Effects of four new brominated flame retardants on hepatic messenger RNA expression, *in vitro* toxicity and *in ovo* toxicity in the domestic chicken (*Gallus gallus*)

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Abstract

Brominated flame retardants (BFR) such as hexachlorocyclopentadienyl-dibromocyclooctane (HCDBCO), bis(2-ethylhexyl)tetrabromophthalate (BEHTBP), 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE) and decabromodiphenylethane (DBDPE) are contaminants of environmental concern. These BFRs are replacement alternatives for some of the major production BFRs, which have been restricted from the marketplace due to their adverse health effects. Their presence in environmental matrices, including wild birds, suggests they should be tested for possible toxic effects. BFR alternatives have been detected in the eggs of colonial fish-eating birds, suggesting maternal transfer during ovogenesis and the potential for these chemicals to bioaccumulate through the food chain. However, information regarding the toxicity of HCDBCO, BEHTBP, BTBPE and DBDPE exposure in birds is lacking. This thesis consisted of a combined *in vitro/in ovo* approach to determine: 1) the concentration-dependent effects of these four BFR alternatives in chicken embryonic hepatocytes (CEH), and 2) the dose-dependent effects of HCDBCO and BTBPE in chicken embryos following injection into the air cell of eggs prior to incubation. Changes in the mRNA expression levels of genes previously found to be responsive to other BFRs were assessed in CEH and liver tissue, in addition to examining overt toxicity (i.e. cytotoxicity, pipping success). None of the BFRs tested were cytotoxic up to 60 μM HCDBCO, 60 μM BEHTBP, 1.4 μM BTBPE or 0.2 μM DBDPE in CEH. Injection doses up to 50 μg/g egg HCDBCO and 10 μg/g egg BTBPE had no effect on embryonic pipping success. The accumulation of HCDBCO and BTBPE was variable in liver and did not follow a linear uptake pattern with respect to injection dose, due in part to difficulties with the solubility of these chemicals in the dimethyl sulfoxide (DMSO) vehicle. In, CEH,

HCDBCO caused a decrease in CYP1A4/5 mRNA at all concentrations tested, while CYP2H1 and CYP3A37 were induced only at 10 μM. In contrast, only TTR mRNA was down-regulated in hepatic tissue at all injection concentrations of HCDBCO. The highest concentration of BTBPE induced CYP1A4/5 mRNA to 115- and 18-fold in CEH, and 6.5- and 1.8-fold in liver tissue. *In vitro* and *in ovo* exposure to BTBPE caused a concentration-dependent decrease in DIO3 mRNA, while CYP3A37 was down-regulated 2-fold at 10 μg/g in liver tissue. In CEH, DBDPE induced CYP1A4/5 mRNA to a maximum of 29- and 59-fold at 0.2 μM, and increases in DIO1 mRNA and decreases in CYP3A37 mRNA were also observed. None of the gene targets were responsive to BEHTBP exposure in CEH. This is the first study to report on the toxicological and molecular effects of HCDBCO, BEHTBP, BTBPE and DBDPE in an avian species. Using this combined *in vitro*/*in ovo* approach has permitted the characterization of these four BFR alternatives by defining possible mechanisms of biological action in a model avian species, the chicken.

Résumé

Les produits d'ignifugeants bromés (PIB), comme le hexachlorocyclopentadienyl-dibromocyclooctane (HCDBCO), le bis(2-éthylhexyle)tétrabromophthalate (BEHTBP), le 1,2-bis(2,4,6-tribromophénoxy)éthane (BTBPE) et le décabromodiphényléthane (DBDPE), sont des contaminants préoccupants d'un point de vue environnemental. Ces ignifugeants bromés sont utilisés comme remplaçant chimique à d'autres ignifugeants bromés de haute production, duquel l'usage a été limité à cause de leurs effets néfastes sur la santé. Leur présence dans des matrices environnementales, incluant les oiseaux sauvages, suggère qu'ils doivent être testés pour des effets toxiques possibles. Des produits de remplacement d'ignifugeants bromés ont été détectés dans les œufs d'oiseaux piscivores coloniaux, ce qui laisse supposer un transfert maternel pendant l'ovogenèse et le potentiel de bioaccumulation de ces produits chimiques dans la chaîne alimentaire. Toutefois, on dispose de peu d'information concernant la toxicité du HCDBCO, du BEHTBP, du BTBPE et du DBDPE pour les oiseaux qui y sont exposés. Cette thèse consistait en une approche combinant des essais *in vitro* et *in ovo* pour définir : 1) les effets liés à la concentration de ces quatre produits de remplacement d'ignifugeants bromés dans les hépatocytes embryonnaires de poulet, et 2) les effets liés à la dose de HCDBCO et de BTBPE sur les embryons de poulet à la suite de leur injection dans la chambre à air des œufs avant l'incubation. Les changements dans les niveaux d'expression d'ARNm des gènes qui s'étaient auparavant avérés réactifs à d'autres ignifugeants bromés ont été évalués dans les hépatocytes embryonnaires de poulet et dans les tissus hépatiques d'embryons, et leur toxicité manifeste a été examinée (c.-à-d. cytotoxicité, bêchage). Aucun des ignifugeants bromés soumis à l'essai n'était cytotoxique jusqu'à 60 µM de HCDBCO, 60 µM de BEHTBP, 1,4 µM de BTBPE ou 0,2 µM de DBDPE

dans les hépatocytes embryonnaires de poulet. Les doses d'injection allant jusqu'à 50 µg/g de HCDBCO par œuf et 10 µg/g de BTBPE par œuf n'ont eu aucun effet sur le bêchage des embryons. L'accumulation de HCDBCO et de BTBPE était variable dans le foie et n'a pas suivi de tendance d'absorption linéaire en ce qui a trait à la dose d'injection, en partie à cause des difficultés liées à la solubilité de ces produits chimiques dans du diméthylsulfoxyde (DMSO). Le HCDBCO a entraîné une diminution de l'ARNm du CYP1A4 et du CYP1A5 à toutes les concentrations testées, tandis que seulement 10 µM a induit l'ARNm du CYP2H1 et du CYP3A37 dans les hépatocytes embryonnaires de poulet. En revanche, seul l'ARNm de la transthyrétine était régulé négativement dans les tissus hépatiques à toutes les doses de HCDBCO. La concentration la plus élevée de BTBPE a induit 115 et 18 fois plus d'ARNm du CYP1A4 et du CYP1A5 dans les hépatocytes embryonnaires de poulet, et 6,5 et 1,8 fois plus d'ARNm du CYP1A4 et du CYP1A5 dans les tissus hépatiques. L'exposition *in vitro* et *in ovo* au BTBPE a entraîné une diminution liée à la concentration dans l'ARNm du DIO3, tandis que 2 fois la quantité d'ARNm du CYP3A37 a été régulée négativement à 10 µg/g dans les tissus hépatiques. Dans les hépatocytes embryonnaires de poulet, 0,2 µM DBDPE a induit au maximum 29 et 59 fois plus d'ARNm du CYP1A4 et du CYP1A5. Des augmentations d'ARNm du DIO1 et des diminutions d'ARNm du CYP3A37 ont également été observées. Aucune des cibles génétiques n'a été réactive à l'exposition au BEHTBP dans les hépatocytes embryonnaires de poulet. Il s'agit de la première étude sur les effets toxicologiques et moléculaires du HCDBCO, du BEHTBP, du BTBPE et du DBDPE sur les espèces aviaires. Les concentrations d'exposition de BEHTBP, BTBPE et DBDPE vont au-delà de leur présence dans les espèces sauvages. Cette approche combinant des essais *in vitro* et *in ovo* a permis de caractériser ces produits de remplacement d'ignifugeants bromés

en définissant les mécanismes d'action biologiques possibles chez une espèce aviaire modèle, dont le poulet.

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List of abbreviations

AhR	aryl hydrocarbon receptor
bw	body weight
BEHTBP	bis(2-ethylhexyl)tetrabromophthalate
BFR	brominated flame retardant
BTBPE	bis(2,4,6-tribromophenoxy)ethane
CAR	constitutive androstane receptor
cDNA	complementary deoxyribonucleic acid
CEH	chicken embryonic hepatocytes
CXR	chicken xenobiotic receptor
CYP	cytochrome P450 monooxygenase
DBDPE	decabromodiphenylethane
DIO	deiodinase
DLC	dioxin-like compound
DMSO	dimethyl sulfoxide
DP	dechlorane plus
dw	dry weight
EC50	half maximal effective concentration
EROD	ethoxyresorufin-O-deethylase
GC-MS	gas chromatography mass spectrometry
HBCD	hexabromocyclododecane
HCDBCO	hexachlorocyclopentadienyl-dibromocyclooctane
L-FABP	liver-fatty acid binding protein
LD50	median lethal dose
lw	lipid weight
m/z	mass-to-charge ratio
MLOD	method limit of detection
MLOQ	method limit of quantitation
PAH	polycyclic aromatic hydrocarbon
PBDE	polybrominated diphenyl ether
PBDD	polybrominated dibenzo-dioxin
PBDF	polybrominated dibenzo-furan

PXR	pregnane x receptor
mRNA	messenger ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
T3	triiodothyronine
T4	thyroxine
TBBPA	tetrabromobisphenol-A
TH	thyroid hormone
THRSP-14α	thyroid hormone responsive SPOT 14α
TTR	transthyretin
UGT	uridine diphosphate glucuronosyltransferase
ww	wet weight

Publication plan

Paper: *"In vitro* and *in ovo* effects of HCDBCO, BEHTBP, BTBPE and DBDPE on hepatic mRNA expression in chicken embryos*"*.

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Statement of contributions

All procedures relating to the chemical analysis of livers were adapted from the Organic Research Group Method (SOP): MET-ORGRES-NEW BFR/PBDE method (Revision #2, Mar. 2010). I would like to acknowledge the contributions of Lewis Gauthier for the GC-MS analysis of all tissue samples pertaining to this project.

Chapter 1 – General introduction

1.1 Thesis overview

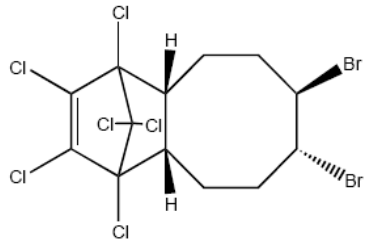
Brominated flame retardants (BFRs) are organic compounds that hinder or reduce the flammability of various materials, thereby enhancing the safety of commercial products to consumers. Hexachlorocyclopentadienyl-dibromocyclooctane (HCDBCO), bis(2-ethylhexyl)tetrabromophthalate (BEHTBP), 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE) and decabromodiphenylethane (DBDPE) are BFRs that have recently been detected in the environment (Figure 1.1). They are alternatives to toxic BFRs (e.g. octa-BDE), which have been largely phased out from the marketplace due to their detrimental effects in animals and potential hazard to humans (Kemmlein *et al*. 2009;Birnbaum and Staskal 2004). Global monitoring studies have reported levels of BTBPE and DBDPE in high trophic-level birds that subsist primarily in aquatic ecosystems (Karlsson *et al*. 2006;Gauthier *et al*. 2009;Verreault *et al*. 2007;Shi *et al*. 2009). The health of water birds is a good indicator of ecosystem health, as they can ingest contaminants accumulated through the aquatic food web. While BEHTBP has not yet been detected in wild avian species, its occurrence in marine mammals (Lam *et al*. 2009) indicates its potential to accumulate up the food chain to top trophic species. Finally, although the bioavailability of HCDBCO is not known, its presence in residential indoor dust suggests its potential to migrate from consumer products, during its manufacture or disposal, and enter the environment (Zhu *et al.* 2008).

As some BFRs are environmentally persistent, bioaccumulative and toxic, it is important to determine the impact that HCDBCO, BEHTBP, BTBPE and DBDPE may have on the health of wild birds that may be at risk of exposure. Previous studies have shown that BFRs affect certain biochemical responses in the liver, which is the primary metabolizing

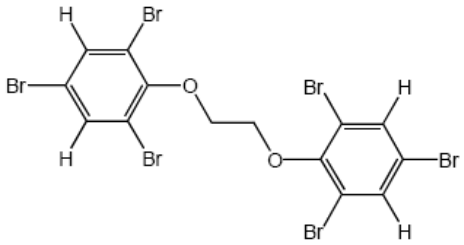
site for the detoxification of xenobiotics. Major production BFRs, such as hexabromocyclododecane (HBCD) and polybrominated diphenyl ethers (PBDEs), have been known to activate aryl hydrocarbon receptor (AhR) –dependent and –independent xenobiotic responses in the liver, while potentially disrupting aspects of endobiotic metabolism. By real time reverse-transcriptase PCR, Crump *et al*. (2008a) identified hepatic genes involved in xenobiotic metabolism, the thyroid hormone pathway and lipid metabolism that were useful molecular markers of HBCD and PBDE exposure. It was suggested that by utilizing these gene targets as molecular markers of BFR exposure, other BFRs of environmental concern could be screened for potential toxic effects. In addition, examining BFR-induced alterations in the expression of these gene targets could provide a mechanistic understanding of the action of that specific BFR in organisms. As the effects of BFR alternatives have not yet been investigated in birds, a series of experiments will look at the toxicological and molecular consequences of HCDBCO, BEHTBP, BTBPE and DBDPE exposure in chicken. The objectives of this thesis were:

- To generate hepatic mRNA profiles for HCDBCO, BEHTBP, BTBPE and DBDPE by assessing molecular markers of BFR exposure in primary cultures of chicken embryonic hepatocytes.
- To validate the *in vitro* results with respect to HCDBCO and BTBPE exposure, by assessing the same hepatic gene targets in developing chicken embryos exposed to these two BFRs.
- To determine the toxic potential of these four BFRs through relative measures of cell viability in primary chicken embryonic hepatocytes and pipping success in developing chicken embryos.

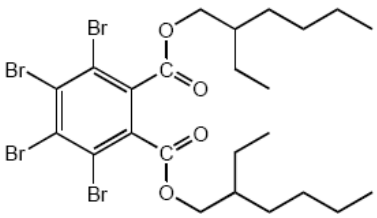
HCDBCO



BTBPE



BEHTBP



DBDPE

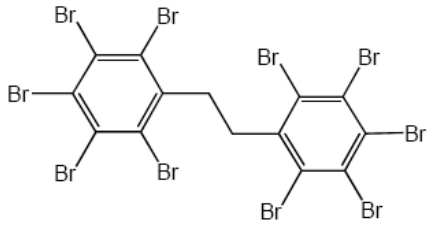


Figure 1.1. Chemical structures of hexachlorocyclopentadienyldibromocyclooctane (HCDBCO), bis(2-ethylhexyl), tetrabromophtalate (BEHTBP), bis(tribromophenoxy)ethane (BTBPE) and decabromodiphenylethane (DBDPE).

1.2 Brominated Flame Retardants (BFRs)

BFRs comprise a large class of structurally diverse compounds consisting of brominated aliphatics, cyclo-aliphatics and aromatics (Figures 1.1 and 1.2). They are incorporated into materials during or after the manufacturing process to increase the fire resistant properties of commercial products. The bromine atoms in BFRs delay or suppress the combustion process of materials by eliminating free radicals, thus inhibiting the progression of fire (BSEF, 2000). BFRs are more effective than other halogenated flame retardants (e.g. organochlorines) due to their thermal stability at high temperatures and less volatile decomposition products (Segev *et al*. 2009). The high performance efficiency and low production cost of BFRs makes these chemicals the largest group of flame retardants marketed globally (Williams and DeSesso 2010;Birnbaum and Staskal 2004).

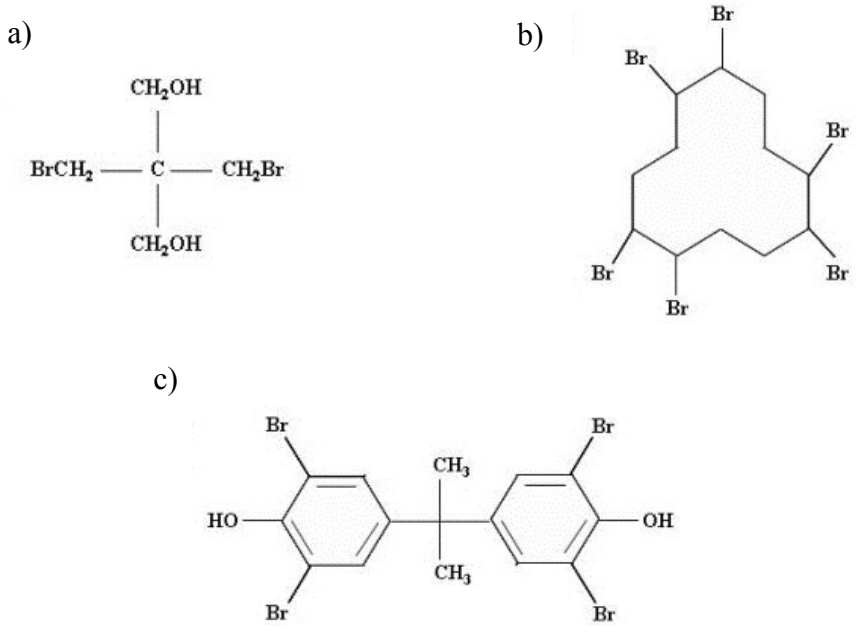


Figure 1.2. Examples of structurally-different BFRs; a) aliphatic dibromoneopentyl glycol, b) cycloaliphatic hexabromocyclododecane (HBCD) and c) aromatic tetrabromobisphenol-A (TBBPA). (*Chemical structures from Segev et al. 2009*)

The manner in which BFRs are integrated into materials can dictate their tendency to leach into the environment. BFRs are commonly used as additives during the manufacture of consumer products, whereby they are physically blended with polymers (Huber and Ballschmiter 2001). Since they are not chemically bound to materials, additive BFRs are more likely to migrate from products over time and enter the environment (Tomy *et al*. 2004).

1.2.1 Sources of BFRs

BFRs are ubiquitous environmental contaminants that originate from various anthropogenic sources. Since the 1960s, the market demand for these organobromines has seen considerable growth due to their high performance efficiency and wide variety of applications (Birnbaum and Staskal 2004). In 2000, the annual market demand for BFRs reached approximately 200 000 metric tons worldwide (BSEF 2000). The polybrominated diphenyl ethers are an important class of BFRs as they are widely used in a variety of consumer products. Major PBDE formulations include penta-, octa- and deca-BDE; although the former two have been restricted in North America and Europe due to their suspected adverse health effects (BSEF, 2009a). Currently in North America, deca-BDE is still unregulated and in high demand, together with hexabromocyclododecane (HBCD) and tetrabromobisphenol-A (TBBPA) (BSEF 2009b,c,d). These major production BFRs are found in numerous domestic and industrial products in order to comply with fire safety standards. They are incorporated into building materials, electronics and components of electrical equipment, thermal insulation foams and textile coatings (BSEF 2009b,c,d). BFRs can be released into the environment during the manufacture, use, destruction or disposal of

consumer products. Facilities that recycle or dispose of BFR-containing materials have high levels of BFR contamination in indoor air and dust, which present potential occupational hazards to humans and possible sources of environmental exposure.

Monitoring studies in wild birds have reflected the extensive use of major production BFRs such as PBDEs. Substantial increases in total PBDE concentrations were reported in wild birds across North America from the mid-1970s to 2007 (Chen *et al*. 2008;Park *et al*. 2009;Elliott *et al*. 2005). This was in line with high production volumes of major PBDE formulations during that time (BSEF, 2000). In addition, increases in total PBDE concentrations in birds were partially attributed to the presence of less brominated PBDE congeners, which were likely the product of enzymatic-mediated processes of debromination (Hakk and Letcher 2003;McKernan *et al*. 2010). Subsequent decreases in the detection of less brominated PBDE congeners in wild birds were observed following the removal of toxic penta-and octa-BDE formulations from the marketplace (Chen and Hale 2010). In contrast, the increasing deca-BDE concentrations in herring gull eggs may reflect the continued commercial use of deca-BDE (Gauthier *et al*. 2008;Chen and Hale 2010). Other types of BFRs, which are unlike the PBDEs in terms of global production volume, have recently been detected in arctic seabirds (Verreault *et al*. 2007;Karlsson *et al*. 2006) and in several herring gull colonies across the Great Lakes at levels even surpassing those of deca-BDE (Gauthier *et al*. 2007;Gauthier *et al*. 2009). The current detection of non-PBDE chemicals in wild birds combined with the phasing out of major PBDE formulations from the global market could indicate the commercial shift towards flame retardant alternatives.

1.2.2 Chemical characteristics

The number of bromines and structure of BFRs can govern their interaction with biological systems, ultimately dictating their biological disposition. As with most classes of halogenated flame retardants, brominated aromatic or aliphatic hydrocarbons are uncharged, nonpolar, lipophilic compounds. These characteristics can influence the solubility of BFRs and therefore their absorption across semi-permeable membranes. The lipid solubility of halogenated aromatic hydrocarbons generally increases with increasing halogenation as well as with increasing ring complexity (Birnbaum 1985). Due to their highly lipophilic nature, BFRs would be expected to diffuse across semi-permeable membranes and partition to lipid-rich tissues. However, bromine atoms are larger than chlorine atoms, which increase the size of brominated molecules; thus, possibly limiting the cellular uptake of larger BFRs and reducing their bioavailability.

1.2.3 BFR toxicity in birds

Knowledge on the toxicity of currently-used BFRs is mostly limited to the PBDEs, HBCD and TBBPA, due to their widespread application and accumulation in biota (reviewed in Birnbaum and Staskal 2004;Darnerud 2003;de Wit 2002); however, studies pertaining to the effects of these BFRs in birds are limited. Acute exposure studies have reported the lethal effects of TBBPA in quail and chicken embryos at 45 µg/g egg (Berg *et al*. 2001), while the pipping success of chickens and American kestrels was negatively affected by 0.1 µg/g egg of HBCD (Crump *et al*. 2010) and ≥10 µg/g egg penta-BDE (McKernan *et al*. 2009), respectively. BFRs have the potential to disrupt the thyroid hormone system (Ucan-Marin *et al*. 2010;Fernie *et al*. 2005;Crump *et al*. 2008a,b,c), affect

neurological processes (Crump *et al*. 2008b,c) and perturb certain aspects of reproduction in birds (Fernie *et al*. 2009;Marteinson *et al*. 2010;Berg *et al*. 2001). The mechanisms of BFR action are not fully understood; however, the TH pathway appears to be highly susceptible to the effects of BFR exposure. Levels of circulating THs can be affected by several mechanisms, such as synthesis, degradation and distribution. Therefore alterations in TH levels due to BFR exposure may be caused by disruptions to normal TH metabolism. PBDEs, in particular, can affect the TH system similar to highly toxic pollutants such as the polychlorinated biphenyls (PCBs) (Hallgren and Darnerud 2002); due to their structural resemblance to TH molecules (Figure 1.2). Similar to PCBs, certain PBDE congeners including hydroxylated metabolites have been shown to disrupt thyroid hormone transport in gulls by displacing the native substrates (i.e. T3 and T4) of transthyretin (TTR), a TH transport protein in serum (Ucan-Marin *et al*. 2009). Hepatic expression of TTR and a deiodinase, which controls circulating TH levels, were also vulnerable to the effects of HBCD exposure in chicken (Crump *et al*. 2008a, 2010). THs are essential to brain growth in vertebrates, through stimulation and coordination of cell proliferation and differentiation (Anderson *et al*. 2003;Ahmed *et al*. 2008). THs and proteins regulating its bioavailability are expressed in the chicken during embryogenesis, at the onset of brain development, and are required for the maturation of tissues (reviewed in Darras *et al*. 2009). TTR transcription was significantly down-regulated in primary cultures of chicken neuronal cells exposed to penta-BDE, in addition to up-regulating gene transcripts associated with signal transduction, neurosteroidogenesis, and neurite and axonal growth (Crump *et al*. 2008b). The growth of kestrel nestlings was affected by PBDE exposure (Fernie *et al*. 2006), whereby reduced

serum T4 levels were observed in *in ovo* and post-hatch American kestrels exposed to this contaminant (Fernie *et al*. 2005).

In addition to affecting the TH pathway, BFR exposure has been shown to elicit xenobiotic responses in chicken (Crump *et al*. 2008a, 2010). Hepatic phase I and II metabolizing enzymes were induced in chicken embryonic hepatocytes and chicken embryos exposed to HBCD. Enzymatic-mediated processes of metabolism are often a means of detoxification, which involves modifying the chemical structure of xenobiotics. This biotransformation can lead to the excretion of contaminants or result in bioaccumulative chemical intermediates. The metabolically-derived transformation of BFRs is suggested as a contributing factor to the presence of methoxylated and hydroxylated PBDE metabolites detected in birds (Verreault et al. 2005;Liu et al. 2010). Debromination of deca-BDE in European starlings was evident in muscle and liver due to the detection of tetra- and nona-BDE congeners in these tissues (Van den Steen *et al*. 2007). Several PBDE congeners were also identified in the egg homogenates of chickens, mallards, kestrels and herons exposed to penta- and octa-BDE, which were not present in dosing solutions (McKernan *et al*. 2010). In fact, *in vitro* exposure of the penta-BDE mixture increased the transcription levels of a hepatic cytochrome monooxygenase (i.e. CYP2H1) in chicken (Crump *et al*. 2008a). Brandsma *et* al. (2009) recently detected a hydroxylated form of HBCD (i.e. monohydroxy-HBCD) in tern eggs from the Netherlands and identified this among four hydroxylated metabolites in several tissues of rats formerly exposed to parent HBCD. Other hepatic genes associated with the lipid regulation/metabolism were also sensitive endpoints of HBCD and PBDE exposure in chicken (Crump *et al*. 2008a). From this study, a series of gene

transcripts were suggested as suitable biomarkers for assessing the effects of emerging BFRs in the environment. These will be described in more detail in the following section.

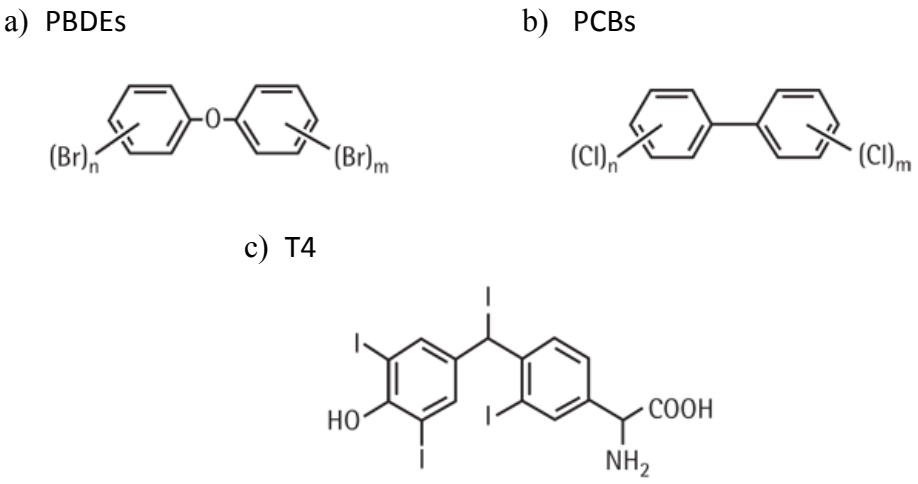


Figure 1.3. Chemical structures of a) polybrominated diphenyl ethers (PBDEs), b) polychlorinated biphenyls (PBCs) and c) the thyroid hormone, thyroxine (T4). (*Images from http://www.sightline.org/maps/charts/toxics_ chemstruct_04. Last accessed October, 2010*)

1.2.4 Biological effects of BFR exposure

The main goal in toxicology is to study the effects of xenobiotic exposure on the state of an organism. Some effects can be measured using biological markers (biomarkers), which consist of "xenobiotically induced alterations in cellular or biochemical components or processes, structures or functions that are measurable in a biological system or sample" (Klassen, 2001). In this thesis, the biomarkers of BFR exposure consist of a series of hepatic gene transcripts associated with the metabolism of xenobiotics, the TH pathway and the metabolism of lipids.

1.2.4.1 Xenobiotic metabolism

Cytochrome P450 enzymes (CYPs) are membrane bound monooxygenases that can metabolize various types of lipophilic compounds, including environmental pollutants, into more soluble intermediates for excretion. The substrates of CYP enzymes include various endogenous substrates such as steroids and fatty acids (Denison and Nagy 2003;Nebert and Gonzalez 1987), in addition to xenobiotics such as polycyclic aromatic hydrocarbons (PAHs) (Conney 2003;Denison and Nagy 2003). The metabolism of xenobiotics is largely performed by phase I metabolizing enzymes of the CYP1, CYP2 and CYP3 families (Dogra *et al*. 1998). The CYP1 response to PAHs is mediated by PAH-induced activation of the AhR and is a biomarker for exposure to DLCs in birds (Kennedy *et al*. 1996;Head and Kennedy 2007a). The mRNA expression of CYP1A4 and CYP1A5 are known to be sensitive measures of avian CYP1A induction by dioxin-like substrates (Head and Kennedy 2007b). In mammals, the CYP1A1 isoform is susceptible to dioxin-like exposure via activation of the AhR, but has also been found to be induced by other PAHs such as the PBDEs (Sanders *et al*. 2005;Szabo *et al*. 2009).

PBDEs in particular have been shown to induce aryl hydrocarbon receptor (AhR)-mediated enzymatic responses (Sanders *et al*. 2005;McKinney *et* al. 2006;Van der Ven *et al*. 2008;McKernan *et al*. 2009), which are commonly attributed to the exposure of dioxin-like compounds such as PCBs. Although PBDEs can bind the AhR similar to DLCs, they do not activate the AhR receptor-AhR nuclear translocator protein-xenobiotic response element complex (Chen and Bunce 2003;Peters *et al*. 2006); a well-defined mechanism of DLC exposure (Poland *et al*. 1976;Mimura and Fuji-Kuriyama, 2003). DLCs are potent AhR

agonists that alter the transcription of phase I and II metabolizing genes (Mimura and Fuji-Kuriyama, 2003;Rifkind *et al*.1994). The AhR, which belongs to the family of bHLH-PAS[1] transcription factors, is usually activated by its binding to DLCs. The DLC-AhR complex subsequently dimerizes with the AhR nuclear translocator (ARNT) protein and binds to xenobiotic responsive elements on DNA; thus, up-regulating the expression of target genes (Figure 1.4).

Pathways independent of AhR regulation have also been targets of BFR exposure (Sanders *et al*. 2005;Pacyniak *et al*. 2007;Crump *et al*. 2008a;Fery *et* al. 2009;Lee *et al*. 2010). PBDEs are activators of the mammalian pregnane X receptor, which is a ligand-activated transcription factor in the nuclear hormone receptor superfamily (Kliewer *et al*. 2002). The mRNA expression of CYP3A11 and CYP2B10, known targets of PXR, were induced in mice exposed to several PBDE mixtures including penta-BDE and deca-BDE (Pacyniak *et al*. 2007). PBDE activation of PXR was confirmed in a reporter gene luciferase assay and further supported by the marked suppression of CYP3A11 and CYP2B10 in PXR-knockout mice (Pacyniak *et al*. 2007). Dose-dependent increases in the hepatic expression of PXR and the constitutive androstane receptor (CAR) were also reported in rats treated with deca-BDE, combined with the induction of specific CYPs (Lee *et al*. 2010). Similar to PXR, CAR is a member of the nuclear receptor superfamily that is involved in the metabolism of endogenous molecules as well as in the clearance of xenobiotics, by inducing proteins involved in detoxification (Kliewer *et al*. 2002). When xenobiotics bind to CAR or PXR, they bind as a heterodimer with the retinoid X receptor (RXR) to DNA elements in the

[1] Basic helix-loop-helix Per-Arnt-Sim homology

nucleus, which promotes the expression of specific CYPs (Figure 1.4) (Tompkins and Wallace 2007).

Related to mammalian CAR/PXR, the chicken xenobiotic-sensing receptor (CXR) is also activated by phenobarbital-type inducers and mediates xenobiotic metabolism through the regulation of CYP2H1 (Handschin *et al*. 2000). In CEH and chicken hepatic tissue, the mRNA expressions of phenobarbital-responsive CYP2H1 and CYP3A37 were induced by HBCD (Crump *et al*. 2008a;Crump *et al*. 2010). CYP3 was determined to be the most sensitive endpoint of HBCD and PBDE exposure (Crump *et al*. 2008a;Sanders *et al*. 2005;Crump *et al*. 2010). CYP1A4, CYP1A5, CYP2H1 and CYP3A37 represent each of the major CYP families of phase I xenobiotic-metabolizing enzymes. Based on this and their responsiveness to structurally unrelated BFRs (i.e. HBCD, PBDEs), these CYP genes are suitable endpoints for characterizing the potential effects of BFR alternatives through AhR or CAR/PXR mechanisms of metabolism.

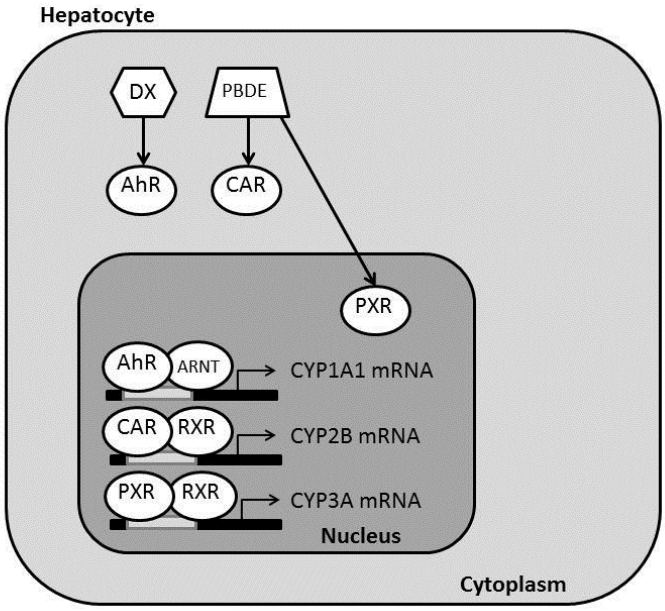


Figure 1.4. Schematic model of aryl hyrdrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) activation as a result of binding with dioxin (DX) or PBDE contaminants leading to induction of cytochrome P450 (CYP) expression in rodents. (*Image adapted from Szabo et al. 2009*).

Uridine diphosphate -glucuronosyltransferase (UGT) is an important phase II metabolizing enzyme mainly involved in detoxification. The removal of xenobiotics is done by UGT glucuronidation of substrates containing nitrogen, sulfur, carboxyl functional groups or oxygen (e.g. phase I metabolites), which make them more polar and hydrophilic for excretion (Bock and Kohle 2005;King *et al*. 2000). CAR/PXR regulates the expression of phase II metabolizing enzymes like UGTs (Mackenzie *et al*. 2003), some of which are inducible by PAHs (Bock *et al*. 1999). In addition to the biotransformation of xenobiotics, UGTs are also responsible for the conjugation of endogenous substrates such as the T4-conjugating UGT1A1 in mammals (Mackenzie *et al*. 2003). BFRs have been shown to affect circulating TH concentrations by inducing hepatic UGTs, which can lead to increased clearance of T4 (Germer *et al*. 2006;Hallgren and Darnerud 2002). In fact, pups originating from PBDE-exposed female rats showed decreases in serum T4 during the post-natal period concurrent with the induction of UGT-T4 activity (Szabo et al. 2009). Induction of UGT1A9 was reported in CEH and chicken hepatic tissue exposed to HBCD (Crump *et al*. 2008a;Crump *et al*. 2010).

1.2.4.2 Thyroid hormone metabolism

In addition to the clearance of T4 through UGT glucuronidation, BFR-associated disruption of TH homeostasis may also involve deiodination and TH transport (Szabo *et al*. 2009). Due to a higher proportion of circulating triiodothyronine (T3) to thyroxine (T4) in avian species, extrathyroidal T3 production is largely dependent on deiodinase activity (McNabb 2007). Three key deiodinase (DIO) pathways are responsible for controlling the levels of circulating T3 and T4 in serum by catalyzing their activation (T4→T3) or

deactivation. DIO1 and DIO2 are involved in the outer ring deiodination of T4 to T3 (McNabb 2007). DIO1 and DIO3 are involved in the inner ring deiodination of T4 and T3, respectively, to inactive forms (McNabb 2007). Hepatic DIO1 enzymatic activity and mRNA expression were down-regulated in pups originating from PBDE-exposed female rats (Szabo *et al*. 2009). Although the deiodinases were not transcriptionally active in CEH, DIO2 was significantly up-regulated in hepatic tissue of chicken embryos exposed to HBCD (Crump *et al*. 2008a;Crump *et al*. 2010). As DIO1, DIO2 and DIO3 are expressed in the liver, they are potential targets of contaminant exposure.

TH homeostasis is also maintained by TH binding proteins in serum, such as transthyretin (TTR). TTR is synthesized in the liver, transports THs to target tissues and provides extra-thyroidal storage of THs in serum (McNabb, 2007). Disruptions in circulating THs may be attributed to the affinity of certain BFRs to bind to TTR, thus displacing its native substrate T4 (Ucan-Marin *et al*. 2009). The mRNA expression of TTR was significantly down-regulated in HBCD-treated CEH concurrent with the induction of UGT1A9 (Crump *et al*. 2008a), an enzyme possibly involved in T4 clearance.

1.2.4.3 Lipid regulation and metabolism

Lipogenesis has been shown in chickens to be affected by alterations in TH status (Rosebrough and McMurtry 2000). Thyroid hormone responsive SPOT14α (THRSP14α) is a transcription factor involved in the TH stimulation of lipogenesis (LaFave *et al*. 2006). Hepatic expression of THRSP14α is restricted to the site of fatty-acid synthesis in the liver and has been shown in mammals to be regulated by the xeno-sensing receptors CAR/PXR (Breuker *et al*. 2010). In CEH, HBCD and PBDE down-regulated the mRNA expression of

THRSP14α and liver fatty acid-binding protein (L-FABP) (Crump *et al*. 2008a). L-FABP is involved in the metabolism and intracellular transport of lipids (Wang *et al*. 2006).

1.3 BFR alternatives – environmental occurrences and current toxicological data

The ubiquitous nature and toxicity of major production BFRs has resulted in the legislative ban or voluntary removal of some of these compounds from commercial production. In order to continue meeting with fire safety standards in consumer products, chemical substitutes possessing similar flame retardant properties to regulated BFRs have been increasingly marketed (U.S. EPA, 2005). HCDBCO, BEHTBP, BTBPE and DBDPE (Figure 1.1) are BFR alternatives that have been detected in various environmental matrices from highly industrialized/urbanized areas to more remote locations, including the Arctic. BEHTBP, BTBPE and DBDPE have recently been detected in several wildlife species and some of the data are summarized in Table 1.1. Although the bioavailability of HCDBCO is not yet known, it has been detected at high levels in house dust, which suggests its release from household products presents a potential source of environmental exposure. The following sections provide further details on the occurrence of these BFRs in the environment and summarize existing toxicological data.

Table 1.1. Summary of reported BFR levels in various biological samples

Compound	Concentrations	Source
BEHTBP	<0.04 – 3859 ng/g lw	Indo-Pacific humpback dolphin and Finless porpoise blubber (Lam *et al*. 2009)
BTBPE	<0.02 – 0.17 ng/g lw	Northern fulmar eggs (Karlsson *et al*. 2006)
	<2.56 – 3.2 ng/g lw	Glaucous gull plasma and egg yolk (Verreault *et al*. 2007)*
	<0.05 – 3.72 ng/g lw	Fish tissue (Law *et al*. 2006)
	0.07 – 2.41 ng/g lw	Watercock tissue (Shi *et al*. 2009)
	0.38 – 7.36 ng/g lw	Herring gull eggs (Gauthier *et al*. 2007)*
	1.71 – 518 ng/g lw	Freshwater food web in Southern China (Wu *et al*. 2010a)
DBDPE	<0.1 – 2.71 ng/g lw	Fish tissue (Law *et al*. 2006)
	<0.1 – 21.2 ng/g lw	Captive panda tissue (Hu *et al*. 2008)
	9.6 – 124 ng/g lw	Watercock tissue (Shi *et al*. 2009)
	118 – 506 ng/g lw	Herring gull eggs (Gauthier *et al*. 2009)*
	<3.8 – 338 ng/g lw	Freshwater food web in Southern China (Wu *et al*. 2010a)

*the reported wet weight concentrations were lipid corrected for these specimens.

1.3.1 Hexachlorocyclopentadienyldibromocyclooctane (HCDBCO)

Public information on HCDBCO usage or production volumes is limited; however, it is thought to be used in styrenic polymers (IPCS, 1997). Unlike other BFRs, HCDBCO consists of a basic norbornene structure and contains chlorine substitutes in addition to being brominated. It has been found at levels ranging from 0.24 ng/g to 93000 ng/g in house dust and in indoor air; surpassing those of major PBDEs (Zhu *et al*. 2008). Several environmental matrices were analyzed for HCDBCO, but no traces of the contaminant could be found in Lake Ontario sediments, mussel and fish tissues (Kolic *et al*. 2009) or in blubber of marine mammals (Lam *et al*. 2009). There is presently no information on the toxicity of HCDBCO; however, it shares structural commonalities with other norbornene chemicals of known

toxicity. Examples of norbornene chemicals include organochlorine pesticides such as heptachlor, which causes developmental toxicity and cancer in animals (US EPA, 2000).

1.3.2 Bis(2-ethylhexyl) tetrabromophtalate (BEHTBP)

BEHTBP comprises one of the brominated chemicals in commercial Firemaster® 550 and BZ54 mixtures (Chemtura Corporation) and is considered a replacement for penta-BDE in polyurethane foams (U.S. EPA, 2005). From 1990 to 2002, 1-10 million pounds of BEHTBP were estimated to have been produced and imported annually in the United States (U.S. EPA, 2002). It was recently found in house dust at levels ranging from 1.5 ng/g to 10,630 ng/g (Stapleton *et al*. 2008). Only one study from China has reported BEHTBP in biota, in which <0.04 - 3859 ng/g lw was measured in blubber samples of Indo-Pacific humpback dolphins and finless porpoises (Lam *et al*. 2009). Toxicological data for this BFR is limited; although, possible adverse health effects that may arise from BEHTBP exposure could be similar to those of its non-brominated analogue di(ethylhexyl)phthalate, which has been reported to cause reproductive and developmental toxicities (Latini *et al*. 2004). The genotoxic effects of commercial Firemaster® BZ54 and 550 mixtures, of which BEHTBP is one of two BFR components, were recently studied in fathead minnows despite the absence of lethality or overt signs of toxicity (Bearr *et al*. 2010). Increased levels of DNA damage were observed in the liver of fish exposed to either of the Firemaster mixtures compared to the control, but levels reduced in significance during depuration suggesting possible hepatic metabolism of BEHTBP (Bearr *et al*. 2010).

1.3.3 Bis(tribromophenoxy)ethane (BTBPE)

BTBPE is commercially known as FF-680 (Chemtura Corporation) and considered a replacement for octa-BDE (Hoh *et al*. 2005). BTBPE has recently been detected in domestic environments (Karlsson *et al*. 2007;Stapleton *et al*. 2008) and reported as the second most abundant BFR in indoor dust from electronic recycling factories, after the PBDEs (Julander *et al*. 2005). Industrial activity releases appreciable amounts of BFRs, where up to 232 ng/g dw BTBPE was detected in outdoor dust from an electronic recycling area in China (Shi *et al*. 2009). Consequently, surrounding ecosystems have been subject to BTBPE contamination from electronic processing sites where levels of 1.71 – 518 ng/g lw were detected among several species of a freshwater food web (Wu *et al*. 2010a) and up to 2.41 ng/g lw were measured in bird tissues (Shi *et al*. 2009). Several studies have reported traces of BTBPE in various sediment samples from lakes across North America (Hoh *et al*. 2005;Kolic *et al*. 2009;Law *et al*. 2006), which present possible sources of exposure to aquatic species. High concentrations of BTBPE were associated with lipid content in several species of a Lake Winnipeg food web (Law *et al*. 2006). Although BTBPE was reported to be highly bioaccumulative among certain aquatic species (Wu *et al*. 2010b), trophic dilution of this contaminant was also observed in a freshwater food web (Wu *et al*. 2010a). The eggs of top trophic level species such as colonial fish-eating birds from the Canadian Great Lakes (Gauthier *et al*. 2007), the Norwegian Faroe Islands (Karlsson *et al*. 2006) and the Norwegian Arctic (Verreault *et al*. 2007) contain <0.02 - 7.36 ng/g lw BTBPE. The presence of BTBPE in eggs suggests it likely accumulates in females and is transferred to the egg during ovogenesis. The acute toxicity of BTBPE in rats is low as an oral LD50[2] over 10g/kg was determined (Nomeir *et al*. 1993). Other dietary studies observed no signs of toxicity in

[2] The dose of a substance required to kill half of the individuals in a test population.

rats fed up to 2 mg/kg bw (Hakk *et al*. 2004) or in fish exposed daily to environmentally relevant doses of BTBPE (Tomy *et al*. 2007). As the majority of parent BTBPE was excreted, it was suggested that BTBPE is poorly absorbed through the gastro-intestinal tract (Hakk *et al*. 2004;Nomeir *et al*. 1993). Nevertheless, hydroxylated metabolites of BTBPE were identified, suggesting its biotransformation by cytochrome P450 enzymes (Hakk *et al*. 2004).

1.3.4 Decabromodiphenylethane (DBDPE)

Also known as Saytex 8010 (Albermarle Corporation), DBDPE is marketed as a replacement for deca-BDE and has been used as an additive flame retardant since the 1990s (Stuart *et al*. 2008). It was first measured in sewage sludge at levels of up to 100 ng/g dw, from several treatment plants across the Netherlands (Kierkegaard *et al*. 2004). From 2004 to 2006, noticeable increases in DBDPE levels were measured in river sediment near an electronic recycling factory and were more abundant than major PBDEs (Shi *et al*. 2009). Wildlife in proximity of these electronic facilities are susceptible to DBDPE contamination as a freshwater food web contained <3.8 to 338 ng/g lw (Wu *et al*. 2010a). DBDPE was also measured in residential indoor air and/or dust (Karlsson *et al*. 2007;Stapleton *et al*. 2008;Stuart *et al*. 2008), suggesting its release from household products and electronics. DBDPE has been detected in more remote areas where industrial activity is minimal. Accordingly, the deposition of this BFR in 11 isolated lakes across Sweden has been attributed to long-range atmospheric transport (Ricklund *et al*. 2010). Concentrations of DBDPE were measured among several aquatic species of a Lake Winnipeg food web and were positively correlated with increasing trophic level, suggesting its potential to

biomagnify up the food chain (Law *et al*. 2006). DBDPE is thought to accumulate in top trophic species, such as predatory birds that depend on aquatic food sources. Particularly, the eggs of herring gulls have measurable levels up to 288 ng/g lw DBDPE; higher than deca-BDE levels detected (Gauthier et al. 2009). The toxicity of DBDPE has been evaluated in certain aquatic species and in rats. DBDPE was acutely toxic to water fleas after 48 hours of exposure, where an EC50[3] of 19 µg/L was determined (Nakari and Huhtala 2009). While DBDPE exposure induced mortality in hatched zebrafish (at 25 µg/L), the hatching rates of zebrafish eggs were also reduced possibly due to delays in embryonic development. The estrogenic potential of DBDPE was observed in a trout hepatocyte bioassay by monitoring levels of vitellogenin, while hepatic metabolism of DBDPE was evident through the induction of ethoxyresorufin-O-deethylase (EROD) and UGT activities (Nakari and Huhtala 2009). In an oral exposure study, no overt signs of toxicity were observed in rats fed 100 mg/kg daily with DBDPE; although, it has been shown to accumulate in the liver, undergo biotransformation and potentially cause hepatotoxicity (Wang et al. 2010).

1.4 Thesis rationale and hypotheses

There is major concern over emerging contaminants in the environment because typically little is known about their biological effects. A large number of BFR alternatives appear to share commonalities with major BFRs (i.e. structure, physical-chemical properties) (U.S. EPA, 2005) and therefore may cause adverse health effects in wild birds at risk of exposure. However, the influx of chemical alternatives in the marketplace makes it difficult to prioritize wildlife risk assessment strategies, as *in vivo* toxicity testing takes time and requires a large number of individuals. A bioassay developed by Crump *et al*. (2008a)

[3] The concentration of a substance that induces 50% of its maximal effect.

was proposed as an *in vitro* screening tool to assess toxicological and molecular consequences of new BFRs in less time than traditional dosing studies. This was followed by an *in ovo* exposure study, which confirmed the *in vitro* screening method as an effective alternative to *in vivo* toxicity testing (Crump *et al*. 2010).

The same *in vitro* method was employed here to evaluate the effects of HCDBCO, BEHTBP, BTBPE and DBDPE in primary cultures of chicken embryonic hepatocytes (CEH) by assessing candidate gene transcripts of BFR exposure (Chapter 2). Although brominated, some of these BFR alternatives are structurally unrelated and would be predicted to produce distinct gene expression profiles. In order to further validate *in vitro* results, the same hepatic gene targets were assessed in a developing embryo exposed to two structurally distinct BFRs, HCDBCO and BTBPE (Chapter 3). It was hypothesized that genes responsive to HCDBCO and BTBPE exposure *in vitro* would display similar patterns of expression in embryonic liver tissue. Finally, the toxic potential of these four BFRs were evaluated through relative measures of cell viability in primary chicken embryonic hepatocytes and pipping success in developing chicken embryos (Chapter 2 and 3). Given the current toxicity data in non-avian species (described in Chapter 1.3), these BFR alternatives were hypothesized not to cause overt lethality in CEH or in chicken embryos.

1.4.1 Species selected

The chicken was selected as a laboratory model based on previous BFR exposure studies by Crump *et al*. (2008a, 2010). Chickens are frequently used in avian toxicity studies because their embryology, physiology, toxicology and genetics have been extensively studied.

1.4.2 Cell culture

Primary cell culture models are useful artificial environments for studying gene expression changes in a controlled setting. They are devoid of physiological and environmental factors (i.e. age, stress, diet) which may influence baseline levels of gene transcription. Cell cultures require a smaller number of individuals, are time-effective and less costly compared to *in vivo* exposure studies. The use of primary embryonic hepatocyte cultures is a well-established method for screening the concentration-dependent effects of xenobiotics.

1.4.3 Egg injection studies

Embryogenesis is a critical period of development, during which an embryo can be highly susceptible to external influences. Chicken eggs are extensively used for screening the dose-dependent effects of environmental pollutants. Egg injection studies can provide measurement endpoints, such as embryo mortality and physical deformations, which cannot be assessed by *in vitro* screenings.

Chapter 2 – The effects of HCDBCO, BEHTBP, BTBPE and DBDPE on mRNA expression in primary cultures of chicken embryonic hepatocytes.

2.1. Introduction

Brominated flame retardants (BFRs) are persistent contaminants in the environment and are suspected of causing adverse health effects in wild birds (Henny *et al*. 2009;Ucan-Marin *et al*. 2009;Verboven *et al*. 2010). While hepatic detoxification of some BFRs has been observed (Hakk and Letcher 2003;Letcher *et al*. 2009;McKinney *et al*. 2006), certain BFRs are bioaccumulative and can perturb endocrine pathways and neurodevelopment (Darnerud 2003;Fonnum and Mariussen 2009;Legler 2008;Williams and DeSesso 2010). Restrictions on the manufacture and use of toxic BFRs, including specific polybrominated diphenyl ether (PBDE) formulations, have resulted in the introduction of chemical alternatives as well as the increased production of non-regulated BFRs still in current use (Kemmlein *et al*. 2009;Hoh *et al*. 2005;de Wit *et al*. 2010). For example, bis(tribromophenoxy)ethane (BTBPE) and decabromodiphenylethane (DBDPE) have been marketed as potential replacements for penta- and octa-BDE, respectively (Hoh *et al*. 2005;de Wit *et al*. 2010). Monitoring efforts worldwide have reported the presence of hexachlorocyclopentadienyl-dibromocyclooctane (HCDBCO), bis(2-ethylhexyl)tetrabromophthalate (BEHTBP), BTBPE and DBDPE in various environmental matrices. The bioavailability of HCDBCO has yet to be reported; however, high levels of this compound detected in residential indoor dust suggest its potential to migrate from consumer products and enter the environment (Zhu *et al*. 2008). Several biological samples are reported to contain traces of BEHTBP, BTBPE and DBDPE (Table 1), demonstrating

the persistence of these chemicals in the environment. Aquatic species have been shown to accumulate BEHTBP, BTBPE and DBDPE (Wu *et al*. 2010a,b;Tomy *et al*. 2007;Law *et al*. 2006;Bearr *et al*. 2010) as well as biomagnify DBDPE (Law *et al*. 2006), which may pose a threat to avian species occupying high trophic levels. The recent detection of BFR alternatives in glaucous gulls of the Norwegian Arctic (Verreault *et al*. 2007), Northern fulmars from the Faroe islands (Karlsson *et al*. 2006) and herring gulls of the North American Great Lakes (Gauthier *et al*. 2007;Gauthier *et al*. 2009) warrants research into the potential toxicity of these chemicals in birds.

There are currently no toxicological data on HCDBCO and only a few studies have investigated the biological effects of BEHTBP, BTBPE and DBDPE in mammals and aquatic species (Hakk *et al*. 2004;Tomy *et al*. 2007;Nakari and Huhtala 2010;Wang *et al*. 2010;Bearr *et al*. 2010). Genotoxic effects were observed in fathead minnows exposed to commercial Firemaster® BZ54 and 550 mixtures, of which BEHTBP is a component, even though traditional endpoints of toxicity were unaffected (Bearr *et al*. 2010). BTBPE was not toxic to rats (Nomeir *et al*. 1993;Hakk *et al*. 2004) or fish (Tomy *et al*. 2007) and considered to be poorly absorbed through the gastro-intestinal tract. However, hydroxylated metabolites were identified in rat feces suggesting cytochrome P450-mediated biotransformation of BTBPE (Hakk *et al*. 2004). DBDPE was found to be acutely toxic to water fleas (Nakari and Huhtala 2010), but harmless to rats; although, DBDPE may be hepatotoxic as it was shown to accumulate and undergo biotransformation in the liver (Wang *et al*. 2010). There are currently no toxicological data on the effects of HCDBCO, BEHTBP, BTBPE and DBDPE in avian species.

Based on previous *in vitro* toxicity studies (Crump *et al*. 2008a;Cwinn *et al*. 2008;Hickey *et al*. 2009), the domestic chicken will be used here as a model species for relating potential effects of BFR exposure in avian species. HCDBCO, BEHTBP, BTBPE or DBDPE were administered to chicken embryonic hepatocytes (CEH) to evaluate potential toxicological and molecular effects of BFR alternatives *in vitro* by measuring molecular markers previously shown to be responsive to BFR exposure (Crump *et al*. 2008a;Sanders *et al*. 2005). This study determined: 1) a relative measure of hepatocyte viability after acute BFR exposure and 2) changes in the transcription levels of genes involved in xenobiotic metabolism, lipid metabolism and thyroid hormone homeostasis by real-time reverse transcription-PCR (real-time RT-PCR).

2.2. Materials and methods

2.2.1. Chemicals

HCDBCO, BEHTBP, BTBPE and DBDPE (>98% pure) were purchased from Wellington Laboratories (Guelph, ON). Stock solutions and serial dilutions were prepared in dimethyl sulfoxide (DMSO) (Sigma–Aldrich, Oakville, ON).

2.2.2. Preparation of chicken embryonic hepatocyte cultures

Fertile, unincubated White Leghorn chicken (*Gallus gallus domesticus*) eggs were obtained from the Canadian Food Inspection Agency (Ottawa, ON) and incubated at 37°C with 60% relative humidity. At day 19 of incubation, chicken embryos were euthanized by cervical decapitation. Whole livers were excised and prepared for cell culture as previously described (Head *et al*. 2006;Kennedy *et al*. 1993). All procedures were conducted according

to protocols approved by the Animal Care Committee at the National Wildlife Research Centre. Chicken embryonic hepatocytes were isolated by collagenase digestion and subsequent filtration of pooled livers (n=50-60). The resulting cell pellets were weighed and suspended in 20 mL of Medium 199 (Sigma-Aldrich) per gram of pellet. CEH cultures were prepared in 48-well Falcon plates by adding 25 µL of the cell suspension to 500 µL of medium. Hepatocytes were incubated at 37°C with 5% CO2 for 24 hours prior to BFR treatment in order to establish a confluent monolayer within the culture plates. The appearance of hepatocytes was monitored regularly using a phase-contrast microscope.

2.2.3. BFR exposure

After incubation for 24 hours, varying concentrations of HCDBCO, BEHTBP, BTBPE and DBDPE solutions were administered to CEH cultures. Hepatocytes were exposed to 2.5 µL of HCDBCO and BEHTBP stock solutions at nominal, in-well concentrations of 0.01, 0.1, 1, 3, 10 and 30 µM. Separate plates were prepared for cell viability and included an additional concentration of 60 µM. This concentration range was adapted from the Crump et al. (2009) study for BFRs. The concentration range for BTBPE and DBDPE was not as broad due to the low solubility of both compounds in the DMSO vehicle at higher concentrations. Therefore, BTBPE and DBDPE suspensions were prepared and allowed to settle in order to sample the supernatant. GC-MS analysis of the supernatant yielded concentrations of 0.28 mg/mL BTBPE and 0.02 mg/mL DBDPE, which were used in the dilution series to treat CEH. A volume of 5 µL of stock solution was administered to hepatocytes to achieve nominal, in-well concentrations of 0.01, 0.03, 0.1, 0.3, 1.4 µM BTBPE or 0.001, 0.003, 0.01, 0.03, 0.1, 0.2 µM DBDPE. Separate plates were prepared for

cell viability and included the same concentration range. All plates contained untreated cells as well as DMSO-treated controls. CEH were incubated for another 36 hours, after which culture medium was removed and cells were either assessed for cell viability or immediately frozen at -80ºC for subsequent RNA isolation.

2.2.4. Cell viability

Cell viability was estimated using the Calcein-AM assay according to the manufacturer's instructions (Molecular Probes, Eugene, OR). The viability of BFR-treated cells was compared to DMSO-treated cells and a negative control comprised of cells killed with 99% ethanol was also included. Fluorescence was measured with a Cytofluor 2350 fluorometer (Millipore, Billerica, MA) using a 485 nm excitation filter and a 530 nm emission filter.

2.2.5. RNA isolation and cDNA synthesis

Total RNA was extracted from hepatocytes using RNeasy kits according to the manufacturer's instructions (Qiagen, Missisauga, ON). Removal of genomic DNA contamination was performed on-column (RNase-free DNase set; Qiagen) and after the extraction process using DNA-free kits (Ambion, Austin, TX). The concentration of isolated RNA was measured on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and RNA purity was estimated by obtaining an absorbance ratio (A260/A280 = 1.8-2.0) for each sample. Complementary DNA (cDNA) was prepared with random hexamers and Superscript II (Invitrogen) as previously described (Hickey *et al*. 2009). Reverse transcriptase was omitted from some samples (no-RT) to control for the

presence of genomic DNA contamination. cDNA and no-RT controls were diluted 1:20 in diethyl pyrocarbonate H_2O for real-time RT-PCR.

2.2.6. Real-time reverse transcription-PCR

Genes of interest to this study (Table 2.1) were assessed by real-time RT-PCR using Brilliant Q-PCR Core Reagent kits (Stratagene). Corresponding primer pairs (Invitrogen) and TaqMan fluorogenic probes (Biosearch, Novato, CA) were designed and optimized for real-time RT-PCR as previously described (Crump *et al*. 2008a;Cwinn *et al*. 2008). Messenger RNA levels were determined in real-time with Stratagene's Mx3000P or Mx3005P PCR systems (Stratagene, La Jolla, CA). All target genes were run with a normalizer gene, β-actin. Each 25 μL TaqMan reaction was prepared with the following components: 1× core PCR buffer, 5 mM $MgCl_2$, 0.8 mM dNTP mix, 8% glycerol (vol/vol), 30 nM of reference dye, primers and probes (refer to Table 2.1 for concentrations), 0.05 U SureStart Taq DNA polymerase and 5 μL diluted cDNA. cDNA from 3 technical replicates were run in duplicate and a no-template control was included in every assay to screen for contamination of reagents. All reactions were incubated according to an enzyme activation step and a two-step thermal-cycling program: 10 min at 95°C, then 40 cycles at 95°C for 30s and 60°C for 1 min. Fluorescence was detected at the annealing step. Standard curves for all multiplex assays were generated from a 1:2 dilution series of cDNA in order to determine reaction efficiencies. Cycle threshold (Ct) data for each target gene was normalized to β-actin and the fold change in mRNA levels of BFR-treated CEH relative to that of DMSO-treated cells was calculated using the $2^{-\Delta\Delta Ct}$ equation (Schmittgen and Livak 2008).

2.2.7. Statistical analysis

Cell viability and gene expression data were analyzed using Microsoft Excel (Edition 2003, Redmond, WA) and Graphpad Prism (v5.01, La Jolla, CA). Statistically significant differences between treatment groups and control groups were determined using a one-way ANOVA followed by a Bonferroni t-test for multiple comparisons versus the vehicle control. Changes were considered statistically significant if $p<0.05$.

Table 2.1. List of genes assessed in this study, including concentrations and nucleotide sequences of primer pairs and probes used in the real-time RT-PCR assays.

	Gene		5'-Sequence	Final conc. (nM)	GenBank accession#
	β-actin	Forward	AAATTGTGCGTGCATCAAGGA	50	NM_205518
		Reverse	GAGGCAGCTGTGGCCATCT	50	
		Probe	TGCTACGTCGCACTGGATTTGGAGC	200	
Xenobiotic metabolism	CYP1A4	Forward	TAAGGACGTCAATGCTCGTTTC	300	NM_205147
		Reverse	CGTCCCGAATGTGCTCCTTAT	300	
		Probe	TGCCTTCGTACAGAAAATTGTCCAGAAC	300	
	CYP1A5	Forward	ACAGCTGTGGAAGAGCACTACCA	300	NM_205146
		Reverse	TCTCCACGCACTGCTCGAT	300	
		Probe	CCGAGACGTCACCGACTCCCTCA	200	
	CYP2H1	Forward	TGACCAGAACCACACTTGACTTG	900	NM_001001616
		Reverse	CCAACCACACGGTCAATCTCC	900	
		Probe	CGGGAACGGGGACAACCAGCACCA	200	
	CYP3A37	Forward	AGCCTGCGGTTGTTGTCATG	900	NM_001001751
		Reverse	CTTCAGCTAATGAGACAGCGTTTC	900	
		Probe	CCCTGCTAGATCCGTGCGCCTGC	200	
	UGT1A9	Forward	CCCTGGTCCTTCCTTCTCATCC	900	XM_421883
		Reverse	CTCCCACCTACTGGCACTACC	900	
		Probe	CTCGGTGTCGCTGCTTCTGCTGCT	200	
Thyroid hormone regulation	DIO1	Forward	TCTTTGTGCTGAAGGTGAAGTGG	900	NM_001097614
		Reverse	AGGTCGGTTATCTCGCATGAAAC	900	
		Probe	AAGACGAAGCCCACGAGGGACGCC	200	
	DIO2	Forward	GTGTTGCAGCACCTGGTAGC	900	NM_204114
		Reverse	TGTTTCTCGGCTATTTAAGCACTG	300	
		Probe	CAGACCTCCCGCTCCCGTGTCAGT	200	
	DIO3	Forward	CCTCCTCTTTCCCCGCTTCC	900	NM_001122648
		Reverse	GTGGGCATCGTCAGCATCTTC	300	
		Probe	CCGCTGTGATGCTCTGGCTCCTGG	200	
	TTR	Forward	TTCTTGTTTTCTTAGCTGGACTGG	900	NM_205335
		Reverse	CATGAGAGGGCATTTGGAATCAAC	900	
		Probe	CCGAAGCTGCACCACTGGTCTCCC	200	
Lipid metabolism	L-FABP	Forward	CCCTCACACTGCACCTTATCC	300	AF380998
		Reverse	CAGTGCAAGAGCTTTCAGAAATTC	300	
		Probe	CATAATGGCATTCAGTGGCACCTGGCA	200	
	THRSP14α	Forward	GCCTCCGTCACCGATCAGAG	300	NM_213577
		Reverse	CAGCCGCTCCTCCAGATTCC	300	
		Probe	CACCAGCAATGCCGACGCCGACAC	200	

2.3. Results

2.3.1. Cell viability

The viability of CEH treated with either the vehicle control (DMSO) or HCDBCO, BEHTBP, BTBPE or DBDPE for 36 hours was not affected up to the highest concentrations tested (refer to the Appendix for figures). Similarly, in cell cultures involving the exposure of hepatocytes to a higher volume (5 µL vs. 2.5 µL) of BTBPE or DBDPE solutions, the viability of cells was the same as that of untreated and DMSO-treated cells. Real-time RT-PCR analysis was performed on all CEH since exposure to varying concentrations of BFRs did not affect cell viability.

2.3.2. mRNA expression

Although no overt signs of cytotoxicity were observed, the relative mRNA expression of β-actin in three treatment groups was affected by high concentrations of BFR exposure (refer to Figure 2.1 for a representative example); therefore, real-time RT-PCR data from the following groups were excluded: 30 µM HCDBCO, 0.3 and 1.4 µM BTBPE. In addition, only the 0.2 µM DBDPE group was excluded from final analysis of CYP3A37 as β-actin was also variable. Despite the lack of overt cytotoxicity, high concentrations of BFRs could have had an impact on the integrity of CEH; thus, affecting the expression of β-actin. None of the remaining BFR treatments affected β-actin expression in hepatocytes and therefore, changes in mRNA levels were due to a change in expression of the gene of interest and not the normalizer gene. The results below describe a select number of genes that were responsive to HCDBCO, BTBPE and DBDPE exposure. BEHTBP, in particular, did not affect the mRNA expression of any of the genes of interest in this study.

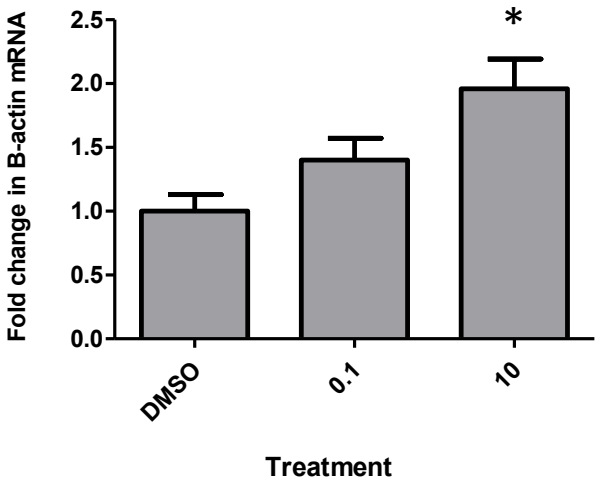


Figure 2.1. Concentration-dependent effect of BFR exposure on mRNA expression of the normalizer gene, β-actin. In this example, β-actin was multiplexed with DIO1 and assessed in CEH treated with HCDBCO. *$p<0.05$ indicates differences compared to DMSO-treated cells based on a one-way ANOVA.

The mRNA expression of CYP1A4 and CYP1A5 was significantly repressed by ≥0.01 µM HCDBCO, reaching a maximal 2.5-fold down-regulation at 3 µM (Figure 2.2a). CYP2H1 mRNA expression increased with concentration to a maximum induction level of 3-fold at 10 µM (Figure 2.2b). Finally, HCDBCO significantly up-regulated CYP3A37 by 4-fold at 10 µM (Figure 2.2c).

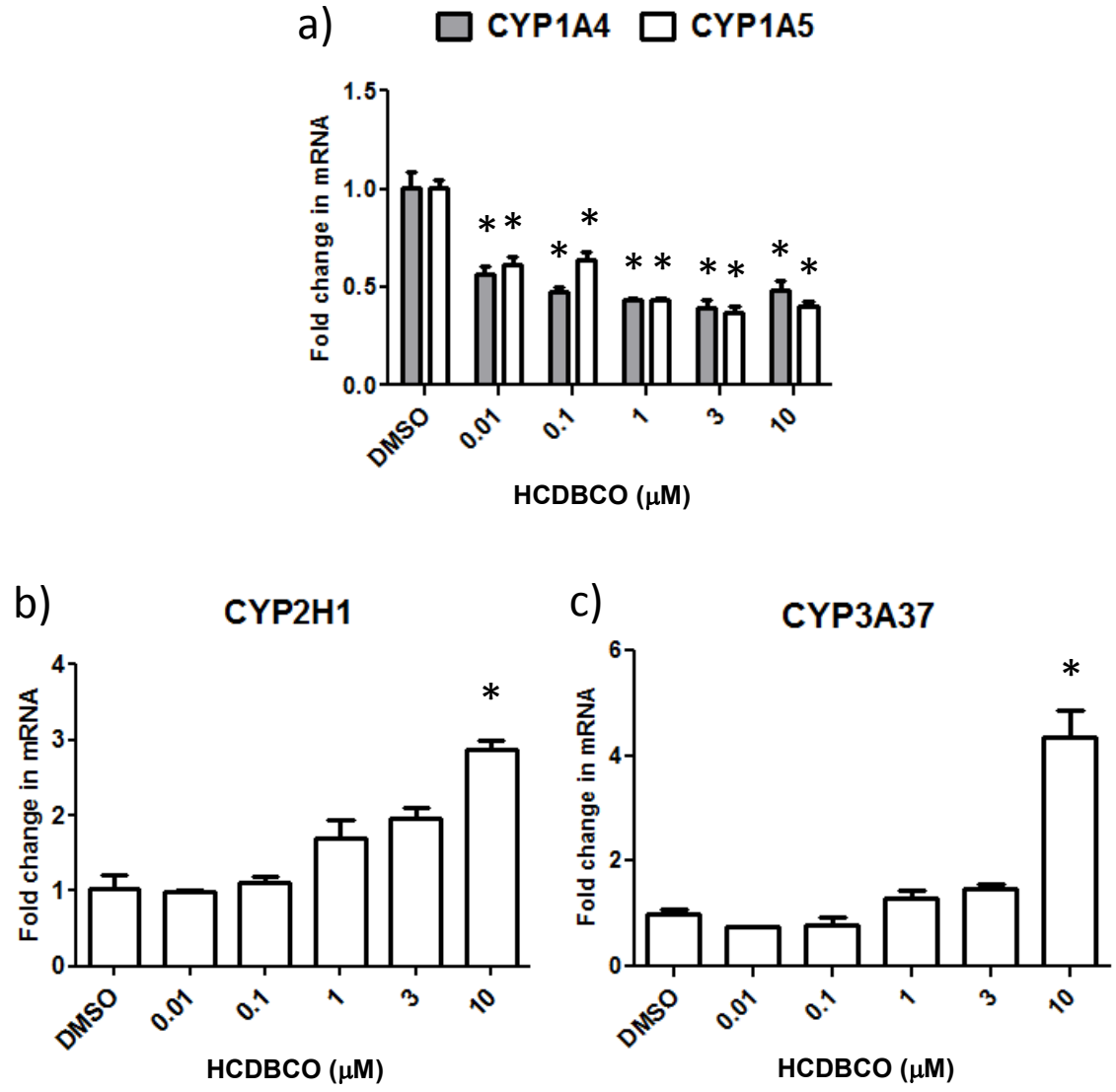


Figure 2.2. Effects of HCDBCO on the expression of a) CYP1A4/5, b) CYP2H1 and c) CYP3A37 levels in CEH. Means and SEs were calculated based on data obtained from 3-4 technical replicates for each concentration of HCDBCO. * indicates significant differences compared to DMSO-treated cells based on a one-way ANOVA (p<0.05).

BTBPE strongly induced CYP1A4 and CYP1A5 mRNA levels at ≥0.03 µM to a maximum of 115- and 18-fold, respectively, at 0.1µM (Figure 2.3a). DIO3 expression decreased in a concentration-dependent manner following exposure to 0.01 – 0.1 µM BTBPE; down-regulation of DIO3 was significant at ≥0.03 µM, down to a maximum 2.5-fold at the highest test concentration (Figure 2.3b).

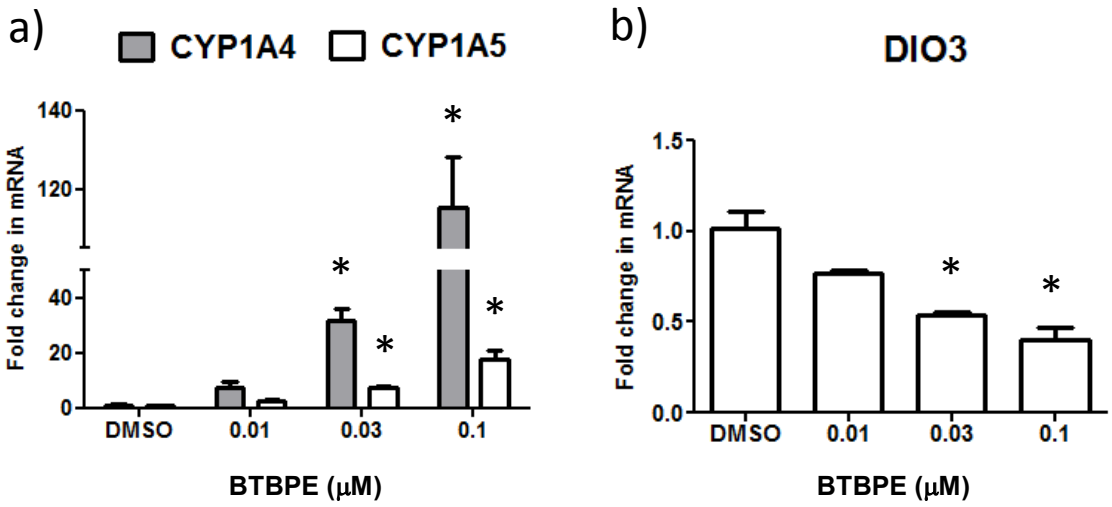


Figure 2.3. Effects of BTBPE on the expression of a) CYP1A4/5 and b) DIO3 mRNA levels in CEH. Means and SEs were calculated based on data obtained from 3-4 technical replicates for each concentration of BTBPE. * indicates significant differences compared to DMSO-treated cells based on a one-way ANOVA (p<0.05).

DBDPE significantly up-regulated the expression of CYP1A4/5 at 0.1 and 0.2 µM to a maximum of 29- and 53-fold, respectively, at the highest concentration tested (Figure 2.4a). Significant decreases in CYP3A37 mRNA levels were observed in CEH at ≥0.01 µM down to 1.8-fold at 0.03 µM DBDPE (Figure 2.4b). A significant 1.8-fold increase in DIO1 mRNA was observed in CEH treated with 0.1 µM DBDPE (Figure 2.4c).

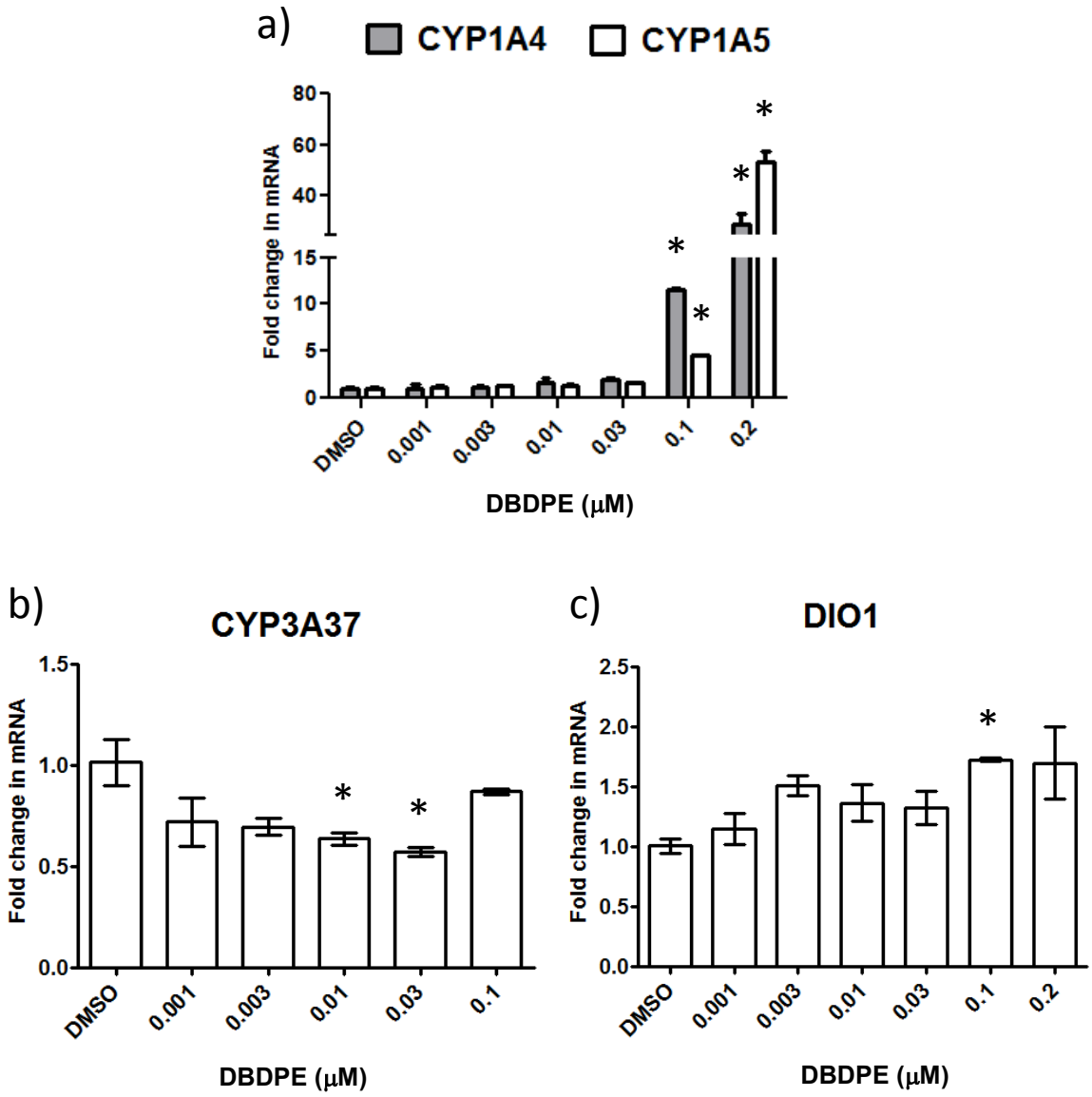


Figure 2.4. Effects of DBDPE on the expression of a) CYP1A4/5, b) CYP3A37 and c) DIO1 mRNA levels in CEH. Means and SEs were calculated based on data obtained from 3-4 technical replicates for each concentration of DBDPE. * indicates differences compared to DMSO-treated cells based on a one-way ANOVA (p<0.05).

No significant changes were observed in the remaining genes involved in thyroid hormone regulation and lipid metabolism as a result of HCDBCO, BTBPE or DBDPE exposure.

2.4 Discussion

This is the first study to evaluate the effects of HCDBCO, BEHTBP, BTBPE and DBDPE in an avian species. Results indicate that these four BFRs are not cytotoxic to CEH at the concentration range administered and the length of time exposed. The avian toxicity data for BEHTBP, BTBPE and DBDPE are in concordance with previous mammalian and fish studies. BEHTBP-containing Firemaster® mixtures were not lethal to fathead minnows and did not cause any adverse effects on growth (Bearr *et al*. 2010). The acute toxicity of BTBPE in rats was determined to be low with an oral LD50>10g/kg (Nomeir *et al*. 1993). In addition, no obvious signs of toxicity were observed in rats (Hakk *et al*. 2004) or in fish exposed to environmentally-relevant doses of BTBPE (Tomy *et al*. 2007). DBDPE was acutely toxic to water fleas with an EC50 of 19 µg/L after 48 hours of exposure (Nakari and Huhtala, 2010), while being non-toxic to rats administered daily doses of DBDPE (Wang *et al*., 2010).

The mRNA expression of genes associated with drug-metabolizing enzymes, the TH pathway and lipid metabolism were assessed in CEH to determine the effects of HCDBCO, BTBPE, DBDPE and BEHTBP. Real-time RT-PCR analysis revealed variable changes in the mRNA levels of certain target genes in CEH as a result of HCDBCO, BTBPE or DBDPE exposure. BEHTBP, on the other hand, had no impact on the suite of mRNA targets assessed. DNA damage induced by Firemaster® BZ54 and 550 mixtures in hepatic fish tissue returned to control levels during depuration, suggesting possible hepatic metabolism of brominated components including BEHTBP (Bearr *et al*. 2010). However, brominated metabolites identified in extra-hepatic tissues of chemically-treated fish were not characteristic of the BEHTBP parent compound (Bearr *et al*. 2010).

The cytochrome monoxygenases are a large class of enzymes capable of metabolizing various environmental pollutants and endogenous compounds. CYP1A4 and CYP1A5 mRNA levels were increased in CEH when exposed to BTBPE and DBDPE. The induction of CYP1A mRNA is a traditional indicator of dioxin-like compound (DLC) exposure in birds, which occurs via activation of the aryl hydrocarbon receptor (AhR). The AhR is a ligand-binding transcription factor which, when activated, binds to xenobiotic response elements within the promoter regions of metabolizing enzymes such as CYP1A isoforms (Whitlock, Jr. 1993). Generally, DLCs bind with high affinity to the AhR, potentiating CYP1A activity and the induction of other dioxin-responsive genes (Bock *et al*. 1990;Head *et al*. 2006;Poland *et al*. 1976). CYP1A4 and CYP1A5 are two constitutively expressed isoforms in chicken that are inducible by DLCs (Rifkind *et al*. 1994). BTBPE and DBDPE are comprised of a double aromatic structure similar to DLCs. Due to this structural resemblance, they may be inducing CYP1A4/5 via a mechanism of action similar to that manifested by DLCs. PBDEs, which also possess a double-ringed structure, were found to induce AhR-mediated CYP1A1 expression in rat liver, albeit at a much lower magnitude than DLCs (Sanders *et al*. 2005). The conformation (or spatial arrangement) of chemicals can influence the magnitude of CYP1A induction by affecting their affinity to bind the AhR (Birnbaum 1985;Mimura and Fujii-Kuriyama 2003). In the present study, BTBPE elicited a greater CYP1A4/5 response in CEH compared to DBDPE-treated CEH at similar concentrations. Although these two BFRs look similar, structurally distinct features such as an ethyl bond linking the two aromatic moieties in BTBPE versus an ethane bond in DBDPE may give each of these chemicals a unique conformation that could result in differential CYP1A4/5 induction. Sanders *et al*. (2005) observed weaker CYP1A1 induction in rats

treated with non-coplanar PBDEs or DLCs (e.g. PCB153) compared to a potent AhR agonist (i.e. PCB126) exhibiting a coplanar conformation. Furthermore, the magnitude of CYP1A4 induction was greater than CYP1A5 in both BTBPE- and DBDPE-treated CEH (i.e. at 0.01, 0.03 and 0.1 μM). The preferential induction of CYP1A4 compared to CYP1A5 is an observation characteristic of chickens exposed to DLCs (Head and Kennedy 2007b).

The induction of CYP1A4 in DBDPE-treated CEH is in accordance with findings from a study by Nakari and Huhtala (2009). The corresponding endpoint was EROD (ethoxyresorufin-O-deethylase) activity, which is a catalytic measurement of CYP1A induction and commonly used as a bioindicator of chemical exposure (Numata *et al*. 2008;Whyte *et al*. 2000). A significant dose-dependent increase in EROD activity was observed in rainbow trout hepatocytes in response to DBDPE exposure (at concentrations as low as 6 μg/L or ~6 nM) that reached maximal induction at 12.5 μg/L (Nakari and Huhtala 2010). In this study, the minimum concentration of DBDPE required to elicit a statistically significant induction in CYP1A4 mRNA was 0.1 μM and is approximately 15 times greater than the lowest effective EROD concentration tested in trout hepatocytes. Contaminant-induced EROD responses have been shown in avian species to closely mirror CYP1A4 responses at the mRNA level (Head and Kennedy 2007a). Thus, it would be interesting to measure EROD activity in CEH following DBDPE exposure and observe if the same concentrations can elicit a similar induction pattern to that obtained for CYP1A4 mRNA. Nakari and Huhtala (2009) further reported inhibition of EROD activity at >12.5 μg/L, suggesting DBDPE was toxic to trout hepatocytes at higher concentrations. In CEH, DBDPE did not cause any overt signs of toxicity up to the highest concentration tested (i.e.

0.2 μM), which is ~8 times the concentration at which EROD activity was completely inhibited in trout hepatocytes.

The possibility that CYP1A4/5 induction may be due to trace levels of potent AhR agonists contaminating the BTBPE and DBDPE stocks should also be considered. Polybrominated dibenzo-dioxins (PBDDs) and polybrominated dibenzo-furans (PBDFs) are low-level contaminants found in some commercial BFR mixtures, including BTBPE (WHO, 1998), and are known to induce hepatic CYP1A-mediated responses similar to DLCs (Birnbaum *et al*. 2003). The chemical analysis of PBDD and PBDF levels in our stock solutions was beyond the scope of this study; however, if detected, BFR stocks could be purified of dioxin and furan contaminants by solvent extraction methods and analysed by high-resolution gas chromatography-mass spectrometry as described by Mundy *et al*. (2010). Highly purified stocks could then be used in exposure studies and the response of CYP1A4/5 mRNA expression could be compared to the current findings.

Significant increases in CYP2H1 and CYP3A37 mRNA levels were observed in CEH exposed to 10 μM HCDBCO only. Related to the mammalian xenobiotic receptors CAR/PXR, the chicken xenobiotic receptor (CXR) has been shown to regulate the induction of CYP2H1 in response to xenobiotic exposure (Handschin *et al*. 2000). CYP3A was considered in other studies to be the most sensitive endpoint of BFR exposure (Canton *et al*. 2008;Sanders *et al*. 2005), including Crump *et al*. (2008a) who reported 30- and 7-fold induction levels in CYP3A37 and CYP2H1, respectively, upon exposure to 10 μM HBCD. This was also observed in HCDBCO-treated CEH, as the highest test concentration elicited a 4.34-fold induction in CYP3A37 mRNA and a 2.87-fold induction in CYP2H1 mRNA.

Given these results, HCDBCO appears to be a less potent activator of these enzymes than HBCD in CEH cultures.

While toxic responses to polyhalogenated pollutants are usually mediated through increased expression of CYP1A and other genes encoding drug-metabolizing enzymes, down-regulation of CYP genes has also been observed in response to this type of exposure (Riddick *et al*., 2004). Although the molecular mechanisms of xenobiotic-mediated CYP induction have been extensively studied (Conney 2003), far less is known about the mechanisms underlying the down-regulation of CYPs in response to toxicants. In the present study, a significant down-regulation of CYP1A4/5 mRNA in CEH was observed following HCDBCO exposure (at 0.001 – 10 μM). DBDPE also caused a slight down-regulation of CYP3A37 mRNA, but was only significant at 0.01 and 0.03 μM ($p<0.05$). The down-regulation of drug-metabolizing enzymes is thought to be a protective response to the action of chemical stressors, such as inflammatory mediators (e.g. cytokines) and toxicants (Renton 2001). Reactive oxygen species, typically produced during inflammation, have been shown to mediate the suppression of CYP1A1 and CYP1A2 transcription in rat hepatocytes, thus possibly preventing additional oxidative damage in the cell (Barker *et al*. 1994). CYP down-regulation is believed to primarily occur through suppression of CYP transcription, but can involve multiple molecular mechanisms (Riddick *et al*. 2004). Down-regulation of constitutively expressed hepatic CYP2C11 mRNA has been shown in rats exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, in addition to inducing CYP1A, and is thought to be mediated via a mechanism of negative regulation that may involve the AhR (Riddick *et al*. 2004). The potential for oxidative stress to occur in the liver as a result of DBDPE accumulation was recently reported in an oral exposure study, following the analysis of

several clinical indices of hepatotoxicity (Wang *et al*. 2010). In addition, Wang *et al*. (2010) observed a significant 1.24-fold increase in rat CYP3A2 mRNA expression, while other CYPs (i.e. CYP1A2, 2B1, 2B2, and 2C6) were unresponsive in DBDPE-exposed rats. Further research would be needed in order to determine whether HCDBCO- or DBDPE-induced down-regulation of CYPs is associated with oxidative stress.

Several major production BFRs are known to have endocrine disrupting effects, particularly affecting the TH system. Several gene targets involved in maintaining TH homeostasis were assessed in CEH to examine the TH disrupting potential of the four test BFRs. Only BTBPE and DBDPE significantly altered the transcription of two deiodinases. These two BFRs are similar in structure to THs (Figure 1.2C). It has been shown that BFRs with structural similarity to THs have a high affinity for TH receptors and could potentially disrupt the TH axis (Meerts *et al*. 2000;Ucan-Marin *et al*. 2010). Deiodinases are responsible for controlling the levels of circulating THs in serum by catalyzing their activation or deactivation. BTBPE caused a down-regulation of DIO3 expression in CEH at 0.03 and 0.1 μM (p<0.05). DIO3 transform sT3 to diiodothyronine and degrades T4 to an inactive reverse-T3 molecule (McNabb 2007). Tomy *et al*. (2007) measured TH endpoints in juvenile rainbow trout during and after exposure to environmentally-relevant doses of BTBPE, but found no resulting perturbations in TH levels or T4 outer ring deiodinase activity; however, it was suggested that the length of exposure *in vivo* may have been too short (i.e. 49 days) for any observable effects on the thyroid hormone axis to be manifested. Moreover, deiodinase expression was not monitored in the latter study and changes in mRNA levels induced by BTBPE exposure may have occurred upstream before having any effect on circulating THs in exposed trout. The mRNA expression of DIO1, which converts

T4 to T3 (McNabb 2007), was up-regulated in CEH upon exposure to 0.1 μM DBDPE (p<0.05). Up-regulation of this particular deiodinase could result in increased T3 concentrations. Significant increases in T3 levels were measured in rat serum after 90 days exposure to 100 mg/kg bw/day of DBDPE; although, T4 levels remained unchanged (Wang *et al*. 2010). Such disturbances in TH homeostasis could have an impact on TH-dependent processes, such as growth, brain development and thermoregulation in birds (McNabb 2007). DIO1 and DIO3 were also assessed in the Crump *et al*. (2008a) study, but found to be unresponsive to BFR exposure.

The remaining gene targets in this study (i.e. UGT1A9, DIO2, TTR, L-FABP, THRSP14α) were unresponsive to HCDBCO, BTBPE and DBDPE. Particularly, genes involved in lipid metabolism (i.e. L-FABP, THRSP14 α) were unaffected by any of the BFRs tested here, although they were down-regulated in the HBCD study (Crump *et al*. 2008a). BFRs are inherently lipophilic and have been shown to preferentially accumulate in adipose tissues, including BTBPE (Hakk *et al*. 2004) and DBDPE (Wang *et al*. 2010). However, Wang *et al*. (2010) assessed clinical endpoints of fat metabolism in serum of rats exposed to high doses of DBDPE (i.e. 100 mg/kg bw/day) and reported levels similar to the control. In this study, CEH were exposed to at least 5 times the amount of DBDPE reported in watercock tissues (Shi *et al*. 2009) and 600 times the amount of BTBPE found in herring gull eggs (Gauthier *et al*. 2007). Therefore, the lack of transcriptional change in UGT1A9, DIO2, TTR, L-FABP, and THRSP14α in response to high concentrations of BTBPE and DBDPE makes them unsuitable targets for these types of exposure. Of the 11 gene targets assessed in CEH, none were affected by BEHTBP. It is possible that the test range is too narrow and the concentration required to elicit a change in gene expression may exceed our

maximum test concentration; however, environmentally realistic levels are near our lowest test concentration as the maximum reported level of BEHTBP in biota was 3859 ng/g lw (Lam *et al*. 2009). If assuming 100% uptake of BEHTBP by hepatocytes and taking into account the mass of cells/culture well, exposure to 0.01 μM BEHTBP is approximately equivalent to a wet weight concentration of 4500 ng /g cell.

In conclusion, HCDBCO, BEHTBP, BTBPE and DBDPE were not cytotoxic at current laboratory exposure levels. Of the 11 gene targets, responsive to HBCD and PBDE exposure in earlier studies (Crump *et al*. 2008a;Sanders *et al*. 2005), 6 were transcriptionally responsive to BFR alternatives. HCDBCO, BTBPE and DBDPE affected genes involved in phase I metabolism and the TH pathway. None of the BFR alternatives tested here altered genes associated with lipid metabolism and regulation as determined by the assays used. Although HCDBCO has not yet been reported in biota, this study shows candidate genes in CEH that are vulnerable to this particular BFR. HCDBCO appears to affect phase I metabolism via mechanisms of CXR-mediated up-regulation and possible AhR-related down-regulation of drug-metabolizing enzymes. BTBPE and DBDPE seem to manifest their effects through AhR-mediated phase I metabolism, while potentially having an impact on TH homeostasis. Of the 11 genes assessed in this study, none were suitable targets for BEHTBP exposure. Further research is required to elucidate these mechanisms and the importance and relevance from a toxicological point of view. The subsequent assessment of these BFR-responsive gene targets *in vivo* would provide further evidence on the mechanisms of action of these BFR alternatives. Due to the increasing global market demand for flame retardants and their persistent nature, alternative BFRs are expected to

become more prevalent in the environment. The data collected in this study will hopefully contribute to understanding the toxicological and molecular biological implications of these BFR alternatives in wild birds at risk of exposure.

Chapter 3 – The effects of HCDBCO and BTBPE on pipping success and hepatic gene expression in the developing chicken embryo.

3.1. Introduction

HCDBCO and BTBPE are chemical alternatives to toxic, regulated BFRs and have become contaminants of environmental concern due to their prevalence in wildlife and/or domestic environments. BTBPE can be taken up in aquatic food webs (Law *et al*. 2006;Wu *et al*. 2010a,b;Tomy *et al*. 2007) and has the potential to accumulate in species at high trophic levels, including fish-eating birds. Traces of BTBPE have been found in the eggs of top predatory birds, suggesting its maternal transfer to the embryo during ovogenesis (Gauthier *et al*. 2007;Karlsson *et al*. 2006;Verreault *et al*. 2007). The bioavailability of HCDBCO is not yet known; however, its detection in residential indoor dust at high levels (i.e. 93 μg/g) suggests its potential to migrate out of consumer products during their production or disposal and make its way into the environment (Zhu *et al*. 2008). The effects of these two BFRs were previously evaluated in chicken embryonic hepatocytes (CEH) via the molecular screening of specific gene markers determined to be sensitive to BFR exposure (Crump *et al*. 2008a;Sanders *et al*. 2005). A number of hepatic genes encoding xenobiotic metabolizing enzymes and proteins involved in TH homeostasis were transcriptionally responsive to HCDBCO and BTBPE exposure, despite the absence of overt cytotoxicity. Identifying such mechanisms of biological action *in vitro* can contribute to understanding the effects of BFR alternatives in organisms.

In this study, HCDBCO and BTBPE were injected into fertile chicken eggs to validate the *in vitro* technique as a sensitive and rapid method for evaluating emerging

pollutants, such as BFR alternatives. The study determined: 1) a relative measure of embryonic viability (e.g. pipping success), 2) HCDBCO and BTBPE accumulation in the liver by GC-MS, and 3) changes in the transcription levels of hepatic genes involved in xenobiotic metabolism, lipid metabolism and thyroid hormone homeostasis by real-time RT-PCR.

3.2. Materials and methods

3.2.1. Chemicals

Technical mixtures of HCDBCO and BTBPE (>97.6% purity) were graciously provided by Dr. Jiping Zhu (Health Canada, Ottawa, ON) and Pamela Martin (Environment Canada, Burlington, ON), respectively. HCDBCO was prepared in DMSO to yield nominal concentrations of 0.1, 1, 5, 50 mg/mL, while BTBPE was prepared in DMSO to yield nominal concentrations of 0.1, 1, 10 mg/mL. Due to the low solubility of these two BFRs in DMSO, suspensions were obtained at the highest doses (i.e. 50 mg HCDBCO/mL and 10 mg BTBPE/mL). The actual concentrations of solutions injected into eggs were determined by GC-MS as described later in this section and are shown in Tables 3.1 and 3.2.

3.2.2. Egg injection

A total of 275 unincubated White Leghorn chicken (*Gallus gallus domesticus*) eggs were obtained from the Canadian Food Inspection Agency (Ottawa, ON) for the egg injection studies. All procedures involving the handling of animals were conducted

according to protocols approved by the Animal Care Committee at the National Wildlife Research Centre. Two main egg injection studies were performed to assess pipping success and hepatic mRNA expression following HCDBCO or BTBPE exposure. The treatment groups for the HCDBCO study included the untreated control (n=20), DMSO vehicle (n=20), 0.1 µg/g (n=20), 1 µg/g (n=20) and 5 µg/g (n=20). The treatment groups for the BTBPE study included DMSO vehicle (n=24), 0.1 µg/g (n=25), 1 µg/g (n=25) and 10 µg/g (n=25). An additional egg injection study was performed to reassess the effects of HCDBCO exposure on embryo pipping success and included the following treatments groups: DMSO vehicle (n=25), 5 µg/g (n=25) and 50 µg/g (n=26).

The eggs were weighed and candled to locate the air cell. A Dremmel® tool was used to drill a 1.5 mm hole in the middle of the air cell. The tip of an Eppendorf repeater pipette was inserted into the hole of the egg, puncturing the membrane to relieve air pressure and to ensure delivery of the solution to the embryo during injection. Based on the average weight of the eggs, a pre-determined volume of DMSO or BFR working solution (i.e. ~1 µL/g egg) was injected into the eggs to achieve the concentrations indicated above. The holes of the eggs were sealed with melted paraffin soon after drilling (i.e. for the untreated group) or injection with BFR solution. The eggs were placed in an incubator (Petersime, Model XI) at 37.5°C and 60% humidity until pipping (i.e. ~ 20-22 days). By the first week of incubation, eggs that did not show any signs of vascularization during candling were deemed infertile and discarded. Pipping success was measured by dividing the number of embryos that pipped by the number of fertile eggs.

3.2.3. HCDBCO and BTBPE liver concentrations

Whole livers were weighed and ground with ~3g diatomaceous earth until a homogeneous mixture was obtained. The sample mixtures were spiked with 25 uL of 1 ppm $^{13}C_{10}$-*syn*-Dechlorane Plus (DP) (Cambridge Laboratories, USA) or 25 uL of 1 ppm $^{13}C_{12}$-BTBPE (Wellington Laboratories, Canada) internal standard solutions for quantification of HCDBCO and BTBPE, respectively. Samples were extracted via accelerated solvent extraction with dichloromethane-*n*-hexane (50V/50V) according to the following parameters during 3 cycles of heat (5 min) and static (5 min): flush% 100 vol; purge 60 sec; pressure 1500 psi; temperature 100ºC. 10% of the resulting eluent was collected for measuring the %lipid concentration in the liver. The remaining volume was cleaned with 50% sulphuric acid silica, concentrated and solvent-exchanged into isooctane to obtain a final 250 µL solution for GC-MS analysis.

The chemical components of the eluent were separated and analyzed on an Agilent gas chromatograph (GC) 6890 using a 15 m × 0.25 mm i.d. DB-5 HT capillary column with a film thickness of 0.10µm (Chromatographic Specialties). 1 µL of the eluent was injected into the injector chamber, heated at 240ºC, with pulsed-splitless injection mode (injection pulse at 25.0 psi until 1.10 min; purge flow to split vent of 96.4 mL/min to 2.10 min; gas save flow of 20 mL/min at 2.0 min) and processed according to the following oven parameters: 100°C for 2 minutes, 25 °C/min to 260 °C, 1.5°C/min to 280 °C and finally 25 °C/min to 325 °C, held for 7 minutes.

Target compounds were analyzed via a 5973 quadrupole mass spectrometer (MS) detector according to the following operation parameters: source temperature 160 °C,

quadrupole temperature 150 °C and auxiliary temperature 280 °C. Ionization was performed in electron capture negative ionization mode using methane as the reagent gas. Selected ion-monitoring mode was used for quantification of the target compounds based on the most selective or abundant mass fragment. The monitoring ions for HCDBCO were 79, 81 and 310 (m/z) and the monitoring ions for BTBPE were 251 and 330 (m/z). Ion selection for internal standards were 257 and 336 (m/z) for $^{13}C_{12}$-BTBPE and 468 and 662 (m/z) for $^{13}C_{10}$-*syn*-DP. Identification of target compounds and internal standards in the samples was accomplished by comparing the relative abundance of monitored ions and retention time with its corresponding standards. Quantification was performed using an internal standard method with a six point calibration curve spanning the range of anticipated analyte concentrations in the samples. Data analysis was performed using ChemStation software (Agilent Technologies).

A method blank was included together with each batch of samples (n=8) to monitor for any contamination during all steps of the analysis. No significant contamination was reported. In addition, pork liver (Ottawa market) homogenates were spiked with target compounds as a method control and included in each batch of samples (n=8) analyzed to test for reproducibility. An acceptable coefficient of variation of less than 10% was obtained. The method limit of detection (MLOD) was measured by performing replicate analyses (n = 8) of pork liver samples, which were spiked with analytes and calculated as 3 times the standard deviation of the detected concentration of target compounds. The MLOD values were 0.15 ng/g ww for BTBPE and 0.01 ng/g ww for HCDBCO. Likewise, the method limit of quantification (MLOQ) was calculated as 10 times the standard deviation of the detected concentration of target compounds. The MLOQ values were 0.44 ng/g ww for BTBPE and

0.03 ng/g ww for HCDBCO. The recovery efficiencies of the internal standards averaged 85±18% for $^{13}C_{10}$-*syn*-DP and 52±14% for $^{13}C_{12}$-BTBPE. Despite the low recovery efficiency for $^{13}C_{12}$-BTBPE, recovery loss was completely accounted for and corrected by using this mass-labeled internal standard. Since an internal standard method was used for quantification, the concentrations of target compounds were inherently recovery-corrected.

3.2.4. Tissue collection

Embryos that pipped successfully were euthanized by cervical decapitation and portions of the liver were sampled from 8 individuals/treatment group, flash-frozen in liquid nitrogen and stored at -80ºC for subsequent RNA extraction. 5 whole livers from the remaining individuals in each treatment group were excised for HCDBCO or BTBPE analysis.

3.2.5. RNA extraction and real-time reverse transcription-PCR

RNA was extracted from livers (n=8) using the RNeasy kit according to the manufacturer's instructions (Qiagen, Missisauga, ON). Removal of genomic DNA contamination was performed on-column (RNase-free DNase set; Qiagen) and after the extraction process using DNA-free kits (Ambion, Austin, TX). The concentration of isolated RNA was measured on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE), while RNA purity was estimated by obtaining an absorbance ratio (A260/A280 = 1.8-2.0) for each sample. A standard concentration of 50 ng/µl RNA was reverse transcribed to cDNA using SuperScript II and random hexamer primers as described by the manufacturer (Invitrogen). Reverse transcriptase was omitted from some samples

(no-RT) to control for the presence of genomic DNA contamination. cDNA and no-RT controls were diluted 1:20 in diethyl pyrocarbonate H2O for real-time RT-PCR.

Genes of interest to this study (refer to Table 2.1) were assessed by real-time RT-PCR using Brilliant Q-PCR Core Reagent kits (Stratagene). Corresponding primer pairs (Invitrogen) and TaqMan fluorogenic probes (Biosearch, Novato, CA) were designed and optimized for real-time RT-PCR as previously described (Crump *et al*. 2008a;Cwinn *et al*. 2008). Messenger RNA levels were determined in real-time with the Stratagene Mx3000P or Mx3005P PCR system (Stratagene, La Jolla, CA). All target genes were run with a normalizer gene, β-actin. Each 25 μL TaqMan reaction was prepared with the following components: 1× core PCR buffer, 5 mM MgCl2, 0.8 mM dNTP mix, 8% glycerol (vol/vol), 30 nM of reference dye, primers and probes, 0.05 U SureStart Taq DNA polymerase and 5 μL diluted cDNA. cDNA from 8 individual embryos per treatment group were run in duplicate for each assay. No-template and no-RT controls were included in every assay to screen for DNA contamination. All reactions were incubated according to an enzyme activation step and a two-step thermal-cycling program: 10 min at 95°C, then 40 cycles at 95°C for 30s and 60°C for 1 min. Fluorescence was detected at the annealing step. Standard curves for all multiplex assays were generated from a 1:2 dilution series of cDNA in order to determine reaction efficiencies. Cycle threshold (Ct) data for each target gene was normalized to β-actin and the fold change in mRNA levels of BFR-treated groups relative to that of DMSO-treated groups was calculated using the $2^{-\Delta Ct}$ equation (Schmittgen and Livak 2008).

3.2.6. Statistical analysis

Pipping success and gene expression data were analyzed using Microsoft Excel (Edition 2003, Redmond, WA) and Graphpad Prism (v5.01, La Jolla, CA). Statistical differences in mRNA expression were identified by performing a one-way ANOVA to $2^{-\Delta Ct}$-transformed data followed by a Bonferroni's t-test for multiple comparisons versus the vehicle control. In some cases where *in ovo* data did not follow a normal distribution, statistical differences in mRNA expression of $2^{-\Delta Ct}$-transformed data were identified by performing a Kruskal-Wallis test followed by Dunn's comparison of dose groups to the vehicle control. Changes were considered statistically significant if $p<0.05$.

3.3. Results

3.3.1. Pipping success

HCDBCO and BTBPE were not lethal to embryos up to the highest doses administered (Figures 3.1a and b). In the first HCDBCO injection study, a high number of infertile eggs and dead embryos were observed in the 5 µg/g group (13/20 viable), compared to any other treatment group. Eggs are generally deemed infertile upon visual inspection of their contents and subject to the experimenter's interpretation; thus, considering that these infertile eggs were possibly aborted early on in development as a result of HCDBCO exposure, the egg injection study was repeated. However, all embryos were viable in the second exposure study up to 50 µg HCDBCO/g, which suggested early-aborted embryos found in the first study were more likely infertile; thus, raising the proportion of viable eggs to 80% in the 5 µg/g group. In addition, the viability of DMSO-injected embryos was comparable to that of embryos in the untreated group, confirming this solvent as a suitable vehicle for introducing BFR compounds into the embryo (Figure 3.1a).

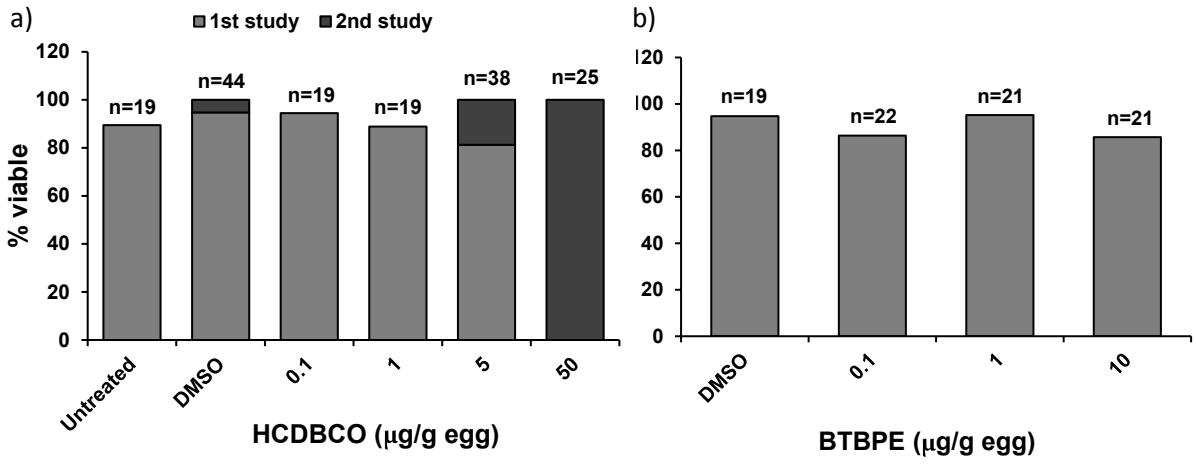


Figure 3.1. Percent viability of untreated, DMSO-injected, HCDBCO-injected (a) or BTBPE-injected (b) chicken embryos at the time of pipping. A second HCDBCO exposure study was performed to validate apparent dose-dependent decreases in viability observed at 5 µg/g during the first study. *n* represents the total number of fertile eggs in each treatment group.

HCDBCO and BTBPE did not cause any delays in embryonic development as individuals within each treatment group reached their pipping stage at times comparable to that of embryos injected only with DMSO (Figures 3.2a and b). In addition, DMSO- or BFR-treated embryos reached the pipping stage at a similar time as untreated embryos (Figure 3.1a), confirming that embryos were unaffected by the injection procedures.

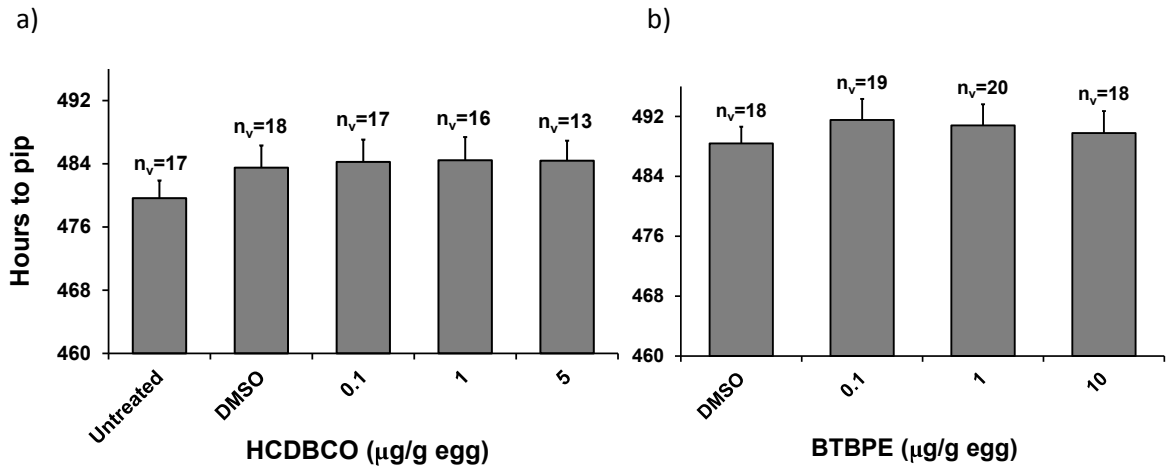


Figure 3.2. Average time to pip in hours of untreated, DMSO-injected, HCDBCO- (a) or BTBPE-injected (b) chicken embryos from day 0 of incubation. n_v represents the number of viable chicken embryos. A one-way ANOVA was performed to verify any significant differences in pipping time compared to untreated and/or DMSO-injected chicken embryos.

3.3.2. HCDBCO liver concentrations

HCDBCO concentrations in the working solutions were 0.064, 0.78, 1.5 and 65 mg/mL. The actual concentrations of the first three dose groups are lower than the target nominal concentrations of 0.1, 1 and 5 mg/mL, while the actual concentration of the highest dose group is near its target nominal concentration of 50 mg/mL (Table 3.1). Except for the highest dose group (i.e. 50 mg/mL), actual concentrations deviate further away from the

targeted nominal concentrations with increasing dose. Inconsistencies between actual and expected stock concentrations could be due to difficulties in solubilizing HCDBCO at higher concentrations in DMSO. HCDBCO was not completely dissolved at the expected 50 mg/mL dose group and therefore, a vortexed suspension was injected into embryos. Chemical analysis of the supernatant and the suspension yielded substantially different final concentrations of 5.3 and 65 mg/mL, respectively. However, serial dilutions for egg injection were prepared from the 1.5 mg/mL stock solution in which HCDBCO did appear to be completely dissolved.

Mean HCDBCO concentrations increased in the liver with increasing dose to a maximum of 2428.0 ng/g ww, except for livers from the 1.5 mg/mL dose group (Table 3.1). However, the extent to which HCDBCO accumulated in the liver with increasing dose did not parallel initial concentrations injected into eggs; livers from the highest dose group had only 4 times the concentration of HCDBCO detected in the first dose group, whereas the highest concentration injected into eggs was 1000 times the lowest injected concentration. Hepatic (ww) levels of HCDBCO in the low dose group were 11.22 times the initial concentration (ng/g) injected into eggs. Following injection with actual concentrations of 746, 1434, 62142 ng/g, hepatic HCDBCO enrichment was 1.36, 0.39 and 0.04 times the initial injection concentration, respectively. Due to the lipophilic nature of BFRs, it is assumed a larger amount of BFR would be associated with the lipid fraction of the liver. Based on a liver lipid content of 3-12%, the lipid weight concentrations followed a similar accumulation trend to that of the wet weight concentrations; however, only the wet weight concentration at the highest dose was significantly greater than the first detected sample in the 61 ng/g group ($p<0.05$).

Table 3.1. Concentration of HCDBCO in hepatic tissue of pipped embryos that were injected with a range of HCDBCO doses prior to incubation. (Actual stock concentrations and hepatic HCDBCO concentrations were measured by GC-MS; the method detection limit for HCDBCO was 0.01 ng/g ww).

HCDBCO dose group (ng/g)	Nominal stock conc. (mg/mL)	Actual stock conc. (mg/mL)	Injected concentration (ng/g)	Mean hepatic [HCDBCO] ng/g ww ±S.E. (n=3-5)	Hepatic enrichment	Mean hepatic [HCDBCO] ng/g lw ±S.E. (n=3-5)
Untreated	-	-	-	0	-	0
DMSO	0	0	0	<0.01	-	0
100	0.1	0.064	61	687 ± 118^{A}	11.22x	6882 ± 1325
1000	1.0	0.78	746	1012 ± 259^{AB}	1.36x	17371 ± 5214
5000	5.0	1.5	1434	566 ± 293^{A}	0.39x	8021 ± 5448
50000	50	65 (5.3)	62142 (5067)	2428 ± 511^{B}	0.04x	36514 ± 15251

Values in brackets are the concentrations of HCDBCO in the supernatant after the suspension was allowed to settle. Injected concentrations are based on a 50-uL injection volume and average egg weight of 52.3g. Different letters indicate statistically significant different values based on ANOVA with Bonferroni's post-hoc test $p<0.05$. Hepatic enrichment values indicate the fold increase in hepatic HCDBCO concentration compared to the initial, actual concentration injected into eggs. Wet weight concentrations were lipid corrected for individual samples.

3.3.3. BTBPE liver concentrations

BTBPE concentrations in the working solutions were 0.051, 0.36 and 3.2 mg/mL. These are below the target nominal concentrations of 0.1, 1 and 10 mg/mL (Table 3). In addition, they deviate further from the targeted nominal concentration with increasing dose. This could be explained by the partial insolubility of BTBPE in organic solvents such as DMSO. During the preparation of dosing solutions, BTBPE did not dissolve completely at the highest dose and a vortexed suspension was injected into eggs. Serial dilutions were subsequently made from the 3.2 mg/mL stock solution. Mean hepatic concentrations of BTBPE remained relatively constant at 53 – 59 ng/g ww, regardless of the initial concentration injected into eggs (Table 3). Hepatic enrichment of BTBPE was 1.10, 0.17, and 0.02 times the initial concentration injected into eggs for the 48, 342 and 3008 ng/g dose

groups, respectively. BTBPE concentrations were also invariable in hepatic tissue after lipid correction (lipid content of 4-12%). Similar to the wet weight concentrations, no statistically significant differences were found between lipid corrected concentrations of the different dose groups.

Table 3.2. Concentration of BTBPE in hepatic tissue of pipped embryos that were administered a range of BTBPE doses prior to incubation. (Actual stock concentrations and hepatic BTBPE concentrations were measured by GC-MS; the method detection limit for BTBPE was 0.15 ng/g ww).

BTBPE dose group (ng/g)	Nominal stock conc. (mg/mL)	Actual stock conc. (mg/mL)	Injected concentration (ng/g)	Mean hepatic [BTBPE] ng/g ww ±S.E. (n=4-5)	Hepatic enrichment	Mean hepatic [BTBPE] ng/g lw ±S.E. (n=4-5)
DMSO	0	0	0	<0.15	-	0
100	0.1	0.051	48	53 ± 9	1.10x	909 ± 263
1000	1.0	0.36	342	59 ± 20	0.17x	705 ± 256
10000	10	3.2 (0.70)	3008	57 ± 22	0.02x	730 ± 320

Values in brackets are the concentrations of HCDBCO in the supernatant after the suspension was allowed to settle. Injected concentrations are based on a 50-uL injection volume and average egg weight of 53.2g. Hepatic enrichment values indicate the fold increase in hepatic BTBPE concentration compared to the initial, actual concentration injected into eggs. Wet weight concentrations were lipid corrected for individual samples.

3.3.4. mRNA analysis

The Ct values for the normalizer gene, β-actin, were invariable across all treatment groups in the HCDBCO study. In the BTBPE study, significant differences in β-actin amplification were observed for the 1 μg/g group compared to the (DMSO) control group (Figure 3.3) and this data set was omitted from final mRNA analysis.

Of the 11 hepatic genes assessed in this study, only TTR was significantly affected by HCDBCO. TTR mRNA was suppressed down to 2-fold in embryonic livers at all doses (p<0.05) (Figure 3.4).

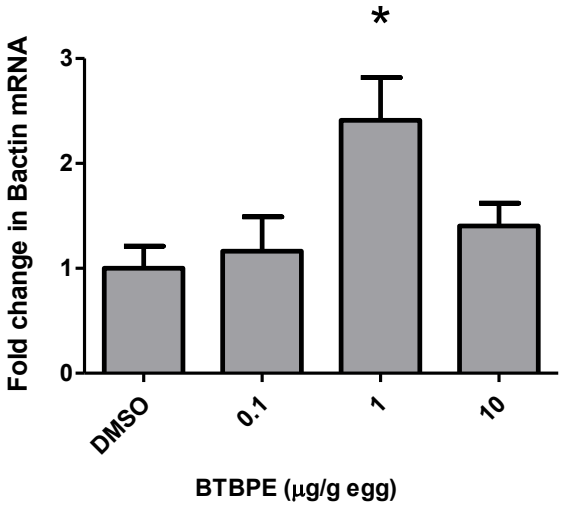


Figure 3.3. Variable mRNA expression of the normalizer gene, β-actin, in embryonic liver tissue at 1 µg/g dose of BTBPE. In this example, β-actin was multiplexed with DIO3. * indicates differences compared to the DMSO-treated group based on a one-way ANOVA (p<0.05).

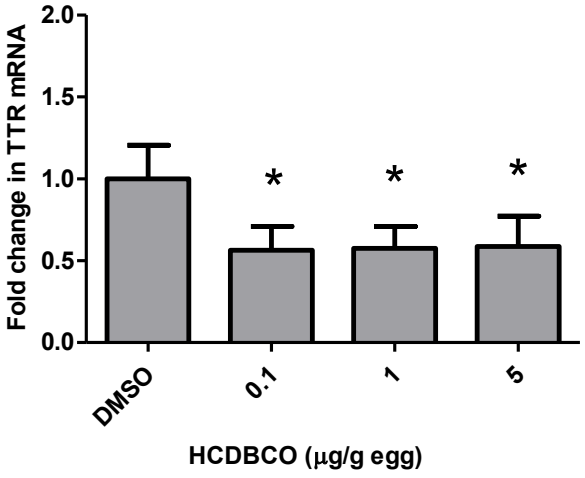


Figure 3.4. The effects of HCDBCO on the mRNA expression of hepatic TTR in chicken embryos. Means and SEs were calculated based on data obtained from 6-8 individuals per dose group. * indicates significant differences compared to the DMSO-treated group based on a Kruskal-Wallis test (p<0.05).

BTBPE significantly increased CYP1A4 and 1A5 mRNA levels 6.5- and 1.8-fold, respectively, at 10 µg/g (Figure 3.5a,b). The same dose elicited a 2-fold down-regulation in CYP3A37 mRNA levels (Figure 3.5c; $p<0.05$). Finally, an apparent dose-dependent decrease in DIO3 mRNA was observed in embryonic livers, with a statistically significant 3-fold down-regulation at 10 µg/g (Figure 3.5d).

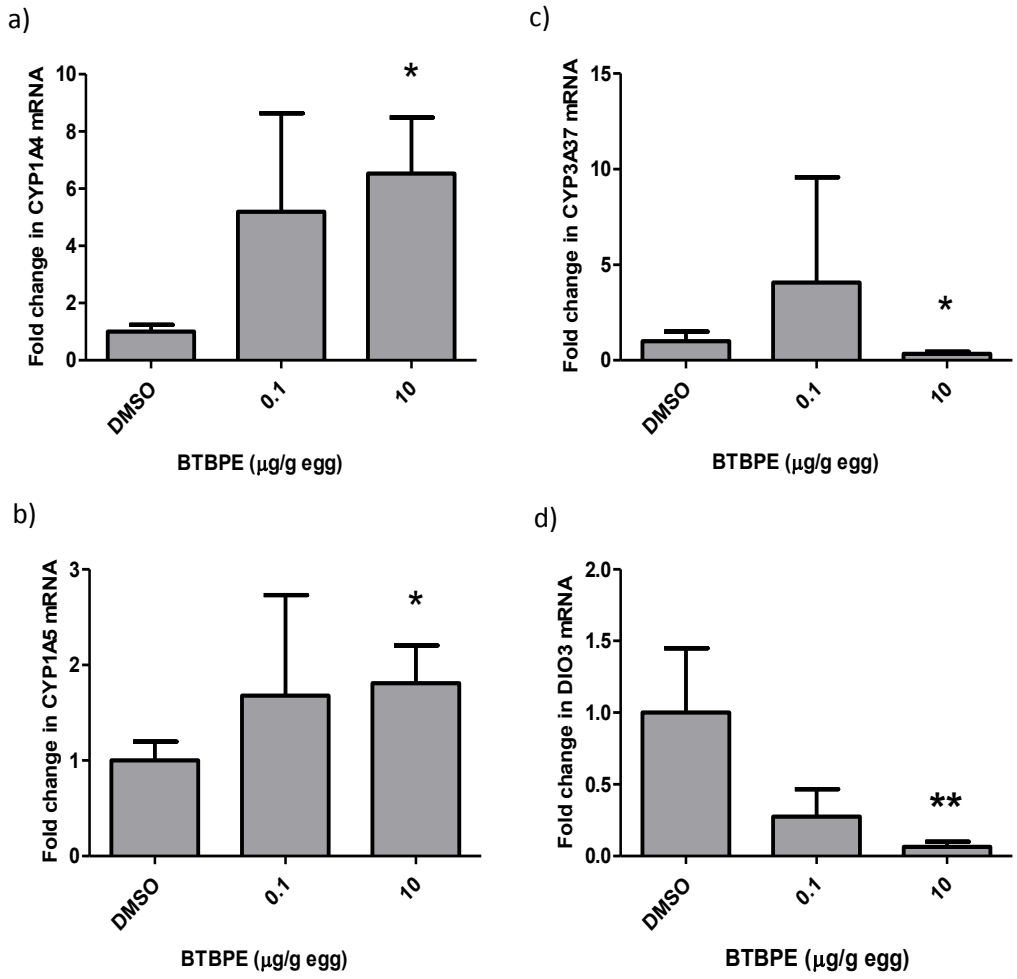


Figure 3.5. The effects of BTBPE on the mRNA expression of hepatic a) CYP1A4, b) CYP1A5, c) CYP3A37 and d) DIO3 in chicken embryos. Means and SEs were calculated based on data obtained from 6-8 individuals per dose group. Each dose group is compared to the DMSO-treated group and significant differences are indicated by * based on a Kruskal-Wallis test or by ** based on a one-way ANOVA ($p<0.05$).

3.4. Discussion

This is the first study to report the effects of HCDBCO and BTBPE in developing chicken embryos. Pursuant to the *in vitro* effects of these two BFRs on hepatic gene expression (Chapter 2), it was important to validate the *in vitro* results in whole organisms to confirm the cell culture method as an effective screening tool for emerging environmental pollutants, such as BFR alternatives.

High doses of HCDBCO and BTBPE did not have an impact on the normal development of chicken embryos as pipping success was not affected by these chemicals. The lowest concentration of BTBPE injected into eggs was approximately 6 times the highest level reported in herring gull egg homogenates (i.e. 7.36 ng/g) from the Great Lakes (Gauthier *et al*. 2007). Moreover, the avian toxicity data for BTBPE are in concordance with previous mammalian and fish studies. 2 mg/kg bw BTBPE was not lethal to rats in an oral exposure study (Hakk *et al*. 2004) and an oral LD50 >10 g/kg was reported in rats suggesting BTBPE was not acutely toxic (Nomeir *et al*. 1993). A chronic dietary study with rainbow trout also reported the absence of toxicity following daily exposure to environmentally relevant doses of BTBPE (Tomy *et al*. 2007).

3.4.1. Hepatic accumulation of HCDBCO and BTBPE

Due to difficulties in dissolving HCDBCO and BTBPE, it was important to determine the actual concentrations that were injected into the eggs; particularly at the highest doses, where both BFRs exceeded their solubility in DMSO. At the highest expected dose, the actual HCDBCO concentration of the supernatant was 5.3 mg/mL, while the

suspension was considerably higher at 65 mg/mL. At the 10 mg BTBPE/mL dose, the supernatant was only 0.70 mg/mL and the suspension was 3.2 mg/mL. This variability within the same dosing solution would lead to highly variable injection concentrations and result in lower hepatic accumulations that did not follow a linear uptake pattern based on the actual concentrations injected. In oral dosing preparations, BTBPE was reported to be fairly insoluble in common vehicle solvents such as peanut oil, which needed to be warmed in order to achieve 0.5 mL of a 2 mg/kg dose without precipitate (Hakk *et al*. 2004).

The average concentration of HCDBCO in livers from embryos that received an actual concentration of 1434 ng/g was less than that of the two lower dose groups (i.e. 61, 746 ng/g). It is likely that HCDBCO was not completely solubilized in DMSO in the 1.5 mg/mL stock solution; the solution that yielded 1434 ng/g in the egg. In the event that precipitate formed at this dose, variable concentrations would have been injected into eggs resulting in highly inconsistent hepatic accumulation of HCDBCO. With the exception of the 1434 ng/g dose group, the remaining groups (i.e. 61, 780, 62142 ng/g) caused HCDBCO concentrations to increase in the liver with increasing dose to a maximum of 2428.0 ng/g ww.

The concentration of BTBPE in liver remained relatively constant among all dose groups. The extent to which a chemical accumulates in the liver is dependent on its uptake by the embryo and its sequestration in extra-embryonic or extra-hepatic tissues. Chicken embryos have previously been shown to be exposed to a very small percentage of the total chemical burden during development, where at least 80% is found instead in lipid-rich tissues such as the yolk sac and chorioallantois membrane (Bargar *et al*., 2001). Accordingly, the probable partitioning of BTBPE (Log Kow 11.95) to more hydrophobic

compartments within the egg would limit its absorption by the embryo and consequently the liver. In orally-exposed rats, lipophilic tissues were found to contain the highest amounts of BTBPE, while 0.2% of the administered dose was found in the liver (Hakk *et al*., 2004).

3.4.2. Hepatic gene expression

Although a large proportion of the initial dose injected into eggs could have remained in extra-hepatic environments, a fraction of HCDBCO and BTBPE did accumulate in the liver; exhibiting potential thyroidogenic effects, while being subject to hepatic metabolism. Transthyretin (TTR), which is a TH binding protein involved in maintaining TH homeostasis, was down-regulated in hepatic tissue of chicken embryos exposed to 1 – 5 µg/g HCDBCO. The accumulation of HCDBCO in the liver generally increased with increasing dose; however, differences in hepatic HCDBCO accumulation between dose groups that were sampled for mRNA analysis were not statistically significant. This observation is in line with TTR being consistently down-regulated at these doses in the liver. A depletion in TTR, particularly at the protein level (not measured here as it was outside the scope of the study), could impact the levels of circulating TH, resulting in disruption of TH-dependent processes such as brain development and post-hatch growth (McNabb 2007). Levels of circulating THs would need to be assessed in the future to investigate the impact of TTR depletions on TH status. Unlike effects observed *in ovo*, TTR was not responsive to HCDBCO in CEH after 36 hours of exposure (Chapter 2). *In ovo* effects on TTR were also absent in embryos exposed to HBCD (Crump *et al*. 2010); however, significant repression of TTR mRNA was observed in HBCD-treated CEH after 24 hours (Crump *et al*. 2008a),

which could be an ideal time point for assessing this particular gene in future *in vitro* exposure studies with HCDBCO.

Compared to the number of gene transcripts in CEH affected by HCDBCO exposure, only one gene was responsive in embryonic livers to this BFR. The mRNA expression levels of CYP1A4/5, CYP2H1 and CYP3A37 were affected by HCDBCO *in vitro* (Chapter 2), while only TTR was significantly affected in livers. The disparity between gene expression results from these two studies reflects the difficulties in capturing complex *in vivo* metabolic processes within a cell culture assay. Where drug-metabolizing enzymes may be induced in hepatocytes upon acute exposure to contaminants for a short period of time (i.e. 36 h), these same enzymes could appear unresponsive in livers (from day 22 embryos) possibly due to the contaminant having already been subject to early enzymatic metabolism during embryonic development. The above underlines the importance of validating *in vitro* results in more biologically realistic conditions.

The highest dose of BTBPE injected into eggs induced CYP1A4 and CYP1A5 mRNA to 6.5- and 1.8-fold, respectively. Compared to concentrations administered *in vitro*, this dose would be equivalent to a nominal concentration of 0.02 µM BTBPE. As discussed in chapter 2, the induction of CYP1A mRNA in chicken embryos may be the result of possible brominated dibenzo-dioxin or -furan contamination. Therefore, repeating the *in ovo* exposure study with highly purified BTBPE stock would clarify this uncertainty. Nevertheless, hepatic accumulation of BTBPE appears to induce AhR-mediated CYP1A4 and CYP1A5 expression, and corroborates the gene expression results *in vitro* for CYP1A (Chapter 2). Although, BTBPE is considered to be poorly absorbed through the gastro-intestinal tract in oral exposure studies (Nomeir *et al*. 1993;Hakk *et al*. 2004), the presence

of several metabolites were reported. Particularly, CYP oxidation of the aromatic rings in BTBPE can produce arene oxides and this was suggested as a plausible mechanism for the formation of mono- or di-hydroxylated metabolites detected in rat feces (Hakk *et al*. 2004).

The down-regulation of CYP3A37 mRNA was observed in embryonic livers as a result of BTBPE exposure. CYP down-regulation is believed to be a protective mechanism initiated by tissues in response to stressors such as inflammation or oxidative stress (Riddick *et al*. 2004). Oxidative stress can result from the production of reactive oxygen species, such as H_2O_2, via the induction of specific CYPs (Morel and Barouki 1999). Contaminant-induced oxidative stress can damage lipid membranes, cellular proteins, DNA (Hoffman and Heinz 1998), and lead to hepatotoxicity (Albina *et al*. 2010). The suppression of CYP transcription is not a typical response to contaminant exposure; however, this has been encountered in hepatocytes, albeit by different BFRs (Chapter 2), which may have been a means to minimize oxidative damage caused by the CYPs. However, CYP3A37 was not responsive in CEH administered concentrations of BTBPE up to 900 times the highest injected dose in eggs. This could be explained by the fact that, in the case of chemical-induced liver injury, inflammatory mediators *in vivo* may be non-existent in isolated hepatocytes. Hepatocellular responses to injury provoke the release of inflammatory proteins, such as cytokines, to mediate healing mechanisms on the affected site (Billiar *et al*. 1992). At the onset of liver injury, inflammatory proteins are produced by non-parenchymal cells (i.e. Kupffer cells) and released to neighbouring hepatocytes (Billiar *et al*. 1992); however, such cell types are excluded from primary cultures of hepatocytes, which could explain the absence of CYP3A37 response to BTBPE *in vitro*. Nonetheless, CYP down-regulation had been observed in CEH exposed to other types of BFRs (Chapter 2).

Therefore, CYP down-regulation in CEH may be a direct response to the presence of reactive oxygen species rather than be attributed to its involvement in inflammatory mechanisms *in vivo*.

The mRNA expression of deiodinase (DIO) 3 was significantly down-regulated in embryonic livers as a result of BTBPE exposure supporting earlier findings in CEH with respect to this gene (Chapter 2). Deiodinases play a crucial role in TH homeostasis by controlling local TH availability to tissues and regulating circulating levels of THs. In chicken, DIO3 is the major TH deactivating enzyme in the liver. During embryonic development, DIO3 converts T4 and T3 to their inactive forms (McNabb 2007). As the embryo reaches the perihatch period, which is a T3-dependent and metabolically demanding process, DIO3 activity is reduced and DIO1 activity increases to convert T4 to T3. The presence of TH deactivating activity during embryonic development is thought to prevent T3 toxicity in the embryo (McNabb 2007). Thus, the marked decrease in DIO3 in response to BTBPE exposure may be associated with a similar hepatic response to prevent embryotoxicity by eliminating a TH-like chemical.

BTBPE-induced changes in mRNA expression were only significant at the highest dose even though hepatic accumulation of BTBPE was constant among all dose groups. As discussed in the results section, the highest dose (i.e. 3.2 mg/mL) was a suspension and may have resulted in highly variable injection concentrations; thus, contributing to the uneven distribution of BTBPE among eggs of the same dose group, which would account for differences in transcriptional response compared to the vehicle control. Furthermore, no real dose-dependent effects on mRNA expression were observed as differences in transcription levels between doses groups were not found statistically significant; thus, possibly the result

of the similar BTBPE concentrations measured in the liver. In order to directly compare dose-related changes in hepatic mRNA expression to the accumulation of BFR in the liver, mRNA data would need to be obtained from the same liver tissue sampled for chemical residue analysis; an important endpoint to consider in future *in ovo* exposure studies. Nevertheless, there is concordance between the BTBPE-induced transcriptional responses *in vitro* (Chapter 2) and *in ovo*; thus, identifying CYP1A4/5 and DIO3 as suitable markers for this particular BFR.

In conclusion, this is the first study to on potential effects of HCDBCO exposure in the chicken. Together with the few available toxicity data on BTBPE (Nomeir *et al*. 1993;Hakk *et al*. 2004;Tomy *et al*. 2007), this study also reports no overt toxic effects in chicken embryos exposed to this contaminant at doses exceeding levels found in wildlife. Of the 11 gene targets, previously identified as markers of BFR exposure (Crump *et al*. 2008a;Crump *et al*. 2010), genes associated with xenobiotic metabolism and the TH pathway were most sensitive to HCDBCO and BTBPE exposure. Due to the occurrence of BTBPE in birds, it would be important to determine if these same gene markers are candidate endpoints for this particular BFR in wild avian species. Accordingly, results from this study will contribute valuable information on HCDBCO-and BTBPE-induced genes to predicting the potential effects of these BFR alternatives in wild species at risk of exposure.

Chapter 4 – General discussion, conclusions and suggestions for future research

In this thesis, the effects of HCDBCO, BEHTBP, BTBPE and DBDPE on selected hepatic gene targets in chicken were investigated by an *in vitro* molecular screening method previously described by Crump *et al*. (2008a). Several findings in this study will contribute useful information regarding these BFRs, for which toxicological data had been limited or unavailable.

Exposure of CEH or chicken embryos to structurally-unrelated BFRs resulted in distinct gene expression profiles (Table 4). Many of the genes assessed here had been robust targets of HBCD exposure as indicated by the similar expression patterns both *in vitro* and *in ovo* (Crump *et al*. 2008a;Crump *et* al. 2010). The BFRs tested in this study were structurally different and therefore expected to affect these gene targets differently. BTBPE and DBDPE elicited changes in the mRNA expression of a similar set of genes possibly due to their common double-aromatic structure, which closely resembles TH molecules. These two BFRs induced AhR- and CXR- mediated CYP responses, in addition to affecting deiodinase transcription. Structurally dissimilar from BTBPE and DBDPE, HCDBCO had the opposite effect on the mRNA expression of mixed-function oxidases, while repressing TTR transcription. However, the norbornene structure of HCDBCO cannot account for the transcriptional changes in these genes as another norbornene-structured flame retardant, dechlorane plus, was determined to be transcriptionally inactive using the same molecular screening method in CEH and embryonic livers (Crump *et al*. 2011, manuscript submitted).

Genes responsive to BTBPE exposure *in vitro* did elicit similar patterns of expression in the hepatic tissue of embryos exposed to this particular BFR. BTBPE significantly induced the expression of CYP1A4/5 genes and suppressed the expression of

DIO3 in both hepatocytes and embryonic livers, which identifies the AhR pathway and the TH hormone pathway as targets of BTBPE exposure. In contrast, genes that were responsive to HCDBCO in CEH were unresponsive in embryonic liver. HCDBCO affected CYP expression in CEH, while down-regulating TTR expression in embryonic livers. As discussed in chapter 3, HCDBCO may have been rapidly metabolized during embryonic development due to the absence of change in CYP transcription levels in day 20-22 embryos. In this case, analyzing the liver for the presence of possible HCDBCO metabolites could further validate this claim.

Table 4.1. Comparison of transcriptional responses of genes in chicken embryonic hepatocytes (H) and embryonic hepatic tissue (E) exposed to HCDBCO, BTBPE, DBDPE and HBCD.

	C1A4/5		C2H1		C3A37		UGT1A9		D1		D2		D3		TTR		LFABP		THRSP14	
	H	E	H	E	H	E	H	E	H	E	H	E	H	E	H	E	H	E	H	E
HCDBCO	↓		↑		↑											↓				
BTBPE	↑	↑											↓	↓						
DBDPE	↑	-		-	↓	-		-	↑	-				-		-		-		-
HBCD*	-	-	↑	↑	↑	↑	↑	↑				↑			↓	↓	↓	↓	↓	

Some gene names were truncated to fit in table, such as CYP (C) and DIO (D). *Gene expression results cited from Crump *et al*. 2008a, 2010**.** (-) Not determined.

One of the main issues encountered in this study was the difficulty in solubilizing some of these BFRs in the vehicle solvent, DMSO. BFRs are non-polar, highly lipophilic molecules and are larger than other halogenated flame retardants due to the bromine atom

(e.g. $Br^- > Cl^- > F^-$). DBDPE was more difficult to solubilize in DMSO than BTBPE as determined by chemical analysis during the cell culture studies. At the highest concentrations prepared for egg injection studies, HCDBCO and BTBPE exceeded their solubility in DMSO. Using non-polar organic solvents, in which these BFRs would have been more soluble, would have been necessary to prepare more concentrated stock solutions. For example, toluene is known to completely solubilize these BFRs, but this solvent was found in earlier studies to be toxic to hepatocytes (unpublished data). Regardless of these solubility issues, most stock preparations that had completely dissolved BTBPE and DBDPE were administered to CEH or chicken embryos and exceeded levels detected in wild birds. However, caution is needed when comparing laboratory exposure levels to concentrations measured in wildlife. As discussed previously (Chapter 1), increased bromination and structural complexity can increase the lipid solubility of BFRs as well as their molecular size; thus, possibly limiting their bioavailability to hepatocytes or hepatic tissues of embryos. The amount of test BFR that had accumulated in hepatocytes after exposure was not quantified in this study and may not reflect the initial concentrations administered. A study by Mundy *et al*. (2004) demonstrated this inconsistency by observing the partial accumulation of radiolabelled-PBDE in primary cultures of rat neocortical cells. After 1 hour of exposure, 15% of the initial PBDE concentration was associated with the cells, whereas 55% remained in the medium and 30% adhered to the plastic culture dish. Therefore, relying on nominal exposure concentrations in cell culture may overestimate concentrations actually found in hepatocytes. Likewise, as discussed in chapter 3, embryos may not be exposed to initial concentrations injected into the egg prior to incubation possibly due to the highly lipophilic nature of BFRs. As BFRs are more likely to partition to

lipid-rich tissues, analyzing extra-hepatic tissues (i.e. adipose tissues) of the embryo and extra-embryonic tissues (i.e. yolk sac) for BFR content would provide a more accurate exposure value. Therefore, quantifying BFR concentrations in hepatocytes and embryos would benefit cell culture and egg injection studies when relating environmentally-relevant levels of exposure in wildlife.

HCDBCO, BTBPE and DBDPE exposure in the chicken elicited changes in the transcription of specific genes encoding functional proteins (i.e. enzymes, transport proteins). Because quantifying gene transcript levels by real-time RT-PCR involves measuring mRNA abundance, the proportion of mRNA that is translated into protein is not accounted for. All RNA that is transcribed from DNA is not necessarily translated into protein since post-transcriptional modifications to the RNA molecule can modulate protein expression. Therefore, assessing protein levels subsequent to BFR-induced changes in gene transcription would further confirm the effects of BFR exposure on specific gene targets. A two-dimensional gel electrophoresis protein assay is a well-established technique for identifying low abundance proteins at high resolution. In this type of assay, proteins extracted from cells or tissue samples are separated according to their overall charge and molecular weight. The separated proteins can then be detected by antibody probes, which specifically bind to the protein of interest, or by mass-spectrometry. Using a protein assay in combination with the determination of mRNA abundance by RT-PCR would strengthen the relationship between the effects of BFR alternatives on the transcription of selected genes and their corresponding proteins.

Several genes in this study were vulnerable to HCDBCO, BTBPE and DBDPE exposure; however, none of the gene targets assessed were determined suitable targets of

BEHTBP exposure. The 11 genes assessed here were only a few of the many genes encoding proteins involved in xenobiotic metabolism, the TH pathway or lipid metabolism. As the molecular screening method employed here was limited to the series of genes that were determined as suitable endpoints of BFR exposure, it could not provide information on the behaviour of other genes involved in the same pathways. Indeed, BEHTBP could be affecting different genes involved in the pathways mentioned above or manifesting its effects through different biological mechanisms. A DNA microarray can offer a more comprehensive transcriptional profile in the chicken by screening thousands of genes simultaneously and identifying alternate pathways that may be responsive to BFR exposure. For example, the estrogen pathway was affected in fish hepatocytes exposed to DBDPE as demonstrated by the production of vitellogenin (Nakari and Huhtala, 2009) and may be a potential target to consider in birds at risk of exposure to this particular BFR.

Despite certain limitations, this study demonstrated that HCDBCO, BEHTBP, BTBPE and DBDPE were not toxic to the chicken at current laboratory levels and identified xenobiotic metabolism and the TH pathway as the main targets of HCDBCO, BTBPE and DBDPE exposure. The molecular screening method employed here was useful in assessing the effects of BFR exposure on specific gene transcripts, which cover three important biological mechanisms (i.e. xenobiotic metabolism, TH pathway and lipid metabolism). Currently, toxicological information on numerous flame retardants in the marketplace is still limited or unknown. Several of these chemicals could be rapidly screened via the *in vitro* method to identify toxic effects or gene transcripts susceptible to such exposure. Information gathered from this screening process could determine future endpoints of research and prioritize which chemicals to pursue in *in vivo* toxicity testing.

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Appendix

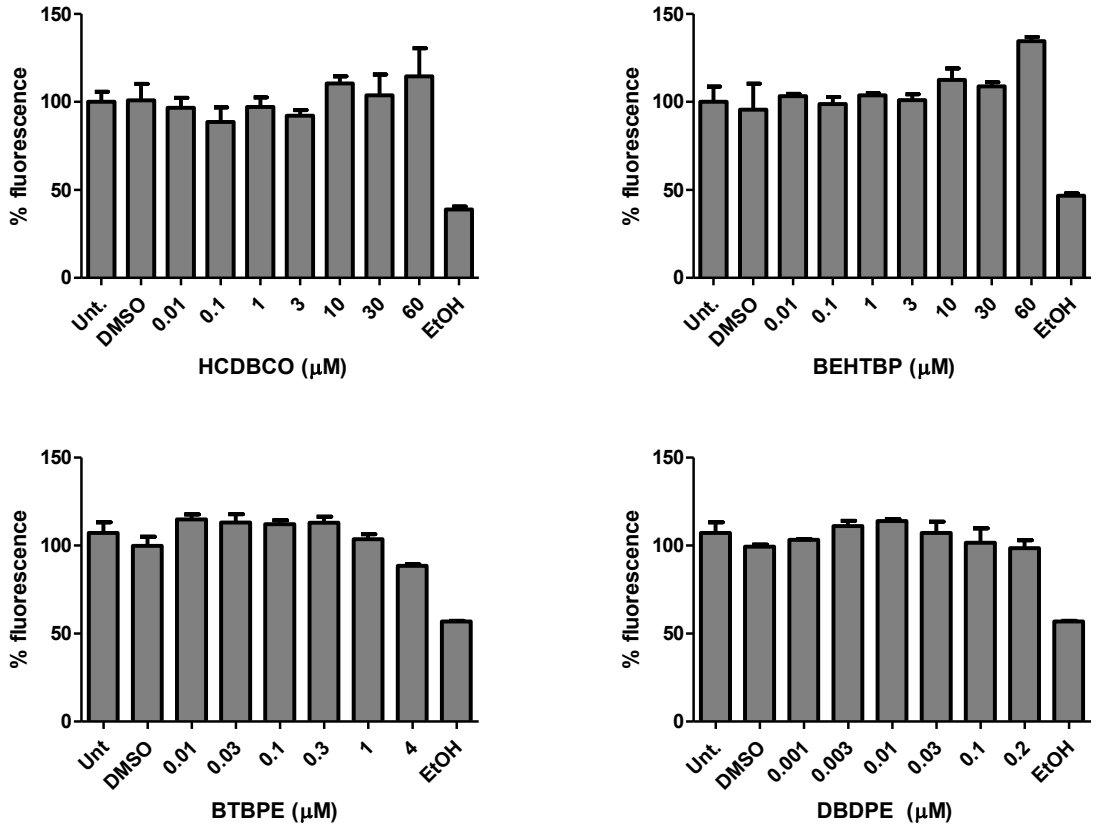

Figure A – Relative measure of cell viability using a Calcein-AM assay, where fluorescence emitted from live cells from each treatment group is compared to the fluorescence emitted from live cells in the untreated group. Ethanol-killed cells were included as the negative control. Means and SEs were calculated based on data obtained from 3 replicates for each concentration of a) HCDBCO, b) BEHTBP, c) BTBPE and d) DBDPE. Significant differences were compared to untreated cells based on a one-way ANOVA ($p<0.05$).