked into late log phase cultures with or without chloramphenicol. Samples were taken over a period of six days and analyzed for the presence of TBBPA MME or TBBPA DME. Analytics made for some difficulties, but it is apparent that in the cultures with chloramphenicol, a protein synthesis inhibitor, O-methylation can occur in a near identical fashion indicating a constitive reaction in M. vanbaalenii PYR1.

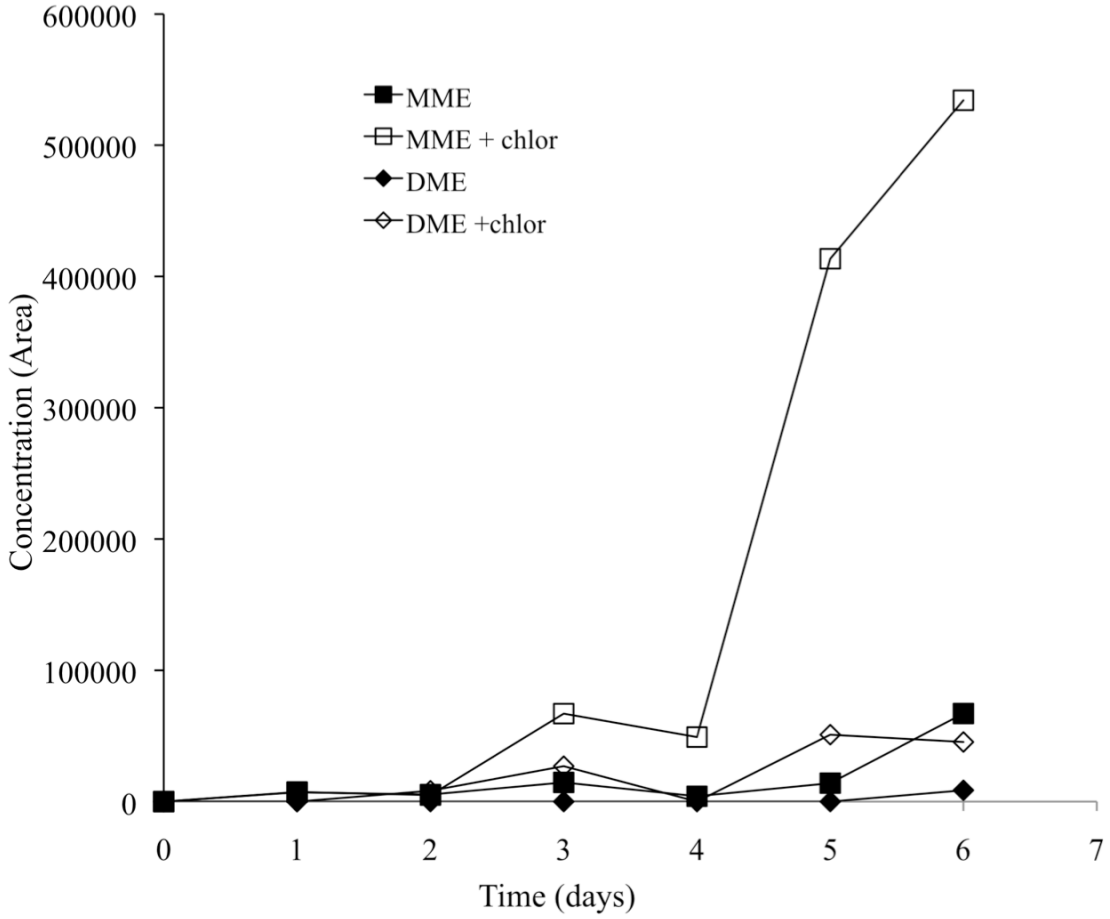

Figure A5: TBBPA O-methylation by *M. vanbaalenii* PYR-1 in the presence of chloramphenicol

8.0 Curriculum Vitae

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Education

Sept. 2005- May 2010 — **Rutgers, The State University of New Jersey & University of Medicine and Dentistry of New Jersey**
Doctor of Philosophy Microbiology and Molecular Genetics
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Sept. 1996- May 2000 — **Ohio Wesleyan University**
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May 2002- Aug. 2005 — **University of Medicine and Dentistry of New Jersey-New Jersey Medical School**
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Sept. 2000- 2002 — **University of Medicine and Dentistry of New Jersey-New Jersey Medical School**
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2002-2005	**Center for International and Security Studies at Maryland** Consultant for Dangerous Pathogens Project
2002	**Medical Repair Laboratories, New York City, NY** Bio-Safety Consultant

Publications

McCormick, J.M., Häggblom, M.M., Cooper, K.R, and L.A. White. The Brominated Flame Retardant, Tetrabromobisphenol A (TBBPA) and its metabolites, Bisphenol A (BPA) and Tetrabromobisphenol A dimethyl ether (TBBPA DME), cause chemical specific lesions in the developing zebrafish (*Danio rerio*) embryo (accepted to Aquatic Toxicology, 2010)

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