Structure-related interactions of brominated and organophosphate flame retardants and degradation products with thyroxine and human thyroid hormone transport proteins *in vitro*

by

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Abstract

One mechanism of action of thyroid disrupting compounds is the competition for thyroid hormone (TH) binding sites on vertebrate serum transport proteins, including transthyretin (TTR) and albumin (ALB). An *in vitro* competitive binding assay was optimized for use with thyroxine (T4) and human TTR or ALB. This assay was applied for two classes of novel and environmentally relevant flame retardant (FR) chemicals, and/or some degradation products; organophosphate (OP) triesters, and tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz). Structure-related differences in the binding of these ligands with TTR or ALB were observed, including a newly discovered apparent allosteric interaction of OP esters with TTR that enhances binding of T4. Degradation products of TeDB-DiPhOBz are ligands for human TTR and ALB *in vitro*, as well as gull TTR *in silico*, with *para*-hydroxylated lower brominated congeners being the strongest binders. Overall, results indicate potential interference of these novel FR contaminants with human TH transport, in a structure-dependent manner.

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Preface

This section contains the complete bibliographical details and statements of contributions of three manuscripts included in this thesis. The content of the manuscripts have been adapted to fit in the thesis structure.

In accordance with Carleton University's Integrated Thesis policy, the supervisors (Drs. Robert J. Letcher and William G. Willmore) and the author of this thesis (Katie L. Hill) confirm that the student was fully involved in designing, setting up, and conducting the research, collecting and obtaining the data, analyzing results, and preparing and writing the co-authored articles and manuscripts in the thesis.

Chapter 2

This chapter is based upon the following manuscript:

Hill, K.L., Hamers T., Kamstra J.H., Willmore, W.G., and Letcher, R.J. Optimization of an *in vitro* assay methodology for competitive binding of thyroidogenic xenobiotics with thyroxine on human transthyretin and albumin. Submitted to the journal *MethodsX* March 2017.

For this manuscript, Katie L. Hill designed and conducted the experiments and data analyses, and wrote the manuscript. Dr. Timo Hamers and Jorke Kamstra provided valuable guidance and technical expertise on the storage and handling of reagents to ensure proteins remained intact. Drs. Robert Letcher and William Willmore provided input into the manuscript. This study was financially supported by the Chemicals Management Plan (CMP; Environment and Climate Change Canada) (to R.J.L.), a Discovery Grant from the Natural and Engineering Science Research Council (NSERC)

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Chapter 3

This chapter has been adapted from the following manuscript:

Hill, K.L., Hamers T., Kamstra J.H., Willmore, W.G., and Letcher, R.J. Organophosphate triesters and selected metabolites enhance binding of thyroxine to human transthyretin *in vitro*. Submitted to the journal *Toxicology Letters* September 2017.

For this manuscript, Katie L. Hill designed and conducted the experiments and data analyses, and wrote the manuscript. Dr. Timo Hamers and Jorke Kamstra participated in discussing the potential implications of the observed results. Drs. Robert Letcher and William Willmore provided input into the manuscript. This study was financially supported by the Chemicals Management Plan (CMP; Environment and Climate Change Canada) (to R.J.L.), a Discovery Grant from the Natural and Engineering Science Research Council (NSERC) of Canada (to R.J.L. and W.G.W.), and the NSERC CREATE Program (to R.J.L. and W.G.W.).

Chapter 4

This chapter has been adapted from the following manuscript:

Hill, K.L., Mortensen, Å.K., Teclechiel, D., Willmore, W.G., Sylte, I., Jenssen, B.M., and Letcher, R.J. *In vitro* and *in silico* competitive binding of brominated polyphenyl ether contaminants with human and gull thyroid hormone transport proteins. Submitted to the journal *Environmental Science & Technology* September 2017.

For this manuscript, Katie L. Hill designed and conducted the *in vitro* experiments and all data analyses, and wrote the manuscript. Åse-Karen Mortensen conducted the *in silico* modelling. Daniel Teclechiel synthesized the test chemical standards. Drs. Robert Letcher and William Willmore provided input into the manuscript. The *in vitro* study was financially supported by the Chemicals Management Plan (CMP; Environment and Climate Change Canada) (to R.J.L.), a Discovery Grant from the Natural and Engineering Science Research Council (NSERC) of Canada (to R.J.L. and W.G.W.), and the NSERC CREATE Program (to R.J.L. and W.G.W.). The *in silico* portion of this study was financed by a PhD Grant from the Norwegian University of Science and Technology (to Å.K.M.) and a grant from the Research Council of Norway (to B.M.J; grant No. 268419/E10).

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4-OH-BDE-49	4-hydroxy-2,2’,4,5’-tetrabromodiphenyl ether
4-OH-CB-187	4-hydroxy-2,2',3,4',5,5',6-heptachloro-biphenyl
AChE	Acetylcholinesterase
ALB	Albumin
ANOVA	Analysis of variance
ANSA	8-anilino-1-naphthalenesulfonic acid ammonium
AOP	Adverse outcome pathway
AUC	Area under the curve
BDPB-402	2,2’,2”,4-tetrabromodiphenoxybenzene
BFR	Brominated flame retardant
CAS No.	Chemical Abstract Service registry number
CMP	Chemicals Management Plan
cpm	Counts per minute
CREATE	Collaborative Research and Training Experience Program
DPHP	Diphenyl phosphate
EDTA	Ethylenediaminetetraacetic acid
FITC-T4	Fluorescence probe fluorescein isothiocyanate associated to T4
FR	Flame retardant
HBDPB-401	4”-hydroxy-2,2’,2”,4-tetrabromodiphenoxybenzene
HPT	Hypothalamic-pituitary-thyroid
IC50	50% inhibitory concentration

ICM	Internal coordinate mechanics software
K_d	Equilibrium dissociation constant
K_i	Inhibition constant
MeO	Methoxylated
MOBDPB-401	4”-methoxy-2,2’,2”,4-tetrabromodiphenoxybenzene
NIS	Sodium/iodide symporter
NSAID	Nonsteroidal anti-inflammatory drug
NSERC	National Science and Engineering Research Council
NWRC	National Wildlife Research Centre
OH	Hydroxylated
OP	Organophosphate
PBDE	Polybrominated diphenyl ether
PB-DiPhOBz	Polybrominated diphenoxybenzene
PCB	Polychlorinated biphenyl
PDB	Protein data bank
***p*-OH-TPHP**	*Para*-hydroxy-triphenyl phosphate
POP	Persistent organic pollutant
PUF	Polyurethane foam
RBP	Retinol binding protein
ROC	Receiver Operator Characteristics
T3	Triiodothyronine
T4	Thyroxine
TBBPA	Tetrabromobisphenol A

TBG	Thyroxine binding globulin
TBOEP	Tris(2-butoxyethyl) phosphate
TDCIPP	Tris(1,3-dichloro-2-propyl) phosphate
TeDB-DiPhOBz	Tetradecabromo-1,4-diphenoxybenzene
TEP	Triethyl phosphate
TH	Thyroid hormone
TR	Thyroid hormone receptor
TPHP	Triphenyl phosphate
TR-CALUX	Thyroid hormone responsive chemically activated luciferase gene expression
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone; thyrotropin
TTR	Transthyretin

1 Chapter: General introduction

1.1 The mammalian thyroid system

The thyroid is involved in several important processes, including the regulation of metabolism, body temperature, and heart rate in adult organisms, as well as embryonic development (McNabb, 2006; Mendoza and Hollenberg, 2017; Patel et al., 2011). Synthesis of thyroid hormones (THs) occurs in the thyroid gland and is followed by secretion into the bloodstream. Circulating levels of THs are kept within a specific and narrow range by the highly complex hypothalamic-pituitary-thyroid (HPT) axis (Zoeller et al., 2007). In brief, synthesis of thyrotropin-releasing hormone (TRH) in the paraventricular nucleus of the hypothalamus stimulates production of thyrotropin (TSH) in the pituitary, which binds to the surface of thyroid follicular cells to stimulate production of thyroxine and triiodothyronine (T4 and T3; Figure 1-1). In order for THs to circulate throughout the body and reach target cells, they rely on binding to TH transport proteins in the plasma (Feldt-Rasmussen and Rasmussen, 2007; Visser et al., 2008). In human blood, > 99 % of THs are bound to one of the three major TH transport proteins: transthyretin (TTR), albumin (ALB), and thyroxine binding globulin (TBG) (Mendel, 1989). The majority of TH circulating in blood is T4, which is converted to T3 by deiodinase enzymes at the target site. Here T3 binds to the thyroid hormone receptor (TR) and regulates gene expression and tissue differentiation (Patel et al., 2011). A negative feedback mechanism is also involved in TH regulation, whereby T3 and T4 presence in the pituitary prevents TSH expression as well as TRH neural activity in the hypothalamus (Chiamolera and Wondisford, 2009; Zoeller et al., 2007).

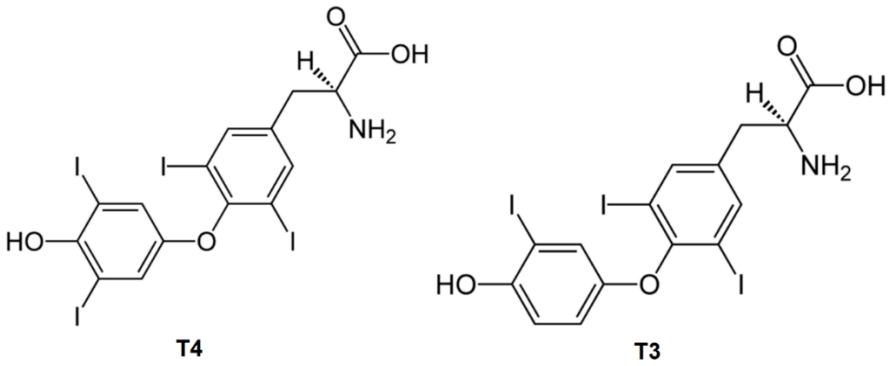


Figure 1-1 Molecular structures of T4 (thyroxine) and T3 (triiodothyronine). Hydrogen atoms on the phenyl rings have been omitted for clarity.

1.1.1 Thyroid hormone transport in humans

In human serum there are distinct differences in the circulating concentrations, TH affinities, and dissociation rates of TH transport proteins. Thyroxine binding globulin has the highest affinity, the lowest plasma dissociation rate, and is present at the lowest concentration (Mendel, 1989). In comparison, ALB is generally present at the highest concentration in the blood, but it has the lowest binding affinity for T4 and a much higher dissociation rate. Transthyretin is believed to be the most important distributor of T4 in humans because its dissociation rate is between those of ALB and TBG (0.094 s^{-1}) (Feldt-Rasmussen and Rasmussen, 2007). Additionally, while all three proteins are synthesized in the liver, TTR is the only TH transport protein synthesized in the choroid plexus and as such it is the major transporter of THs to the brain (Chang et al., 1999; Feldt-Rasmussen and Rasmussen, 2007; Palha, 2002). Transthyretin is also capable of crossing the placental barrier and thus plays an important role in the supply of THs to the developing fetus (Zoeller et al., 2007). Transthyretin and ALB are consistently important across

vertebrate species and as such these two TH transport proteins will be the focus of this thesis.

1.1.1.1 Transthyretin

Transthyretin circulates as a tetramer of four identical subunits (dimer-of-dimers configuration) arranged in a beta sheet structure (Figure 1-2) (Ghosh et al., 2000; Trivella et al., 2011). These subunits come together to form a channel of two funnel-shaped TH binding sites, with inner and outer sites for TH binding (Palha, 2002). In this binding channel, a T3 or T4 molecule generally becomes oriented such that the hydroxyl group and adjacent iodine atoms of one ring enter the inner binding site, and the iodine atoms on the second ring face the outer binding site (Ghosh et al., 2000; Weiss et al., 2009). Halogen interactions are believed to be primarily responsible for binding (Ghosh et al., 2000), however hydrogen bonds formed in the presence of a water molecule may also contribute to this interaction (Tomar et al., 2012). Serine and lysine residues present in the binding channel are also involved in this hydrogen bonding (Tomar et al., 2012; Wojtczak et al., 1996). Negative binding cooperativity has most commonly been observed, and as such only one TH molecule typically binds to TTR at a time (Blake et al., 1978; Chang et al., 1999; Foss et al., 2005; Palha, 2002).

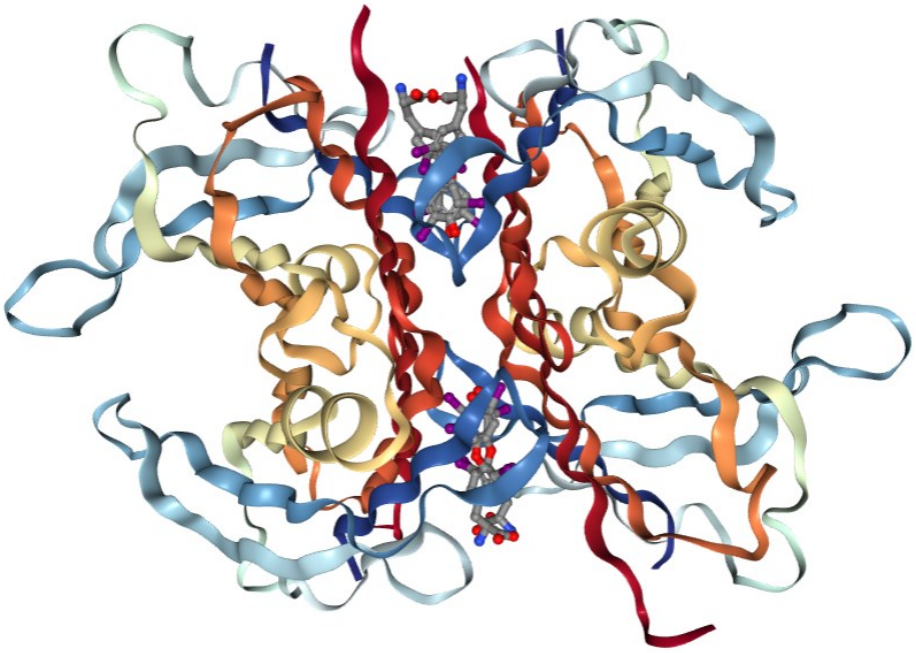


Figure 1-2 Crystal structure of human TTR (transthyretin), showing the binding orientation with thyroxine (T4, with the iodine atoms of T4 in purple, oxygen atoms in red, nitrogen atoms in blue, carbon atoms in grey, and hydrogen atoms omitted for clarity); however note that typically only one T4 molecule is present the binding channel at a time. Image determined by X-ray diffraction and obtained from the RCSB Protein Data Bank (PDB ID 2ROX; Wojtczak et al.).

In addition to THs, TTR is also involved in transporting retinol binding protein (RBP) (which binds retinol; vitamin A). There are four potential RBP binding sites on the surface of TTR, whereby two RBPs may bind to TTR at a time due to steric hindrance (Coward et al., 2009; Vieira and Saraiva, 2014). The binding of RBP to TTR does not affect the TH binding channel. A recently discovered additional function of TTR is proteolysis, with identified substrates to-date including Amyloid β, apolipoprotein A-I, and neuropeptide Y (Vieira and Saraiva, 2014).

1.1.1.2 Albumin

Albumin is a single polypeptide chain that forms three alpha-helical domains, each containing two subdomains (Figure 1-3) (Kragh-Hansen et al., 2016; Yang et al., 2014). There are four main binding sites for T4 across the protein, and hydrogen bonds are the main interactions that occur between ALB and T4, with each binding site resulting in a different ligand orientation (Tarhoni et al., 2008). Albumin also serves as a transport protein for several endogenous compounds other than THs, including fatty acids, bile acids, and metals (Yang et al., 2014).

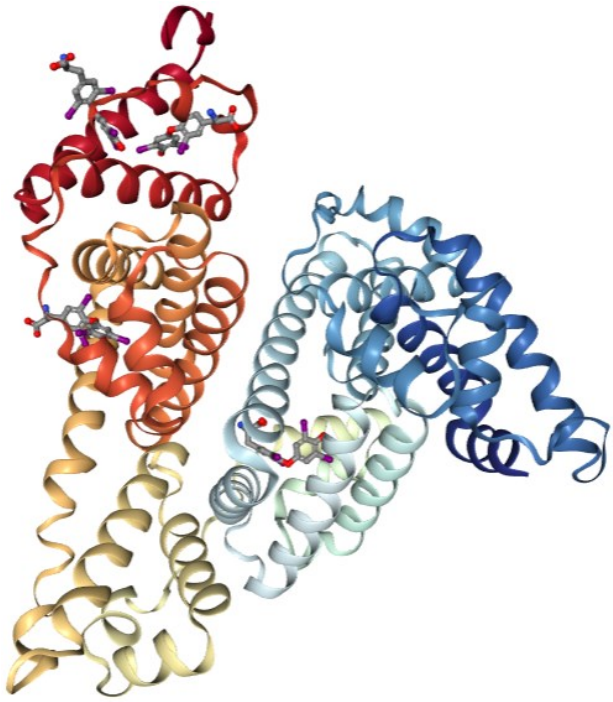


Figure 1-3 Crystal structure of human ALB (albumin), showing the four binding sites for thyroxine (T4, with the iodine atoms of T4 in purple, oxygen atoms in red, nitrogen atoms in blue, carbon atoms in grey, and hydrogen atoms omitted for clarity). Image determined by X-ray diffraction and obtained from the RCSB Protein Data Bank (PDB ID 1HK1; Petitpas et al.).

1.1.2 Thyroid hormone function and transport in other vertebrates

The chemical structures of T3 and T4 are identical in all vertebrate species, and thyroid processes (e.g., synthesis, signaling and regulation) are similar overall across the vertebrate classes (Bergmann et al., 2012; Miller et al., 2009). However, some interesting differences have been observed. For example, while T4 is the predominant TH transported through mammalian blood, T3 is the major circulating TH in all other vertebrate organisms including birds, reptiles, amphibians, and fish (Richardson et al., 2015; Ućan-Marin et al., 2010). This evolutionary change in mammals allows for conversion of T4 to T3 by deiodinases in a tissue-specific manner and provides a greater level of control of TH action compared to other vertebrates (Chang et al., 1999; Prapunpoj and Leelawatwattana, 2009).

Thyroxine binding globulin is not expressed in birds, reptiles, amphibians or fish (Ućan-Marin et al., 2010). In marsupials, birds, fish, and some reptiles and amphibians, TTR is the major TH transport protein in the blood. Like in humans, TTR in other mammals, birds and reptile species is also synthesized in the choroid plexus and as such it is the major transporter of THs to the brain (Chang et al., 1999; Palha, 2002). In fish and amphibians, mRNA for TTR is present in the choroid plexus, but it does not appear to be active (Prapunpoj and Leelawatwattana, 2009).

The sequence of TTR has been determined for numerous species including herring and glaucous gull, duck, chicken, crocodile, frog, zebrafish, rat and human (Schuler et al., 1991; Ucán-Marín et al., 2009). Overall the protein is highly conserved across these species, though some differences exist in the amino acid residues in N-terminal region including shortening of the sequence in eutherian mammals and resulting

in increased hydrophilicity of this region (Chang et al., 1999; Prapunpoj and Leelawatwattana, 2009). These changes to the N-terminal region are expected to be driving factors for the differences in ligand binding affinity between species, whereby mammalian TTR preferentially binds T4 over T3 while TTR in other vertebrates has a higher affinity for T3 over T4 (Chang et al., 1999).

Albumin has also remained fairly well conserved throughout vertebrate evolution, although with 70 % similarities in the amino acid sequences of avian and human ALB, some differences in function may be expected (Ućan-Marin et al., 2010). When comparing between avian species, ALB samples from two gull species were recently purified and were found to have over 85 % similarities in amino acid sequence to chicken (Ućan-Marin et al., 2010). Consistent with TTR there are differences in ALB binding affinities for THs between species; mammalian ALB preferentially binds T4 whereas avian ALB preferentially binds T3 (Ucán-Marín et al., 2009).

1.1.3 Disruption of thyroid hormone transport

A sufficient supply of thyroid hormones to the vertebrate embryo is necessary to allow proper development of muscular and skeletal systems, as well as nervous system functions (McNabb, 2006; Patel et al., 2011). In humans and other mammals TTR is able to cross the placental barrier to deliver THs to the developing fetus. Maternal THs are essential as the fetus is not capable of producing T3 or T4 until the second trimester (Patel et al., 2011). An insufficiency of THs during this critical time of development can lead to detrimental effects including reduced IQ and mental retardation (Richardson et al., 2015). Competition of an exogenous compound at the TH binding site on TTR could

reduce TH delivery, as well as potentially exposing the fetus to these chemicals (Legler and Brouwer, 2003).

Concerns are similar for wildlife species. For example, in birds exogenous chemicals bound to TTR or ALB (and the subsequent reduction in THs) may be transported from the mother to the embryo during egg laying. The degree of embryonic development at hatch naturally differs by avian species across a spectrum from precocial (well-developed and able to leave the nest quickly) to altricial (less-developed and requiring more parental support) (McNabb, 2006). Hypothyroidism in the avian egg is linked to delayed hatching, as THs are necessary in the perihatch period to stimulate pipping (Farhat et al., 2013; McNabb, 2006). Because each species has evolved to develop to a certain degree at hatch, a chick that hatches late or at a less-developed stage may be abandoned or otherwise unable to compete for resources (Farhat et al., 2013).

Certainly the predominant toxicological concern of TH transport disruption is the effect on physiological and neurodevelopment during early life stages. In adult organisms, physiological impacts of this mechanism of action are not well understood to-date, however it is expected that TH displacement from transport proteins may lead to increased excretion and thus potentially perturb regulation of TH synthesis (Miller et al., 2009; Ouyang et al., 2016). Furthermore, if a xenobiotic chemical is able to competitively bind to TH transport proteins *in vitro*, it could possibly exhibit similar effects at other TH-dependent sites of action, e.g., preferentially binding at the TR site and leading to impact on gene transcription activity (Ren et al., 2013).

Several physical-chemical properties have been identified as important factors in the interactions of endogenous and exogenous ligands with TH transport proteins (and

predominantly human TTR). The three most important characteristics identified to-date are 1) presence of an aromatic ring(s), 2) presence of a halogen(s), and 3) presence of a hydroxyl group (and preferably a *para*- or *meta*-OH group) (Papa et al., 2013; Weiss et al., 2015; Zhang et al., 2015). Other notable characteristics include a molecular size of up to 550 g/mol, partial charge, polarity, and hydrophobicity (Lans et al., 1993; Papa et al., 2013; Weiss et al., 2015; Zhang et al., 2015).

1.1.4 Other mechanisms of thyroid disruption

The mechanism of action of interest for this project is TH serum transport, however several other steps within the thyroid regulation pathway could be potentially impacted by xenobiotic chemicals. For example TH synthesis, which is dependent on iodide uptake into the thyroid via the sodium/iodide symporter (NIS), can be inhibited due to a deficiency in dietary iodide or NIS blockage by chemicals, leading to reduced circulating THs (Miller et al., 2009). Metabolism and excretion of THs is an important component of thyroid homeostasis, and this is dependent upon the regulated production and activity of enzymes including deiodinases, glucuronosyltransferases, and sulfotransferases which may be inhibited or up-regulated by xenobiotic contaminants (Miller et al., 2009; Murk et al., 2013). Additionally, cellular transport of THs may be impacted by up-regulation of organic anion-transporting polypeptides which could lead to increased biliary elimination of THs (Murk et al., 2013). Similar to competitive inhibition in the TH binding site on transport proteins, another potential mechanism of xenobiotic action can involve competitive binding at the TR site and lead to alterations in TH-dependent gene transcription (Mendoza and Hollenberg, 2017).

1.2 Flame retardant chemicals

A structure fire is reported once every minute in the United States. In 2015 alone, the result was over 500,000 fires, 2,500 deaths, and $10 billion in property damage (National Fire Protection Association, 2017). While these figures are disconcerting, the number of fires occurring in structures in the US has been reduced by half since 1977 (National Fire Protection Association, 2013). This decline in the incidence of fires is at least in part due to the use of flame retardant (FR) chemicals and application to consumer products in order to meet fire safety regulations (Birnbaum and Staskal, 2004).

Flame retardants are applied either additively (mixed in) or reactively (chemically bonded) to a multitude of products including wood, building materials, textiles, polyurethane foams, and electronics. The major classes of FR chemicals include brominated, chlorinated, nitrogen-containing, phosphorus-containing, and inorganic FRs. The specific mechanism of flame retardancy varies based on chemical composition. For example, halogen-containing FRs interact with the gas phase of the fire cycle trapping free radicals and interrupting the chemical chain reaction leading to flame formation, thus preventing or delaying the spread of flames. Conversely, phosphorus-containing FRs interfere with the combustion process by releasing phosphoric acid, causing the material to char and preventing the release of flammable gases.

1.2.1 Exposure of humans and wildlife to flame retardants

Flame retardants are a unique class of environmental pollutants because the sources for human exposure are largely indoors. While effective at hindering combustion

and delaying the spread of fire, the release of FRs (particularly additive compounds) through volatilization, leaching, and abrasion can be substantial over the lifetime of a product (Bergmann et al., 2012; Birnbaum and Staskal, 2004; Marklund et al., 2005). Inhalation of FRs in house dust is a substantial route of exposure for humans, and especially so for young children who spend ample time on the floor and exhibit hand-to-mouth behaviour (Fang and Stapleton, 2014; Stapleton et al., 2014). The life cycle of FRs in commerce includes chemical synthesis, incorporation into products, consumer use, disposal to landfill, and export of e-waste to e.g., China (Jonkers et al., 2016). Release of FRs into the environment can occur at any of these stages, including into wastewater effluent and sewage sludge following the uptake and subsequent elimination from humans exposed to FRs (Davis et al., 2012; O'Brien et al., 2015; Schreder and La Guardia, 2014; Venkatesan and Halden, 2014). Many FR chemicals are also volatile and have been found to be present in air samples taken from urban and remote environments including the arctic (Möller et al., 2011; Shoeib et al., 2014). As a result, aquatic and terrestrial wildlife species are also exposed to FR chemicals and their degradation products.

1.2.2 Polybrominated diphenyl ethers

Polybrominated diphenyl ethers (PBDEs) are a group of additive brominated FRs produced in three commercial mixtures based on the degree of bromination: penta-BDE, octa-BDE and deca-BDE (Figure 1-4). The substantial use of PBDEs over several decades (starting in the 1970s) has led to high exposures of humans and wildlife, including presence in human blood and breast milk, and ubiquitous detection in e.g.

birds, fish, soil, sediment, water, and air, including Arctic air (Gentes et al., 2015; Poma et al., 2014; Stapleton et al., 2011; Venkatesan and Halden, 2014; Wu et al., 2007). Due to widespread exposure (especially in children), and concerns of persistence and bioaccumulation of PBDEs all three commercial mixtures have recently been characterized as persistent organic pollutants (POPs) under Annex A of the Stockholm convention (United Nations Environment Programme, 2017).

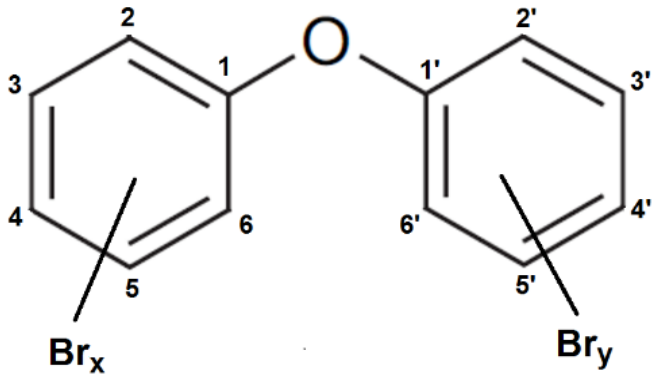


Figure 1-4 The general molecular structure of a polybrominated diphenyl ether (PBDE) congener, whereby x + y ≤ 10 bromine atoms. Hydrogen atoms have been omitted for clarity.

The basic molecular structure of PBDEs shares several similarities to THs, and as such there is concern for their potential to interfere with normal thyroid function. Some biotransformation products of PBDEs include hydroxylated metabolite products with varying degrees of bromination, which share further resemblance to THs. Indeed, existing literature suggests the ability of PBDEs and metabolites to disturb thyroid function. Several PBDE congeners and some metabolites have been tested for TTR and ALB binding competition with T4 *in vitro*, and for example tetra-BDE congener BDE-47 and related tetra-BDE metabolites 6-MeO-BDE-47, 6-OH-BDE-47 and 4-OH-BDE-49 were found to be increasingly potent ligands for human and gull proteins (Hamers et al., 2006;

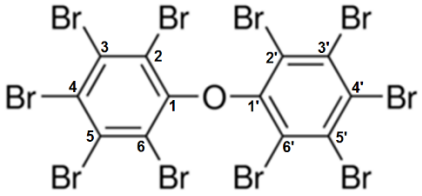
Ucán-Marín et al., 2009). Additionally, Ren et al. (2013) found varying effects of OH-PBDE exposure on human TR based on degree of bromination whereby lower brominated OH-PBDEs had an agonistic effect on TR while higher brominated congeners acted in an antagonistic manner.

The concerns for PBDEs to cause thyroid disruption in humans and wildlife, and recent status listing as POPs have led to the worldwide phase-out of PBDEs from commerce (Dodson et al., 2012; Stapleton et al., 2014). However due to remaining fire safety requirements there has been a steady increase in the production of a number of replacement FRs in the place of PBDEs. Many of the replacement FRs are largely unregulated and with little available information on the environmental fate, occurrence, and toxicity to humans or wildlife (Dishaw et al., 2011; Van den Eede et al., 2013). These are referred to as novel FRs as they have historically been used in small volumes, and/or have only recently started being used. Two types of novel FRs will be discussed herein.

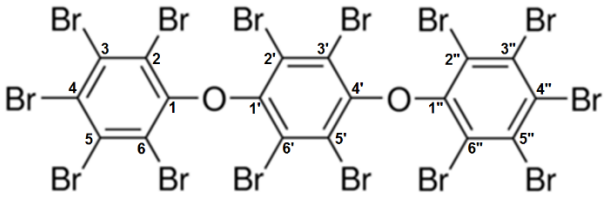
1.2.3 Tetradecabromo-1,4-diphenoxybenzene

Several non-PBDE brominated FRs remain in commerce (Poma et al., 2014; van den Berg, 1990). One of these substances is tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz; CAS No. 58965-66-5), which is a suspected deca-BDE replacement. In fact, the structure of TeDB-DiPhOBz is similar to 2,2′,3,3′,4,4′,5,5′,6,6′-decabromodiphenyl ether (BDE-209; CAS No. 1163-19-5), which is the dominant PBDE congener in the deca-BDE formulation. Both are comprised of fully brominated aromatic rings connected by oxygen atoms, with BDE-209 containing two aromatic rings and TeDB-DiPhOBz containing three (Figure 1-5). These chemicals are both insoluble in

water, and have very low bioavailability with Log K_{ow} values >10 (Howard and Muir, 2011). BDE-209 is known to undergo photolytic debromination leading to increased bioavailability (Su et al., 2014b), and recent photolysis studies have also demonstrated that rapid photolytic debromination of TeDB-DiPhOBz occurs when it is exposed to sunlight irradiation (Chen et al., 2013; Su et al., 2016b, 2014b). The photolysis products of TeDB-DiPhOBz included a variety of brominated DiPhOBz congeners, with the most abundant identified as Br_4- to Br_7-DiPhOBz (Chen et al., 2013). Given the reduction in molecular weight and size, the bioavailability of these lower brominated DiPhOBz compounds is thus increased compared to the parent compound.



BDE-209



TeDB-DiPhOBz

Figure 1-5 Molecular structures of BDE-209 (decabromodiphenyl ether) and TeDB-DiPhOBz (tetradecabromo-1,4-diphenoxybenzene).

TeDB-DiPhOBz is listed as a priority substance for risk assessment under the third phase of the Government of Canada's Chemicals Management Plan (CMP3), although it is currently unregulated worldwide (Environment and Climate Change

Canada, 2015). Several Asian suppliers exist, however, no specific information is available on the use profile of TeDB-DiPhOBz to-date (MHP Chemicals, 2017; TCI Chemicals, 2017). Furthermore, data on the biological fate and effects of TeDB-DiPhOBz and its potential degradation products are extremely limited. Only recently, biomonitoring efforts identified methoxylated (MeO) Br_4- to Br_6-DiPhOBz congeners for the first time in Great Lakes herring gull eggs, and these are suspected metabolite products of TeDB-DiPhOBz (Chen et al., 2012, 2011). The predicted link of these novel contaminants to TeDB-DiPhOBz exposure in gulls is further supported by *in vitro* metabolism studies conducted using both gull and rat microsomes, which confirm that exposure to TeDB-DiPhOBz results in the formation of several phase I Br_4 to Br_7-OH-PB-DiPhOBz metabolites (Su et al., 2016a). The only existing toxicological information on these compounds to-date is an *in vitro* assessment of mRNA expression in chicken embryonic hepatocytes exposed to TeDB-DiPhOBz and lower-brominated photolysis products (i.e., Br_8 to Br_{11} congeners), the latter of which resulted in alterations in CYP1A4 expression (and with similar expression profile to BDE-209 and its photodegradates) (Su et al., 2014b). Currently, nothing is known of potential exposure of humans to TeDB-DiPhOBz, nor any other environmental or biotic media.

1.2.4 Organophosphate triesters

Organophosphate (OP) triesters are a group of novel FRs that have been in use for decades and that have undergone increased production in the wake of PBDE phase-out (van der Veen and de Boer, 2012). These compounds contain a phosphate backbone and three alkyl or aryl moieties, which may be halogenated or non-halogenated. In addition to

their use as FR chemicals, some OP triester compounds are also used in other commercial applications including plasticizers, varnishes and lubricants. Halogenated OP triesters are more often applied as additive FRs, whereas non-halogenated OP triesters are more often used as plasticizers (van der Veen and de Boer, 2012). Due to their widespread use, OP triesters are commonly detected in various biotic and environmental media. Some of the commonly found OP triesters in house dust samples include tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), triphenyl phosphate (TPHP), triethyl phosphate (TEP), and tris(2-butoxyethyl) phosphate (TBOEP), and diester metabolites of each these compounds have also been identified in human urine (e.g., up to 56,080 ng TDCIPP/g dust and 25 ng of the diester bis(1,3-dichloro-2-propyl) phosphate (BDCIPP)/mL urine) (Fromme et al., 2014; Hoffman et al., 2015; Meeker et al., 2013; Schindler et al., 2009; Van den Eede et al., 2015, 2011).

The metabolic pathways of OP triesters in vertebrates are not fully understood to-date, however *in vitro* laboratory tests have also reported the formation of Phase I (involving cytochrome P450 enzymes) diester biotransformation products including for example diphenyl phosphate (DPHP) and *para*-OH-TPHP formed in both human liver hepatocytes and chicken embryonic hepatocytes exposed to TPHP (Figure 1-6) (Su et al., 2014a; Van den Eede et al., 2015). Phase II reactions using S9 fractions (involving glutathione S-transferase enzymes) were also observed to occur for TDCIPP and TBOEP resulting in glutathione conjugates of Phase I products (Van den Eede et al., 2013).

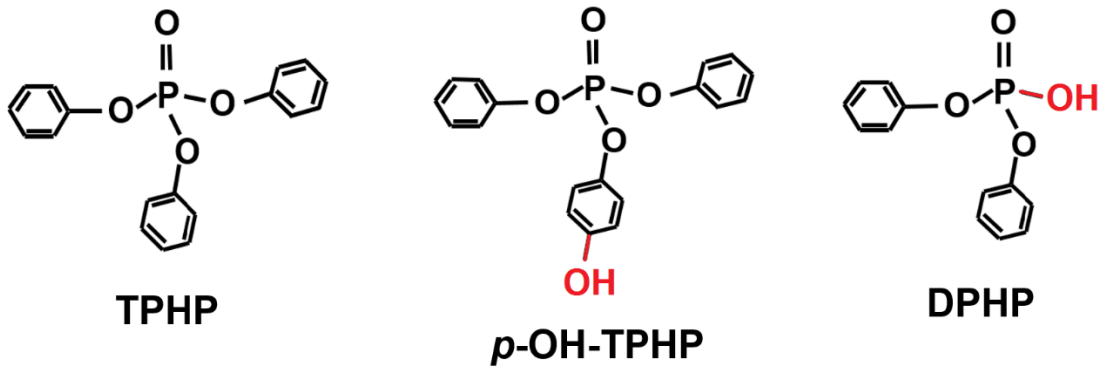


Figure 1-6 Molecular structures of TPHP (triphenyl phosphate) and metabolites *p*-OH-TPHP (*para*-hydroxy-triphenyl phosphate) and DPHP (diphenyl phosphate). Hydrogen atoms have been omitted for clarity.

Exposure of wildlife to various OP triester congeners has been confirmed in several environmental monitoring studies, including sampling herring gulls and fish in the Great Lakes ecosystem (Greaves et al., 2016; Greaves and Letcher, 2014; McGoldrick et al., 2014). For example, TBOEP was detected in the blood of some gulls (e.g., up to 2.25 ng/g in plasma and 4.69 ng/g wet weight in red blood cells), as well as in the eggs indicating maternal transfer (e.g., average of 8.09 ng/g wet weight albumen; 1.89 ng/g wet weight yolk) (Greaves and Letcher, 2014). Similarly, mean TBOEP levels in fish sampled in Lakes Ontario and Erie ranged from 0.71 to 9.40 ng/g wet weight for various species (Greaves et al., 2016).

Concerns of OP triesters to-date include widespread prevalence and persistence in the environment, toxicity to aquatic organisms, and neurotoxicity to wildlife due to their similarities in structure to organophosphate pesticides (Dishaw et al., 2014; van der Veen and de Boer, 2012). With regards to thyroid toxicity, recent studies have identified thyroidogenic effects at various levels of biological activity. Zebrafish embryos and larvae exposed to TDCIPP exhibited developmental toxicity including mRNA expression

in the HPT axis, circulating T4 and occurrence of malformations (Wang et al., 2013). Exposure studies of chicken embryos to various OP triesters have been conducted *in ovo* and with varying observed effects including delayed pipping, reduced growth and free T4 from TDCIPP (Farhat et al., 2013) and TEP (Egloff et al., 2014), as well as induction of hepatic CYP3A37 mRNA from TBOEP (Egloff et al., 2014). Interestingly, a study of human nuclear receptors exposed to select OP triesters found no impacts on human TR *in vitro* (Kojima et al., 2013).

At the time of selection of this study set of OP triesters and metabolites, no previous studies had yet been published on interactions of OP triester FRs with TH transport proteins. Since then two published studies have observed interactions of select OP triesters with human TTR using a similar *in vitro* assay to the present methodology, as well as some *in silico* analyses (Weiss et al., 2015; Zhang et al., 2015). These studies consistently predicted no competition of OP triesters (including the presently-selected TDCIPP, TBOEP, and TPHP) with T4 for binding at the TH binding channel of human TTR *in silico* or *in vivo*. No OP triester metabolite products were tested in these studies.

1.3 Rationale for the research project

There has been increased market use of several novel FRs to meet flammability standards in the wake of PBDE phase-out. Recent biomonitoring studies have detected many of these compounds including OP triesters and replacement brominated FRs in humans and wildlife, as well as in various environmental media (Chen et al., 2011; Dodson et al., 2012). Increasing evidence suggests that some OP triester FRs may have an impact on the thyroid system, and known metabolite products have been identified for

several congeners including e.g. diester and *para*-OH triester conjugates in the case of TPHP (Farhat et al., 2013; Meeker and Stapleton, 2010; Preston et al., 2017; Su et al., 2015a). For this thesis project, a study set of four OP triester FRs was selected, including TDCIPP, TEP, TBOEP, TPHP, as well as two known metabolites of TPHP, diphenyl phosphate (DPHP) and *para*-OH-TPHP. As a secondary study set, degradation products of a deca-BDE replacement FR, TeDB-DiPhOBz, were selected including a tetra-brominated (TB)-DiPhOBz congener, a *para*-OH TB-DiPhOBz, and a *para*-MeO TB-DiPhOBz. Nothing is known about this FR or its degradation products with regards to volume of use, exposure to humans and wildlife (apart from recent findings of MeO-polybrominated-DiPhOBzs in Great Lakes herring gull eggs), or potential toxic effects. The parent compound is not expected to be bioavailable due to its large molecular size, however the lower brominated degradation products are more likely to be bioavailable. The similarity in molecular structure of these compounds to PBDEs raises the concern of a shared mechanism(s) of action, which could include thyroid disruption. The FR compounds selected for testing have been listed as priority substances for human health and ecological risk assessment under phases two and three of the CMP (Environment and Climate Change Canada, 2015).

1.4 Hypothesis and thesis objectives

The null hypothesis for the research conducted in this thesis project is that there will be no observed *in vitro* competition of xenobiotic ligands with T4 for the TH binding sites on human TTR or ALB, and that there will be no structure-related differences in the binding interactions.

The overall goal of this thesis project was to apply a fully optimized and validated *in vitro* competitive protein binding assay with human TTR and/or ALB to investigate the potential thyroidogenicity of several novel FRs and degradation products with varying molecular structures. Two classes of FR contaminants were selected to allow for a qualitative assessment of the variation in competitive binding potency in relation to molecular structure. The two study sets include OP triesters and metabolites, and TB-DiPhOBz derivatives of a novel brominated FR. The latter study also incorporated an *in silico* binding analysis component, using a previously developed 3D homology TTR model for an avian species, the herring gull, to allow for an interspecies comparison of the binding properties of these chemicals.

The selected study compounds, along with predicted results are as follows:

1) Four OP triesters TDCIPP, TEP, TBOEP, TPHP as well as TPHP metabolites DPHP and *para*-OH-TPHP.

Predictions: Some competitive inhibition of T4 binding to human TTR is expected, and in a structure-related manner. The OP triester TPHP and its metabolites, followed by TDCIPP, are expected have higher binding potencies than the other OP triesters due to the presence of phenyl side groups in the case of TPHP (and the hydroxyl group on *p*-OH-TPHP), and the halogenation of TDCIPP.

2) Three tetrabrominated derivatives of the novel brominated FR TeDB-DiPhOBz including one TB-DiPhOBz congener, one *para*-OH TB-DiPhOBz, and one *para*-MeO TB-DiPhOBz each with bromine atoms at positions 2,2',2",4 (Figure 1-5).

Predictions: Some competitive inhibition of T4 binding to human TTR and ALB is expected, due to the structural similarity of these compounds to PBDEs and metabolites. A similar result is expected for the gull TTR homology *in silico* model, however with some differences in predicted binding affinity due to the variation in the affinity for THs between transport proteins of mammals and birds. The *p*-OH-TB-DiPhOBz congener is expected to be a more potent competitor for TH transport proteins than the *p*-MeO-TB-DiPhOBz analog, followed by the TB-DiPhOBz analog.

Notably, the initial project objectives included running *in vitro* tests with recombinant TTR and ALB from the herring gull (*Larus argentatus*; a Great Lakes bioindicator species) in addition to human TH transport proteins, in order to provide an inter-species *in vitro* comparison of protein-contaminant interactions. However, access to recombinant gull TTR and ALB proteins was not available in the timeframe of this thesis work. Additionally, there were major challenges encountered for the competitive binding assay using human TTR and ALB, which required necessary attention for optimization. For example, specific handling and storage conditions for TTR are necessary to prevent the protein from denaturing (and the subsequent loss of the TH binding channel). The result of this effort is a refined and optimized assay methodology which has been submitted to the open access protocols journal *MethodsX*, as described in Chapter 2.

1.4.1 Outline of the research

To achieve the set objectives, this thesis research was divided into three chapters.

Chapter 2: This chapter involved development and optimization of the competitive protein binding assay, initially with human TTR and subsequently with human ALB. A detailed overview of the sample set up procedure, quality control measures and data analysis are described.

Chapter 3: This chapter studied the interaction of the OP triester and metabolite test set with human TTR *in vitro*. The concentration-response curves are presented along with calibration and positive control data, and IC50s, T4-relative potencies, and K_i values were calculated when possible.

Chapter 4: This chapter studied the interaction of the TB-DiPhOBz test set with human TTR and ALB *in vitro*, and with gull TTR *in silico*. The concentration-response curves from the *in vitro* studies are presented along with calibration and positive control data, and IC50s, T4-relative potencies, and K_i values were calculated when possible. The *in silico* scoring values from the gull TTR homology model are presented for the target TB-DiPhOBz ligands, as well as the natural ligands T3 and T4. Predicted interactions of the TB-DiPhOBz compounds with key residues in the gull TTR binding pocket are also described.

2 Chapter: Optimization of an *in vitro* assay methodology for competitive binding of thyroidogenic xenobiotics with thyroxine on human transthyretin and albumin[1]

2.1 Abstract

Thyroid hormones (THs) are involved in the regulation of many physiological processes in vertebrates. Competition for TH binding sites on serum transport proteins can interfere with delivery of THs to target tissues, and this is a potential mechanism of action of exogenous thyroidogenic substances. To date, detailed accounts of *in vitro* methods for competitive binding with THs on TH transport proteins (human or wildlife) are sparse. In the limited number of published studies on *in vitro* radio-labelled TH-TH transport protein interactions, method descriptions were brief and with insufficient details for successful replication. Furthermore, upon review of these methodologies, we identified several opportunities for optimization. The present study addresses the methodological deficiencies and describes, in detail, a fully optimized and validated competitive T4 radio-ligand binding assay with human transthyretin (TTR) and albumin (ALB).

- Significant improvements were made over previous methods, including better maintenance of protein stability, increased protein affinity for ligands, and enhanced measurement of competition between different ligands.

[1] Adapted from Hill, K.L., Hamers T., Kamstra J.H., Willmore, W.G., and Letcher, R.J. Optimization of an *in vitro* assay methodology for competitive binding of thyroidogenic xenobiotics with thyroxine on human transthyretin and albumin. Submitted to the journal *MethodsX* March 2017.

- Sample size was reduced to allow use of small pre-packed size exclusion chromatography columns, which eliminates the rinsing step during the separation procedure.
- The assay was parameterized for use with T4 and human TTR and ALB.

2.2 Background

The structural resemblance of many anthropogenic chemicals to thyroid hormones (THs) warrants concern for the perturbation of TH-dependent processes in humans and other vertebrates (Meerts, 2000). Thyroxine (T4) and triiodothyronine (T3) are THs involved in the regulation of many important physiological processes in the body including neurological and behavioural development, growth, metabolism, and respiration (Richardson et al., 2015). In mammals (including humans), circulating T4 concentrations are greater in plasma than for T3. Thyroxine is considered the "precursor" TH, and it is deiodinated to T3, which is the main ligand for the TH receptor leading to gene regulation (Richardson et al., 2015). The vast majority (> 99 %) of THs in plasma are delivered to target tissues by binding to TH transport proteins, which include albumin (ALB), transthyretin (TTR), and thyroxine-binding globulin (TBG). The TH binding affinities and dissociation constants of these three proteins vary, with ALB having the lowest relative affinity for T4 and the greatest concentration in plasma; and TBG having the highest affinity for T4, and the lowest concentration in plasma (Cody, 2005). Transthyretin is the only TH transport protein synthesized in the choroid plexus, and it thus plays a role in the movement of THs from the blood into the cerebrospinal fluid (CSF) which is particularly important in early development (Richardson et al., 2015).

One potential mechanism of action of xenobiotic thyroidogenic substances is competition for TH binding sites on serum transport proteins, as this can interfere with delivery of THs to target tissues. A limited number of previously published studies report variations of the *in vitro* radiolabelled T4-TTR binding assay technique, with target chemicals including polychlorinated biphenyls, perfluorinated compounds, polybrominated diphenyl ethers, and some degradation products (Hamers et al., 2006; Lans et al., 1993; Meerts, 2000; Somack et al., 1982; Ucán-Marín et al., 2009; Weiss et al., 2009). However, the methods reported in these studies do not provide sufficient details to successfully replicate the procedure and we faced several challenges in attempting to do so. Therefore, the objectives of the present study were to refine, optimize, validate, and fully describe a competitive *in vitro* radio-ligand-protein binding assay that is used to investigate thyroidogenicity of chemicals via interaction between T4 and human TTR. Furthermore, we expanded this method to include interactions between T4 and human ALB which, to our knowledge, has not been adequately described elsewhere. The model xenobiotic chemical that we used as a competitor ligand is 4-hydroxy-2,2',4,5'-tetrabromodiphenyl ether (4-OH-BDE-49), which is often detected in human serum (Stapleton et al., 2011) and has previously been found to bind to human TTR isolated from human plasma with higher affinity than T4 *in vitro* (Ucán-Marín et al., 2009).

A number of other methods have been developed for the purpose of investigating potential competition of xenobiotics with T4 for TTR, including the ANSA (8-anilino-1-naphthalenesulfonic acid ammonium)-TTR competitive fluorescence displacement assay (Montaño et al., 2012), the TR-CALUX (thyroid hormone responsive chemically

activated luciferase gene expression) assay (Bekki et al., 2009), surface plasmon resonance-based biosensor assays (Marchesini et al., 2006), and FITC-T4 (fluorescence probe fluorescein isothiocyanate associated to T4) assays (Ouyang et al., 2016; Ren and Guo, 2012). Notably, Ouyang et al. (2016) presents an enhanced FITC-T4 assay that has been miniaturized into a 96 well microplate to generate a high throughput method. While the miniaturized FITC-T4 method offers increased efficiency, the radiolabelled T4-protein binding assay described herein is more sensitive than the assay presented in Ouyang et al. (2016), and by an order of magnitude for potent xenobiotic competitors (those with IC50s < 100 nM). This is particularly important for the detection of receptor binding events at low concentrations (e.g., those found in blood or other biological samples) and for reducing the likelihood of a type II error. Additionally, our method extends to include interactions with ALB, which increases the relevance of this assay as ALB is the most abundant protein in plasma and serum.

The study system applied herein represents a simplified serum environment, whereby T4 is combined with one TH transport protein, and one of a series of concentrations of an exogenous ligand in a Tris-EDTA buffer at pH 8.0. This allows for isolated measurement of the ligand competition for the specific TH and protein. The concentration of T4 used in the *in vitro* assay was close to the normal range of total T4 in an adult human (55 nM T4 + 0.5 nM ^{125}I-T4, compared to a normal range of 60 to 140 nM in human serum). Concentrations of TTR and ALB used herein however are lower than the concentrations typically found in human blood. In human serum TTR concentrations may range from 2,800 to 7,400 nM, compared to 30 nM used in the *in vitro* assay. Likewise, the concentration of ALB in human serum generally ranges from

540,000 to 770,000 nM, compared to 600 nM used in this assay. Thus the IC50 values generated in the assay do not necessarily directly correlate to concentrations that would exhibit 50% TH-protein inhibition in human serum. For example, using a similar assay Weiss et al. (2009) compared the T4-TTR IC50 with 30 nM TTR as well as 60 nM TTR. The IC50 of T4 was increased when the TTR concentration was doubled *in vitro* (Weiss et al., 2009). However, the relative potencies derived herein by comparing the *in vitro* IC50s of exogenous ligands to those of the natural ligands are expected to provide a reliable indication of potential competition *in vivo*.

2.3 Sample preparation

The entire assay takes two days to complete for each batch of samples to be processed. The sample preparation occurs on Day 1 and is described as follows. Prepare the solutions of the proteins, THs and competitive ligands to be used in the *in vitro* assay according to Table 2.1 and using materials specified in Table 2.2. First, thaw the working solutions of these components to room temperature if they have been placed in frozen storage. For the protein working solutions, prepare the day they are to be used based on the number of assay aliquots required (one vial is sufficient for approximately 45 assay samples). Take note of the protein handling recommendations in Section 2.5 Precautions and tips. Label a series of polypropylene test tubes corresponding to the samples being prepared. This should include triplicate samples for each ligand competitor concentration. See Figure 2-1 for an example of sample set up for a batch of assays including vial labels and contents.

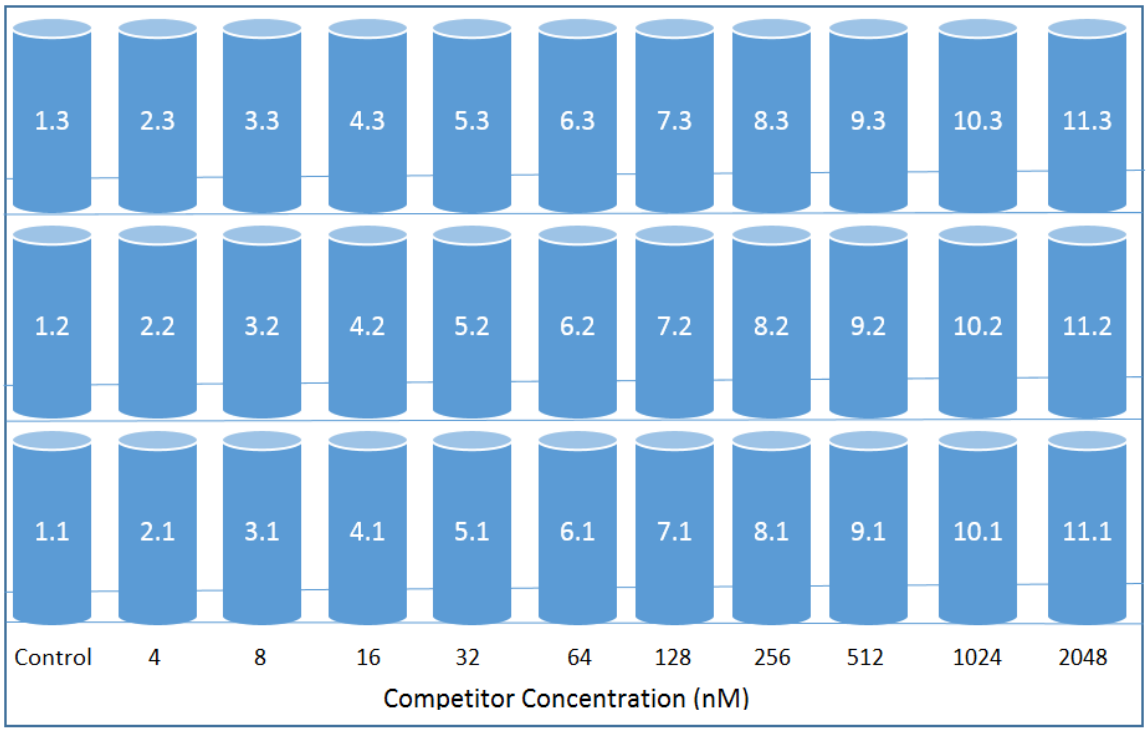


Figure 2-1 Example of the sample set up procedure, including vial labels and concentrations of thyroxine (T4) as a competitor ligand for the calibration exercise.

Prepare the T4/^{125}I-T4/Tris-EDTA buffer solution using one polypropylene test tube per triplicate set of assays. For each, pipette 20 µL of T4 working solution, 200 µL ^{125}I-T4 of stock solution (see Section 2.5 Precautions and tips for details on signal strength required), and 2,580 µL of Tris-EDTA into the test tube(s) and invert to mix (resulting in the addition of 0.5 µL T4, 5 µL ^{125}I-T4, and 64.5 µL Tris-EDTA to each sample).

The following steps describe the order of pipetting components for assay incubation mixtures.

1. Starting with the calibration curve samples (T4 as a competitor), pipette 5 µL of the lowest competitor concentration into all replicate assays. Continue for each increasing concentration of the ligand. Control samples receive 5 µL of DMSO in place of a competitor ligand.
2. For the first "replicate set" (e.g., 1.1 to 11.1 in Figure 2-1), pipette 70 µL of the T4/^{125}I-T4 solution into each test tube, followed by 25 µL of the selected protein (e.g., TTR). This results in a total sample volume of 100 µL.
3. Centrifuge the samples at 300 x g for 30 seconds to force the solution to the bottom of the tube.
4. Measure the initial radioactivity on the gamma counter. Cap the samples immediately, being careful not to disturb solutions, and place in the fridge at 4˚C overnight, which will facilitate reaching protein-ligand binding equilibrium.

While the first replicate set is on the gamma counter, repeat Steps 2 to 4 for the subsequent replicate set(s) (e.g., 1.2 to 11.3). Follow the same preparation sequence for the xenobiotic competitor(s) being analyzed (e.g., 4-OH-BDE-49).

Table 2.1 Preparation and storage details for the stock and final concentrations of the ligands and transport proteins for the thyroid hormone competitive binding assay.

Component	Stock Solution			Working Solution			Final Concentration in Sample
	Concentration	Preparation	Storage	Concentration	Preparation	Volume Added to Sample	
Protein (Select One)							
TTR	3.64 µM	Dissolve 0.5 mg into 2,500 µL Tris-EDTA by inversion and portion into 40 µL aliquots	1,500 µL polypropylene microcentrifuge tubes at -20˚C for up to one year	120 nM	Add 1,160 µL Tris-EDTA to aliquot on day of use	25 µL	30 nM
ALB	72.8 µM	Dissolve 12.1 mg into 2,500 µL Tris-EDTA by inversion and portion into 40 µL aliquots	1,500 µL pp tubes at -20˚C for up to one year	2,400 nM	Add 1,160 µL Tris-EDTA to aliquot on day of use	25 µL	600 nM
Natural Ligand							
T4	1,000 µM	Dissolve 5 mg into 6,435 µL DMSO in a test tube by gentle vortex and transfer to storage vessels	1,500 µL pp tubes at -20˚C for up to three years	11,000 nM	Add 11 µL stock solution to 989 µL DMSO	70 µL (0.5 µL T4, 5 µL ^{125}I-T4, and 64.5 µL Tris-EDTA per sample)[a]	55 nM + ~5%[b]
^{125}I-T4	0.0536 µM (1,000 µCi/g)	Transfer contents of ampoule to storage vessel	1,500 µL pp tube at 4˚C for up to two months	(use stock)			
Competitor Ligand (Select One)							
T4	(see above)			20 to 40,960 nM	Perform two-fold dilution series from 40,960 to 20 nM using T4 stock to prepare standards for calibration curve	5 µL	1 to 2,048 nM
4-OH-BDE-49	19.9 µM	Conduct a solvent exchange for DMSO using nitrogen evaporator	2,000 µL amber glass vials at 4˚C for several years	5 to 5,120 nM	Perform two-fold dilution series from 5 to 5,120 nM	5 µL	0.125 to 256 nM[d]
						Total Volume Per Sample = 100 µL	

[a] See Section 2.3 Sample preparation for details.
[b] Concentration of ^{125}I-T4 varies depending on decay schedule (see Section 2.5 Precautions and tips).
[c] Concentration range of 4 to 2,048 nM for TTR; 1 to 1,024 nM for ALB.
[d] Concentration range of 0.25 to 256 nM for TTR; 0.125 to 128 nM for ALB.

Table 2.2 Description of the reagents, materials and equipment required to conduct the thyroid hormone competitive binding assay.

Component	Details	Source
Reagents		
Transthyretin (TTR)	Lyophilized from human plasma (≥95%)	Sigma-Aldrich P1742
Albumin (ALB)	Lyophilized from human plasma (≥99%)	Sigma-Aldrich A8763
L-thyroxine (T4)	(≥98%)	Sigma-Aldrich T2376
Radio-labeled L-thyroxine (^{125}I-T4)	50 µCi (800 – 1000 µCi/g)	MP Biomedicals 07190128
4'-hydroxy-2,2',4,5'-tetrabromodiphenyl ether (4-OH-BDE-49)	10 µg/mL in acetonitrile (97.8%)	Chromatographic Specialties Inc. AHBDE4002SCN02X
Tris-EDTA buffer solution	10 mM Tris-HCL, 1 mM EDTA, pH 8.0	Sigma-Aldrich 93283
Materials		
Amber glass vials	2 mL with lids	Chromspec C779100A
Eppendorf® LoBind microcentrifuge tubes	1.5 mL with attached caps	Sigma-Aldrich Z666505
Polypropylene test tubes	12 x 75 mm with lids	Sigma-Aldrich T1911
Bio-Spin® P-6 Gel Columns	Tris buffer, sample volume 50-100 µL	Bio-Rad 732-6228
Equipment		
Eppendorf® pipettes various sizes with corresponding tips		
Nitrogen evaporator		
Fridge (4°C) and freezer (-20°C)		
Temperature controlled centrifuge (4°C)		
Gamma counter in radioactivity-licensed laboratory (e.g., Packard Cobra II)		
Analytical balance		

2.4 Separation procedure

The separation procedure occurs on Day 2 of the assay. Label a series of clean test tubes corresponding to the samples prepared on Day 1 and repeat these labels on Bio-Spin® columns. Work on one replicate set at a time, leaving the remaining columns and incubation mixtures in the fridge until ready for processing. Prepare spin columns for use according to Bio-Spin® column instructions (invert column to re-suspend gel, remove cap and tip, centrifuge in test tube at 1,000 x g for 2 minutes, discard drained packing buffer). Process the incubation mixtures as follows:

1. Extract the first sample of the replicate set from the incubation tube using a 100 µL pipette and load the sample to the head of the corresponding column, being careful not to disturb the gel bed. Release the emptied pipette tip into the incubation tube.
2. Repeat for each sample in the replicate set, and centrifuge samples for 4 minutes at 1,000 x g.
3. Measure the radioactivity of the eluate fraction, as well as the residual radioactivity in the incubation tubes containing the discarded pipette tips.

Repeat Steps 1 to 3 for each of the remaining replicate sets. The separation procedure involves size exclusion chromatography, whereby the protein-bound ligand is eluted from the Bio-Spin® columns and unbound ligand remains in the columns. The size exclusion limit of the chromatography columns is 6,000 g/mol, and thus any compound smaller than this size will travel through the polyacrylamide beads (a longer route

through the column) while larger molecules will move around the beads (a shorter route through the column). Measuring the radioactivity of the emptied incubation tubes and pipette tips accounts for nonspecific binding and any potential transfer loss.

Note: Previous versions of this method (Hamers et al., 2006; Meerts, 2000; Ucán-Marín et al., 2009; Weiss et al., 2009) suggest an additional step involving rinsing of the columns with Tris buffer, followed by a second round of centrifugation to elute all protein-bound ligand. This step was found to be unnecessary as the present method is optimized to the sample volumes and Bio-Spin® columns used.

2.5 Precautions and tips

Transthyretin is a labile substance and is susceptible to denaturation. It is thus important to handle proteins with care when preparing solutions and processing samples to ensure they remain intact. Do not vortex solutions containing TTR. Prepare working solutions of TTR or ALB only on the day of use to ensure that the protein structural integrity is maintained. Additionally, keep solutions containing proteins on ice during sample preparation and processing also to prevent denaturing. Incubation mixtures should be left in the fridge until ready for processing, and analyzed quickly so as to reduce time on the column. Use a temperature controlled centrifuge set to 4˚C to maximize protein integrity. Furthermore, it is recommended that Eppendorf LoBind® Polypropylene test tubes be used to prepare incubation samples, in order to minimize potential loss of proteins and ligands from adsorption to the vessel.

Care must also be taken when handling radiolabelled thyroxine. All components of the assay involving ^{125}I-T4 must be completed in a radioactivity-licensed laboratory by

trained personnel. Wear the necessary personal protective equipment, and work under a fume hood for all steps involving pipette work. The proportion of ^{125}I-T4 added to samples can be varied depending on signal strength, aiming for >10,000 cpm per sample for initial readings. Adjust the amount of Tris-EDTA added to each sample accordingly.

2.6 Data analysis

Calculate the percent of protein binding for each sample by dividing the radioactivity of eluate on Day 2 by the initial radioactivity measured on Day 1, minus any transfer loss (i.e., % binding = eluate cpm / [Day 1 cpm – cpm of emptied incubation tube and pipette tip]). Express results with the logarithmic competitor concentration on the X-axis, and mean ± standard deviation of percent binding compared to controls on the Y-axis. Generate 50 % inhibition concentrations (IC50s) for each competitor in GraphPad Prism 7.02 (GraphPad Software, Inc., La Jolla, CA, USA), using the sigmoidal dose-response equation "log[inhibitor] vs. response – variable slope", defined as:

$$Y = Bottom + (Top - Bottom) / (1 + 10^{(LogEC50 - X)*Hillslope})$$

The inhibition constant (K_i) for each competitor can be determined based on Yung-Chi and Prusoff (1973) and following GraphPad guidance (Motulsky, 1996). In a homologous assay (i.e., with T4 as the competitor) the cold ligand and radioligand can be assumed to have the same binding affinities. As such, the K_i and the equilibrium dissociation constant (K_d) for T4 are determined using the IC50 of T4 and the concentration of radioligand in samples:

$$(K_i)_{T4,\ 125I\text{-}T4} = (K_d)_{T4,\ 125I\text{-}T4} = (IC50)_{T4} - [^{125}I\text{-}T4]$$

With these parameters defined, the K_i of other competitors can be determined using the following equation:

$$(K_i)_{competitor} = (IC50)_{competitor} / (1 + [^{125}I\text{-}T4] / (K_d)_{125I\text{-}T4})$$

A simplified method for calculating K_i is as follows:

$$(IC50)_{T4} / (IC50)_{competitor} = (K_i)_{T4} / (K_i)_{competitor}$$

The relative potency for a competitor is defined as $(IC50)_{T4} / (IC50)_{competitor}$, and thus the above calculation can be re-arranged as:

$$(K_i)_{competitor} = (K_i)_{T4} / (\text{relative potency})_{competitor}$$

Note that at low concentrations of radioligand, the K_i for a competitor approximates the corresponding IC50, which is the case herein.

For competitors that do not exhibit a full *in vitro* dose-response competition curve, conduct an analysis of variance (ANOVA) in GraphPad based on percent of specific binding, and followed by Dunnett's test to determine which concentration(s) of a competitor ligand resulted in significant differences from controls.

2.7 Method validation

A method calibration curve should be generated in duplicate each time an assay batch is performed, using the series of prepared concentrations of the natural ligand as the competitor (e.g., 4 to 2,048 nM T4 for TTR, plus negative controls). To ensure instrument performance, the gamma counter should be calibrated prior to each use, and instrument blanks (i.e., empty test tubes) can be included at various points during measurement of sample radioactivity. Serving as a positive control, each assay should include triplicate samples with the IC50 concentration of a known potent competitor. For

example, 4-OH-BDE-49, 4-OH-CB-187, (CAS No. 158076-68-7) and tetrabromobisphenol A (TBBPA, CAS No. 79-97-4) have previously been identified as potent ligands for TTR (Hamers et al., 2006; Meerts, 2000; Ucán-Marín et al., 2009). Negative controls should also be included for each assay batch, where the competitive ligand is absent and replaced with only DMSO. An additional method validation test that can be included in the assay procedure is a test of column performance using a sample prepared as a negative control but containing Tris-EDTA buffer in place of TTR. With this control, the ligands are in an unbound state and therefore should remain in the column after centrifugation, resulting in background radioactivity in the eluate. All assays should include triplicate samples, and should be conducted twice on separate days to include inter-and intra-day replicates (n=6 total). Dose-response curves with the negative control (DMSO) and positive control (4-OH-BDE-49) were generated with T4-TTR interactions as well as T4-ALB interactions (Figure 2-2).

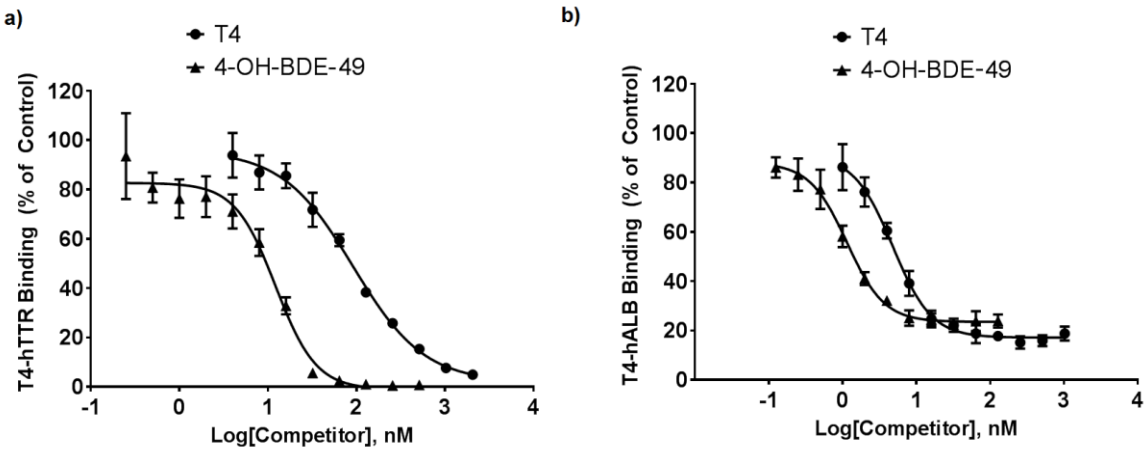


Figure 2-2 Ligand binding curves generated in GraphPad Prism for T4 (thyroxine) interactions with a) TTR (transthyretin) and b) ALB (albumin) in competition with the natural ligand T4 and the positive control 4-OH-BDE-49 (4'-hydroxy-2,2',4,5'-tetrabromo diphenyl ether). Results are

presented as relative percent of T4 bound to TTR compared to controls (means ± standard deviations; six replicates for each concentration tested).

Inter-laboratory reproducibility of this *in vitro* assay was confirmed by comparing T4-TTR calibration data produced in our National Wildlife Research Centre (Ottawa, Canada) laboratory to those produced in the laboratory of Dr. Timo Hamers (Department of Environment & Health, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands) (Figure 2-3).

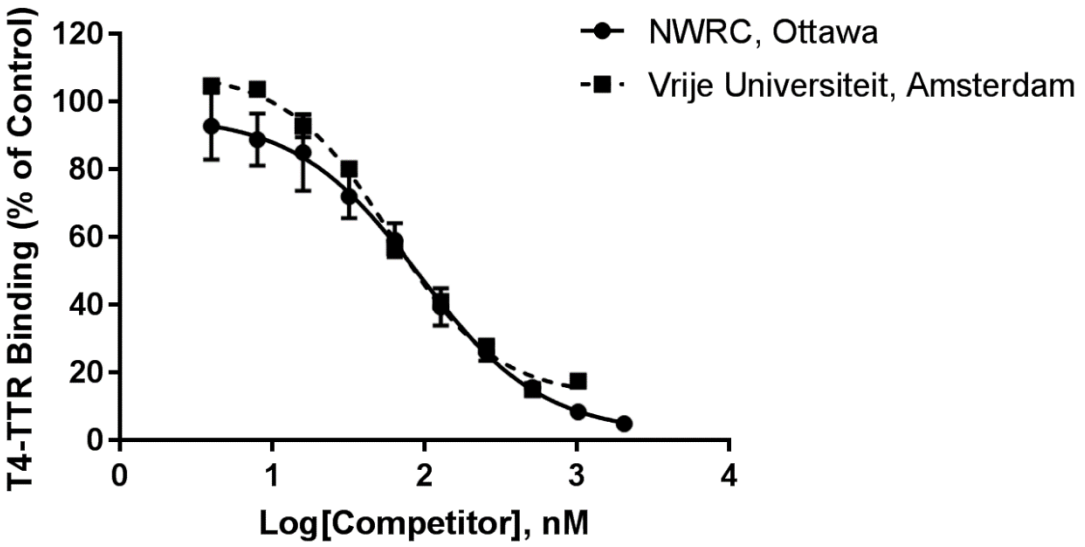


Figure 2-3 Ligand binding curves generated in GraphPad Prism showing the inter-laboratory reproducibility of thyroxine (T4)-transthyretin (TTR) calibration for results produced in our NWRC (Ottawa, Canada) laboratory (n=6) compared to the results produced in the laboratory at the Vrije Universiteit Amsterdam (Amsterdam, The Netherlands) (n=2). Results are presented as relative percent of T4 bound to TTR compared to controls (means ± standard deviations).

3 Chapter: Organophosphate triesters and selected metabolites enhance binding of thyroxine to human transthyretin *in vitro*[2]

3.1 Abstract

The toxicological properties of organophosphate (OP) triesters that are used as flame retardants and plasticizers are currently not well understood, though increasing evidence suggests they can affect the thyroid system. Perturbation of thyroid hormone (TH) transport is one mechanism of action that may affect thyroid function. The present study applied an *in vitro* competitive protein binding assay with thyroxine (T4) and human transthyretin (TTR) to determine the potential for the OP triesters, TDCIPP (tris(1,3-dichloro-2-propyl) phosphate), TBOEP (tris(butoxyethyl) phosphate), TEP (triethyl phosphate), TPHP (triphenyl phosphate), *p*-OH-TPHP (*para*-hydroxy triphenyl phosphate), and the OP diester DPHP (diphenyl phosphate) to competitively displace T4 from TTR. Enhancement of T4 binding to TTR, rather than the hypothesized competition, was observed for the OP triesters and DPHP and in a concentration-dependent manner. For example, T4-TTR binding was significantly increased at concentrations of TBOEP as low as 64 nM, and up to 184 % of controls at 5,000 nM. A plausible explanation of these results, which to our knowledge has not been previously reported, may be allosteric interactions of the OP esters with TTR allowing T4 to access the second site of the TH binding pocket. These *in vitro* results suggest a novel

[2] Adapted from Hill, K.L., Hamers T., Kamstra J.H., Willmore, W.G., and Letcher, R.J. Organophosphate triesters and selected metabolites enhance binding of thyroxine to human transthyretin *in vitro*. Submitted to the journal *Toxicology Letters* September 2017.

mechanism of OP ester toxicity via T4 binding enhancement, and possible dysregulation of T4-TTR interactions.

3.2 Introduction

Organophosphate (OP) triesters are commonly used as plasticizers and as additive flame retardants (FRs) for a variety of products including foams, textiles, electronics, and waxes (van der Veen and de Boer, 2012). With the phase-out and international regulation of polybrominated diphenyl ether (PBDE) FRs, the production and use of several OP triesters as FR chemicals has been increasing in recent years (United Nations Environment Programme, 2017). As of 2004, 14% of the 1.5 million tonnes of global FR use was attributed to OPs (Schindler et al., 2009). For example, tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) is being used as an alternative for penta-BDE in flexible polyurethane foam (PUF), and this OP triester was recently identified as one of the most prevalent FRs in baby products containing PUF (Stapleton et al., 2011).

Organophosphate triesters are susceptible to leaching from the products they are applied to due to abrasion and volatilization (Bergman et al., 2012; Marklund, 2005), and levels of OP triesters measured in house dust commonly exceed those of PBDEs (Meeker and Stapleton, 2010). Humans are primarily exposed through dermal absorption and inhalation of dust particles in indoor environments, and several biomonitoring studies have found detectable concentrations of OP triesters and (OP diester) metabolites in urine and hair samples (Butt et al., 2014; Cequier et al., 2014; Hoffman et al., 2015; Kucharska et al., 2015; Qiao et al., 2016; Su et al., 2016c, 2015c). Consequently, leached OP triesters and degradation products are released into aquatic environments. A food web

study of Lake Ontario and Lake Erie in 2010 detected OP triesters in several fish species as well as in herring gull eggs collected at various sites (Greaves et al., 2016). Organophosphate esters are also capable of long-range transport and have been measured in atmospheric air samples including across the North American Great Lakes (Salamova et al., 2013) and even the European Arctic (Salamova et al., 2014).

The mechanism(s) of toxicity of OP triesters are not well understood to date, however several congeners have been identified as potential neurotoxicants (Meeker and Stapleton, 2010), and TDCIPP is a suspected carcinogen (California Environmental Protection Agency, 2011; World Health Organization, 1998). Increasing evidence suggests OP esters may impact the thyroid system, including recent studies on zebrafish (Kim et al., 2015; Wang et al., 2013; Xu et al., 2015) and chicken (Crump et al., 2012; Egloff et al., 2014; Farhat et al., 2014, 2013). Additionally, biomonitoring studies of human urine and serum thyroid hormone (TH) levels have identified positive associations between triphenyl phosphate (TPHP) exposure and total thyroxine (T4) levels in women (Preston et al., 2017), as well as triiodothyronine (T3) levels in men (Meeker et al., 2013), based on urinary levels of the diester metabolite diphenyl phosphate (DPHP).

The thyroid system serves a multitude of important functions in vertebrate organisms, including regulation of metabolism in adults as well as physiological and neurodevelopment in young (Zoeller et al., 2007). Thyroid hormones T3 and T4 (where T4 is the major circulating TH in mammals) are delivered to target cells via transport proteins transthyretin (TTR), albumin (ALB), and thyroxine binding globulin (TBG), and TTR is particularly important as it is capable of crossing the placental and blood-brain barriers (Vieira and Saraiva, 2014). Several environmental contaminants have been

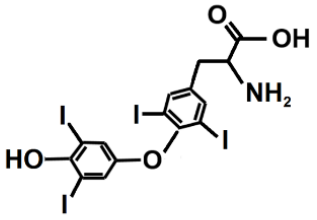
previously identified as competitive ligands for TTR, including PBDEs and polychlorinated biphenyls (PCBs), and especially their hydroxylated, lower halogenated metabolite products (Hamers et al., 2006; Lans et al., 1993; Ucán-Marín et al., 2009; Ućan-Marin et al., 2010). Competitive displacement of T4 from TTR is a mechanism of toxicity of potential concern for xenobiotic chemicals. The objectives of the present study were thus to investigate the potential thyroidogenicity of a suite of OP triesters and some known metabolite products that are relevant for human exposure and as environmental contaminants, using an *in vitro* competitive protein binding assay with T4 and human TTR.

3.3 Materials and methods

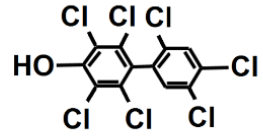
3.3.1 Reagents and chemicals

Tris-EDTA, non-radiolabelled thyroxine (T4; ≥ 98 % purity), and lyophilized transthyretin isolated from human plasma (TTR; ≥ 95% purity) were obtained from Sigma-Aldrich (Oakville, ON, Canada). The radioligand ^{125}I-labelled T4 (50 μCi) was obtained from MP Biomedicals (Solon, OH). The polychlorinated biphenyl (PCB) metabolite 4-hydroxy-2,2',3,4',5,5',6-heptachloro-biphenyl (4-OH-CB-187; > 98 % purity) was obtained from Wellington Laboratories (Guelph, ON, Canada). The OP esters, tris(butoxyethyl) phosphate (TBOEP; 94 % purity), triethyl phosphate (TEP; 99 % purity), triphenyl phosphate (TPHP; 99 % purity), and diphenyl phosphate (DPHP; 99 % purity) were obtained from Sigma-Aldrich (St. Louis, MO). Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP; > 95 % purity) was obtained from TCI America (Portland, OR), and

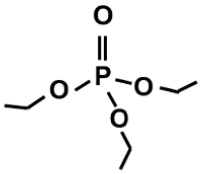
para-hydroxy triphenyl phosphate (*p*-OH-TPHP; 98 % purity) was generously donated by Dr. Heather Stapleton (Duke University, DC) (Figure 3-1).



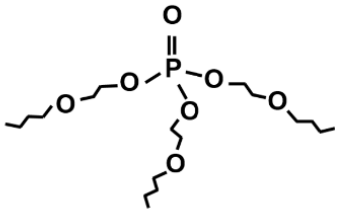
T4
(thyroxine)



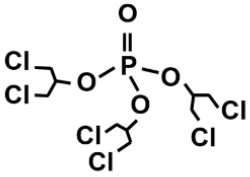
4-OH-CB-187
(4-hydroxy-2,2',3,4',5,5',6-heptachlorobiphenyl)



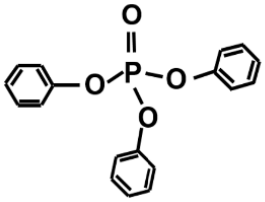
TEP
(triethyl phosphate)



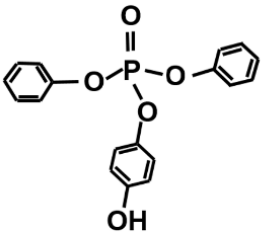
TBOEP
(tris(butoxyethyl)phosphate)



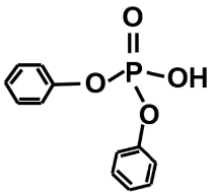
TDCIPP
(tris(1,3-dichloro-2-propyl) phosphate)



TPHP
(triphenyl phosphate)



***p*-OH-TPHP**
(para-hydroxy triphenyl phosphate)



DPHP
(diphenyl phosphate)

Figure 3-1 Molecular structures of the compounds included in the dataset including the natural ligand T4 (thyroxine), the positive control hydroxy-polychlorinated biphenyl metabolite 4-OH-CB-187, and the selected OP (organophosphate) esters. Hydrogen atoms have been omitted for clarity.

3.3.2 Competitive binding assay procedure

The competitive TH binding assay procedure, including reagent preparation, sample set up and separation, as well as data analysis are described in Chapter 2. This study focused solely on interactions with TTR as the ALB component was not developed at the time of these experiments. The PCB metabolite 4-OH-CB-187 was used as a positive control, which has been identified as having a higher affinity for TTR over T4 using a similar assay (Ucán-Marín et al., 2009).

3.4 Results and discussion

An IC50 of 82.8 nM was calculated for the natural ligand T4 and TTR (Table 3.1). This value falls within the range of previously reported IC50s for T4-TTR of 50.9 to 91.5 nM, based on similar assays (Hamers et al., 2006; Lans et al., 1993; Meerts, 2000; Ucán-Marín et al., 2009; Weiss et al., 2009). The positive control 4-OH-CB-187 was a more potent competitor for TTR than T4, with an IC50 of 15.0 nM (Table 3.1, Figure 3-2). This is consistent with the IC50 of 12.5 nM reported for 4-OH-CB-187 from a comparable assay by Ucán-Marín et al. (2009), which was conducted with recombinant herring gull TTR and T4. The resulting potency ratio for the positive control 4-OH-CB-187 is 5.52, which was calculated by dividing the IC50 of T4 by the IC50 of 4-OH-CB-187 (Table 3.1).

Table 3.1 IC50 values and model parameters calculated for interactions of the natural ligand T4 (thyroxine) and the positive control 4-OH-CB-187 (4-hydroxy-2,2',3,4',5,5',6-heptachloro-biphenyl) with transthyretin (TTR) and T4. See Figure 3-1 for the chemical structures of the competitor ligands.

Parameter	T4 Result	4-OH-CB-187 Result
IC50 (nM)	82.8	15.0
Relative Potency[a]	1	5.52
R^2	0.98	0.97
Hill Slope	-1.11	-2.67
Maximum Competition (%)[b]	97.1 ± 1.01	99.7 ± 0.344
Highest Concentration Tested (nM)	2,048	128

[a] Calculated by dividing T4 IC50 by competitor IC50.
[b] Maximum competition occurred at the highest concentration tested.

Hill slope values of -1.11 and -2.67 were generated for T4 and 4-OH-CB-187, respectively (Table 3.1). The slope informs as to whether a compound is interacting with one or more sites on the substrate. A slope of -1 or steeper indicates a single binding site is involved, and a slope shallower than -1 (i.e., closer to 0) indicates the potential for binding to multiple sites. There are two T4 binding sites on TTR in the central channel of the dimer-dimer interface, however the results herein suggest negative cooperativity is observed at these sites. This finding is consistent with previous studies of T4-TTR interactions (Cianci et al., 2015; Lima et al., 2010).

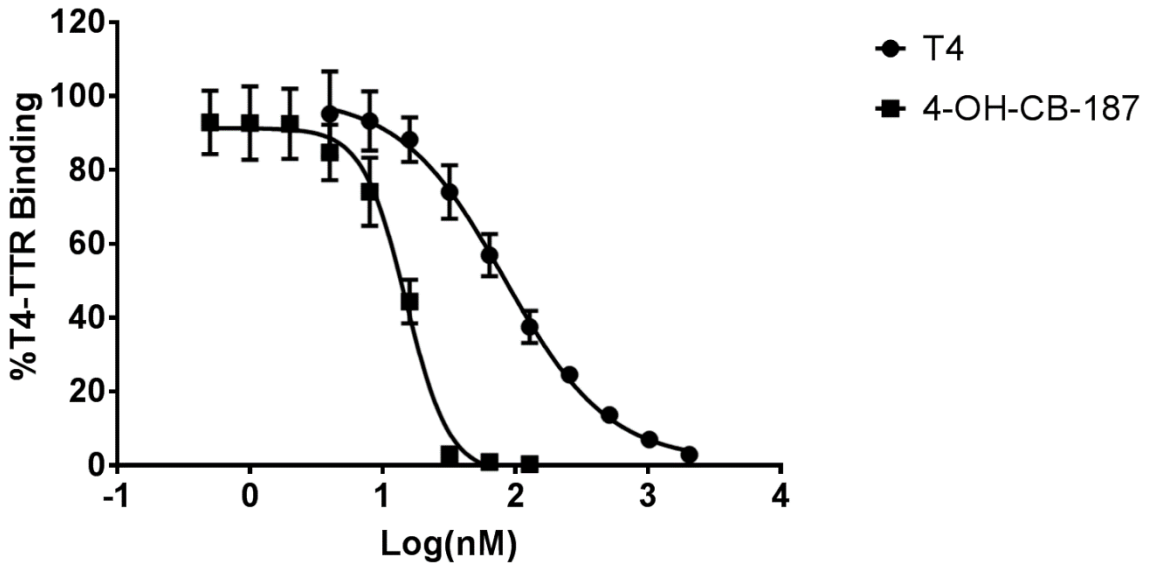


Figure 3-2 Ligand binding curves generated in GraphPad Prism for T4 (thyroxine) interactions with TTR (transthyretin) and in competition with the natural ligand T4 and the positive control 4-OH-CB-187 (4-hydroxy-2,2',3,4',5,5',6-heptachloro-biphenyl). Results are presented as relative percent of T4 bound to TTR compared to controls (means ± standard deviations; six replicates for each concentration tested).

The OP ester experiments were conducted in parallel with the T4 and 4-OH-CB-187 assays. However, the results of the OP triester and diester assays indicate no competitive displacement of T4 from TTR for any of the compounds tested. Rather, the relative percent of T4-TTR binding compared to controls increased in the presence of each OP ester in a concentration-dependent manner (Figure 3-3). Significant differences from controls were found at as low as 64 nM, where TBOEP at this concentration resulted in 119 % T4-TTR binding compared to controls. The greatest relative increase in T4-TTR binding was also observed for TBOEP, with 184 % of controls at 5,000 nM. At concentrations of 512 nM (and up to the highest tested concentrations of 2,048 or 5,000 nM), all six OP esters significantly increased T4-TTR binding compared to controls.

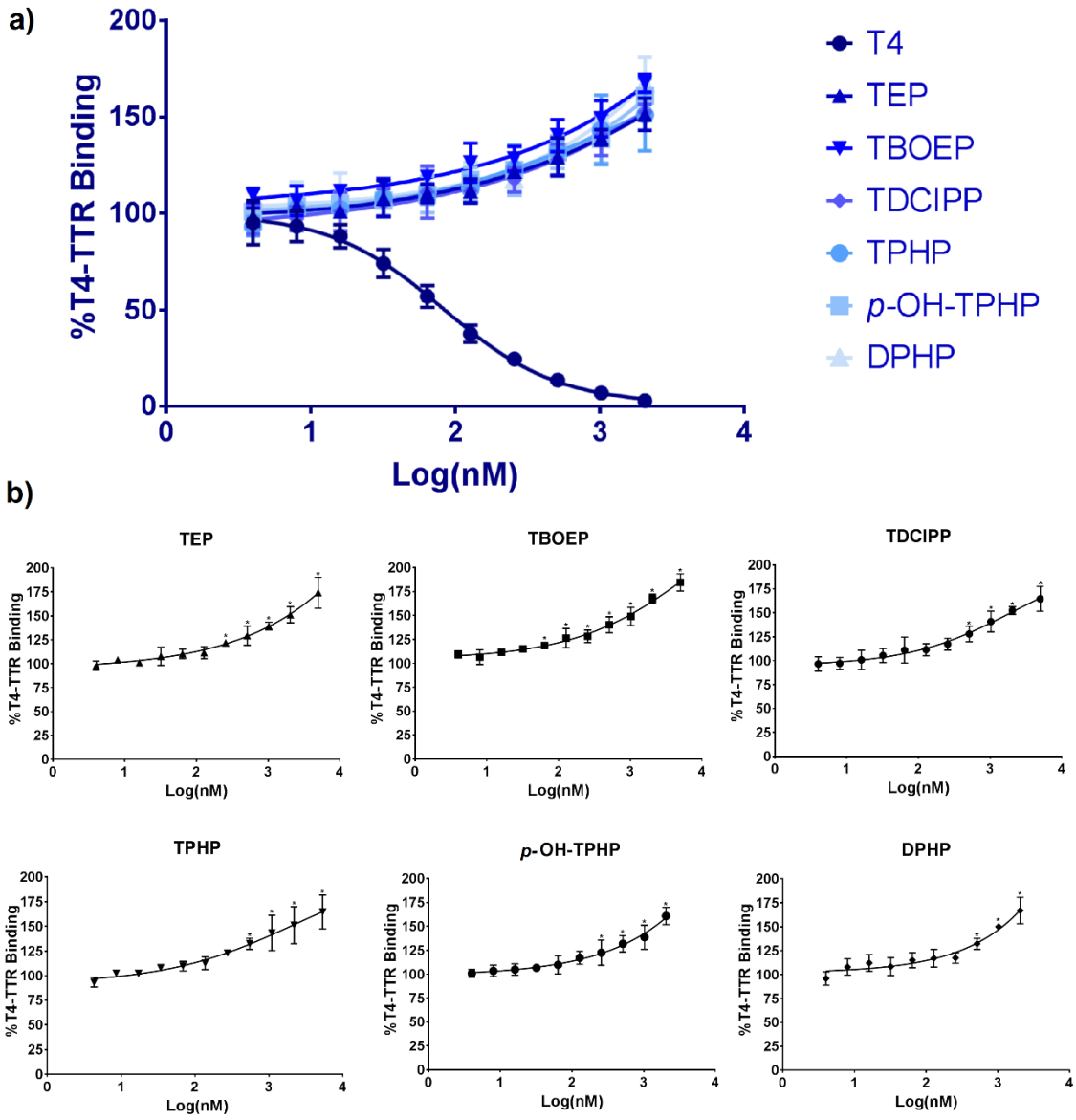


Figure 3-3 Ligand binding curves generated in GraphPad Prism displaying a) the combined results including interactions of the natural ligand T4 (thyroxine) as well as the various OP (organophosphate) esters with T4 and TTR (transthyretin), and b) the individual ligand binding curves for each OP ester tested. Ligands include TDCIPP (tris(1,3-dichloro-2-propyl) phosphate), TBOEP (tris(butoxyethyl) phosphate), TEP (triethyl phosphate), TPHP (triphenyl phosphate), *p*-OH-TPHP (*para*-hydroxy triphenyl phosphate), and DPHP (diphenyl phosphate) (see Figure 3-1 for the chemical structures). Results are presented as relative percent of T4 bound to TTR compared to

controls (means ± standard deviations; three replicates for each concentration tested). Significant differences from controls are indicated by * ($P \leq 0.05$).

Specific binding of T4 to TTR for various study concentrations up to 2,048 nM of OP ester and for controls are displayed in Figure 3-4. The mean specific binding of T4 to TTR in control samples for each ligand assay ranged from 6.26 ± 0.22 % to 7.60 ± 0.33 %. This indicates consistent results between the controls for each OP triester assay that was conducted. Additionally, the level of specific binding in controls is within the range of what is expected for a ligand binding assay (< 10 % based on the assumption of no ligand depletion; Motulsky, 1996). The highest specific binding observed in the OP ester tests was 12.7 ± 1.05 % for DPHP at 2,048 nM. At 5,000 nM, specific binding in the OP triesters ranged from 10.5 ± 1.11 % for TPHP to 12.0 ± 0.59 % for TBOEP (Table 3.2).

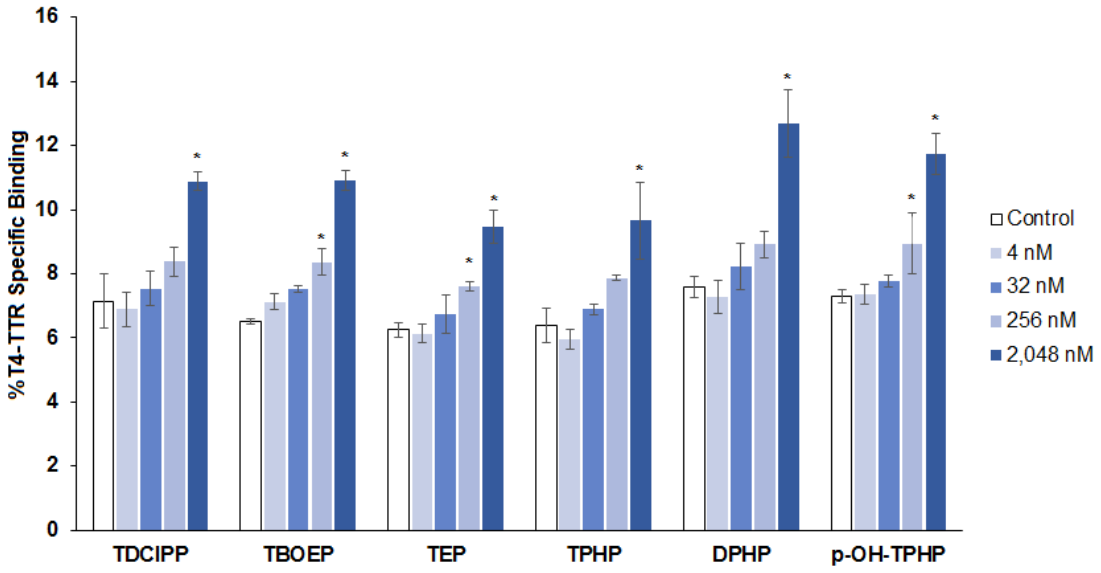


Figure 3-4 Specific binding of T4 (thyroxine) and TTR (transthyretin) at various concentrations of OP (organophosphate) esters. Ligands include TDCIPP (tris(1,3-dichloro-2-propyl) phosphate), TBOEP (tris(butoxyethyl) phosphate), TEP (triethyl phosphate), TPHP (triphenyl phosphate), DPHP (diphenyl phosphate), and *p*-OH-TPHP (*para*-hydroxy triphenyl phosphate). Results are presented as

means ± standard deviations, with three replicates for each OP ester concentration tested. Significant differences from controls for each test indicated by * ($P \leq 0.05$).

Table 3.2 Percent of total specific binding, and percent of T4 (thyroxine) bound to TTR (transthyretin) compared to controls, in the presence of various concentrations of each OP (organophosphate) ester. Results are presented as means of triplicate samples. See Figure 3-1 for the chemical structures of the competitor ligands. Significant differences from control values are indicated with asterisks.

Conc. (nM)	TEP		TBOEP		TDCIPP		TPHP		*p*-OH-TPHP		DPHP	
	SB	RB	SB	RB	SB	RB	SB	RB	SB	RB	SB	RB
0	6.26 ± 0.22	—	6.52 ± 0.09	—	7.15 ± 0.84	—	6.39 ± 0.55	—	7.30 ± 0.21	—	7.60 ± 0.33	—
4	6.13 ± 0.30	98	7.13 ± 0.25	109	6.90 ± 0.54	97	5.96 ± 0.31	93	7.36 ± 0.30	101	7.28 ± 0.53	96
8	6.50 ± 0.08	104	6.94 ± 0.50	106	6.94 ± 0.45	97	6.55 ± 0.05	102	7.56 ± 0.44	104	8.20 ± 0.64	108
16	6.32 ± 0.13	101	7.28 ± 0.21	112	7.21 ± 0.72	101	6.53 ± 0.19	102	7.66 ± 0.43	105	8.52 ± 0.66	112
32	6.74 ± 0.59	108	7.51 ± 0.11	115	7.53 ± 0.53	105	6.90 ± 0.16	108	7.78 ± 0.18	107	8.23 ± 0.71	108
64	6.90 ± 0.31	110	7.74 ± 0.24	119*	7.93 ± 0.97	111	7.01 ± 0.31	110	8.01 ± 0.70	110	8.73 ± 0.60	115
128	6.98 ± 0.39	111	8.23 ± 0.66	126*	7.97 ± 0.45	111	7.19 ± 0.42	113	8.56 ± 0.49	117	8.89 ± 0.71	117
256	7.62 ± 0.15	122*	8.37 ± 0.42	128*	8.38 ± 0.45	117	7.86 ± 0.08	123	8.94 ± 0.96	122*	8.92 ± 0.42	117
512	8.09 ± 0.62	129*	9.14 ± 0.54	140*	9.14 ± 0.57	128*	8.45 ± 0.37	132*	9.62 ± 0.62	132*	10.0 ± 0.44	132*
1,024	8.72 ± 0.25	139*	9.71 ± 0.62	149*	10.1 ± 0.78	141*	9.16 ± 1.15	143*	10.1 ± 0.91	139*	11.4 ± 0.19	150*
2,048	9.46 ± 0.52	151*	10.9 ± 0.31	167*	10.9 ± 0.29	152*	9.66 ± 1.20	151*	11.7 ± 0.66	161*	12.7 ± 1.05	167*
5,000	10.9 ± 1.02	174*	12.0 ± 0.59	184*	11.8 ± 0.93	165*	10.5 ± 1.11	165*	NA	NA	NA	NA

Conc. = OP ester concentration (0 = controls).
NA = not applicable (> maximum concentration of chemical standard available).
SB = % specific binding of T4 to TTR ± standard deviation.
RB = % relative binding of T4 to TTR compared to controls.
Significant differences from controls indicated by * ($P \leq 0.05$).

The *in vitro* assay applied herein is specifically designed to identify the presence or absence of competition for T4 binding to TTR, in the central TH channel of the dimer-dimer interface, when exposed to a xenobiotic compound. The observed enhancement of T4 bound to TTR by the OP esters suggests a separate mechanism of interaction is occurring, and resulting in increased affinity to or availability of T4 at the TH binding site. With the anticipation of structure-related variation in response, a structurally diverse OP ester study set was deliberately selected, which included alkyl, aryl and halogenated side groups as well as diester and *p*-OH-triester metabolites of TPHP. The common structural element across these compounds is the phosphate moiety, thus it is hypothesized that this phosphorous group binds to an allosteric site on TTR causing a conformational change to the protein. In the present study, it is plausible that this interaction may have affected the shape of the second TH binding site and allow for two T4 molecules at a time to bind to TTR (given the near two-fold doubling of bound T4).

As per its name TTR *trans*(ports) *thy*(roxine and) *retin*(ol), i.e., it also serves to transport retinol binding protein (RBP) bound to retinol through the blood. The four RBP binding sites are on the surface of the protein and do not compete with the TH binding channel, and only two RBPs may bind to TTR at a time due to steric hindrance (Coward et al., 2009; Vieira and Saraiva, 2014). While several studies have investigated binding interactions of a variety of ligands with these two known TTR binding sites, to our knowledge there are currently no reports of other potential binding sites or allosteric interactions with TTR. Ishihara et al. (2003) observed enhancement of T3 binding to recombinant bullfrog TTR by the organochlorine pesticide dicofol using a similar assay, by up to 170 % at 400 nM however followed by 17 % inhibition at 40,000 nM. The

authors also suggested a potential allosteric site of interaction to explain this phenomenon. Two recent *in silico* and *in vitro* analysis of the TTR affinity of house dust contaminants included select OP triesters including TBOEP, TDCIPP, and TPHP (Zhang et al., 2015). All three compounds were predicted nonbinders for the TH site on human TTR, based on predicted interactions specifically at the TH binding channel. Interestingly however, the *in vitro* results for these three compounds also identified TTR binding as non-responsive using a similar assay.

Organophosphate pesticides are structurally related to OP triester FRs, with varying side groups and either a phosphate or phosphothioate moiety. The predominant mechanism of action of several OP pesticides is the irreversible phosphorylation of acetylcholinesterase (AChE) in the central nervous system of insects by covalent binding to a nucleophilic serine –OH group (Colovic et al., 2013). Additional target substrates have been investigated for OP pesticides, and for example Medina-Cleghorn et al. (2014) proposed enzyme phosphorylation as the mechanism of action for chlorpyrifos-exposed mice exhibiting widespread inhibition of serine hydrolases in the brain and liver, and resulting in dysregulation of lipid metabolism. Similarly, Morris et al. (2014) reported that TPHP inhibited mouse carboxylesterase enzymes *in vivo*, and irreversible phosphorylation of the serine in the active site was identified as the mechanism of inhibition. In addition to serine, both lysine and tyrosine have been identified as potential motifs for covalent bonding to OP agents (Lockridge and Schopfer, 2010; Yang and Bartlett, 2016). For example, phosphorylation of tyrosine on the surface of human ALB results in the formation of adducts and is a biomarker of OP pesticide exposure (Lockridge and Schopfer, 2010). Adduct formation of ALB was recently investigated for

OP triesters *in vitro* including the four from the present study to investigate whether this mechanism is shared across OP contaminants, and surprisingly none of the eleven OP triesters tested formed ALB adducts (Chu et al., 2017). The authors suggested the observed differences between OP pesticides and OP triester FRs may be related to the former possessing better leaving groups (i.e., *ortho*- and/or *para*- electron-withdrawing substituents), but that protein specificity was likely to have occurred between OPs.

In the present study, a plausible explanation of the observed results is the covalent bonding of OP tri- and di-esters to residues of serine, lysine or tyrosine on the surface of TTR, resulting in a conformational change in the dimer-dimer interface and allowing for both TH binding pockets to be accessible for T4. Regardless, perturbation of normal TH transport may impact homeostasis of TH functions, including metabolism in adults and, importantly, neuro- and physiological development *in utero* and in childhood (Miller et al., 2009). The enhancement of T4 binding to TTR in the presence of OP triesters and metabolites *in vitro* observed herein suggests the potential for an increase in the levels of TH transported by TTR through the blood. Further research into the possibility of an allosteric site of interaction between OP esters and TTR, e.g., using *in silico* modelling and/or x-ray crystallography, would facilitate an increased understanding of this potential mechanism of toxicity.

4 Chapter: *In vitro* and *in silico* competitive binding of brominated polyphenyl ether contaminants with human and gull thyroid hormone transport proteins[3]

4.1 Abstract

Tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz) is a highly brominated additive flame retardant (FR). Debrominated photodegradates of TeDB-DiPhOBz are hydroxylated *in vitro* in liver microsomal assays based on herring gulls (*Larus argentatus*), including one metabolite identified as 4"-OH-2,2',2",4-tetrabromo-DiPhOBz. Chemically related methoxylated tetra- to hexabromo-DiPhOBzs are known contaminants in herring gulls. Collectively, nothing is currently known about biological effects of these polybrominated (PB) DiPhOBz-based compounds. The present study investigates the potential thyroidogenicity of 2,2',2",4-tetrabromo (TB)-DiPhOBz along with its *para*-methoxy (MeO)- and hydroxy (OH)- analogues, using an *in vitro* competitive protein binding assay with the human thyroid hormone (TH) transport proteins transthyretin (TTR) and albumin (ALB). *Para*-OH-TB-DiPhOBz was found to be capable of competing with thyroxine (T4) for the binding site on human TTR and ALB. *In silico* analyses were also conducted using a 3D homology model for gull TTR, to predict whether these TB-DiPhOBz-based compounds may also act as ligands for an avian TH transport protein despite evolutionary differences with human TTR. This

[3] Adapted from Hill, K.L., Mortensen, Å.K., Teclechiel, D., Willmore, W.G., Sylte, I., Jenssen, B.M., and Letcher, R.J. 2017. *In vitro* and *in silico* competitive binding of brominated polyphenyl ether contaminants with human and gull thyroid hormone transport proteins. Submitted to *Environmental Science & Technology* September 2017.

analysis found all three TB-DiPhOBz analogues to be potential ligands for gull TTR, and with similar binding efficacies to THs. Results indicate structure-related differences in binding affinities of these ligands, and suggest there is potential for these contaminants to interact with both mammalian and avian thyroid function.

4.2 Introduction

Polybrominated diphenyl ethers (PBDEs) were until recently some of the most widely used brominated flame retardants (BFRs) worldwide (Bergman et al., 2012; Dodson et al., 2012). Concerns of persistence, long-range transport, exposure of humans and wildlife (e.g., fish and herring gulls from the Great Lakes) and even more remote regions such as the Arctic, as well as toxicological effects, led to a worldwide phase-out of all three commercial mixtures (penta, octa, and deca-BDE) from commerce over the last several years (Dodson et al., 2012; Letcher et al., 2018; Ryan and Rawn, 2014; Su et al., 2017, 2015b; Wu et al., 2007). In 2009 penta- and octa-BDE formulations were added to Annex A of the Stockholm convention on persistent organic pollutants (POPs), and deca-BDE was added in early 2017 (United Nations Environment Programme, 2017). With the phase-out and regulation of the various PBDE formulations, several replacement flame retardants are now being produced including the deca-BDE replacement tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz; CAS No. 58965-66-5). TeDB-DiPhOBz is a highly brominated additive flame retardant applied to polyesters, resins, wires and cables (Trouborst et al., 2015). There are several Asian suppliers of TeDB-DiPhOBz-containing products and commercial formulations, however no regulations

exist for TeDB-DiPhOBz production and little information is available on the magnitude and global range of its use (MHP Chemicals, 2017; TCI Chemicals, 2017).

TeDB-DiPhOBz has been found to undergo photolytic debromination including to degradation products of Br_4- to Br_7-DiPhOBzs (Chen et al., 2013; Su et al., 2016b, 2014b). Recently, methoxylated (MeO) Br_4- to Br_6-DiPhOBz congeners were identified for the first time in herring gull (*Larus argentatus*) eggs, and are shown to have been present in gull eggs for at least the last 30 years and in concentrations up to 100 ng/g wet weight (Chen et al., 2012, 2011). *In vitro* metabolism studies using both herring gull and rat microsomes confirmed that several Phase I Br_4- to Br_7-OH-PB-DiPhOBz metabolites are formed *in vitro* including one identified as 4"-OH-2,2',2",4-tetrabromo-DiPhOBz (Su et al., 2016a). The MeO-Br_4- to Br_6-DiPhOBz contaminants found in herring gulls are thus suspected degradation products of TeDB-DiPhOBz.

Virtually no toxicological data exist for TeDB-DiPhOBz or its potential degradation products, apart from one *in vitro* study that identified alterations in CYP1A4 expression of chicken embryonic hepatocytes (Su et al., 2014b). Effects are expected to be similar to structurally analogous PBDEs including lower brominated hydroxylated (OH) PBDE metabolites, which are suspected to be capable of perturbing thyroid homeostasis. Thyroid hormones (THs) are involved in several important functions including the regulation of metabolism in adult organisms, and tissue differentiation during fetal and embryonic development (Mendoza and Hollenberg, 2017). One potential mechanism of action of thyroid disruption is competition for TH binding sites on serum transport proteins, as THs triiodothyronine (T3) and its precursor thyroxine (T4) rely on binding to TH transport proteins to be delivered to target tissues. There are three major

TH transport proteins in vertebrates including transthyretin (TTR), albumin (ALB), and thyroxine binding globulin (TBG), where TTR and ALB are consistently important in mammalian and avian species (Richardson et al., 2015; Ućan-Marin et al., 2010).

The objectives of the present study were to investigate the interactions between selected and model tetrabrominated (TB) DiPhOBz congeners with human and avian TH transport proteins as per the following approaches. Firstly, *in vitro* competitive binding interactions of the study set of TB-DiPhOBz congeners with T4 and human TTR and ALB using a recently optimized assay. Secondly, *in silico* molecular homology modelling of glaucous gull (*Larus hyperboreus*) TTR, developed by Mortensen (2015), was conducted to investigate competitive binding interactions of the study set of the TB-DiPhOBz congeners with T4 and T3. Glaucous gull TTR and herring gull TTR were previously found to have identical amino acid and nucleotide sequences (Ucán-Marín et al., 2009).

4.3 Materials and methods

4.3.1 Reagents and chemicals

Tris-EDTA, thyroxine (T4; ≥ 98 % purity), and the lyophilized plasma proteins human transthyretin (TTR; ≥ 95 % purity) and albumin (ALB; ≥ 99 % purity) were obtained from Sigma-Aldrich (Oakville, ON, Canada). The radioligand ^{125}I-labelled T4 (^{125}I-T4; 50 μCi) was obtained from MP Biomedicals (Solon, OH, USA). The PBDE metabolite 4'-hydroxy-2,2',4,5'-tetrabromo diphenyl ether (4-OH-BDE-49; 97.8 % purity) was obtained from Chromatographic Specialties Inc. (Brockville, ON, Canada). Pure standards of the diphenoxybenzene-based chemicals of interest for this study were

developed and provided by Daniel Teclechiel at AccuStandard® Inc. (New Haven, CT, USA). These included a TB-DiPhOBz as well as *para*-OH- and MeO-TB-DiPhOBz associated congeners with the same Br- positioning: 2,2',2'',4-tetrabromo-diphenoxybenzene (BDPB-402; 98.1 % purity), 4"-OH-2,2',2",4-tetrabromo-diphenoxybenzene (HBDPB-401; 100 % purity), and 4"-MeO-2,2',2",4-tetrabromo-diphenoxybenzene (MOBDPB-401; 99.5 % purity) (Figure 4-1).

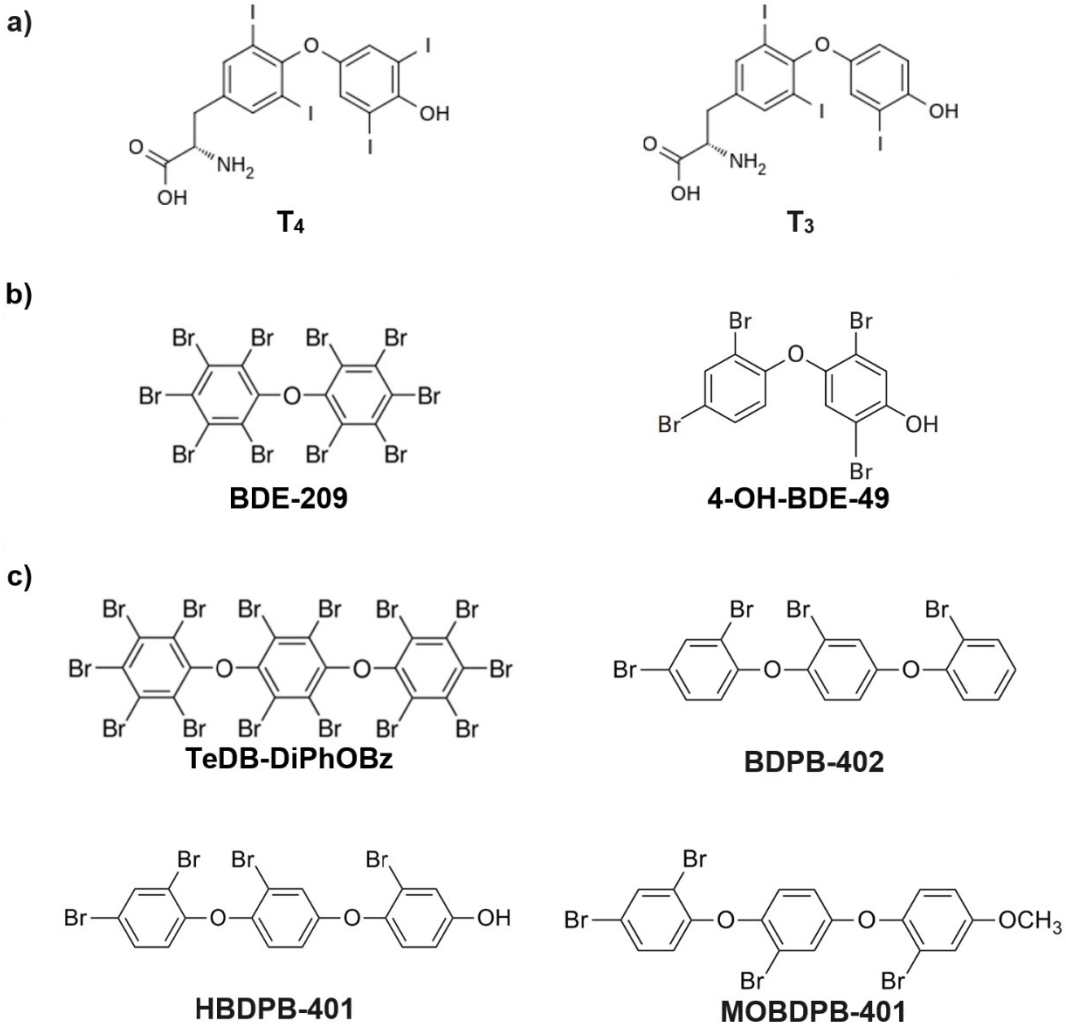


Figure 4-1 Chemical structures of a) thyroid hormones thyroxine (T4) and triiodothyronine (T3); b) decabromodiphenyl ether (BDE-209) and the *para*-hydroxy tetrabrominated metabolite 4-OH-

BDE-49; and c) tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz) and tetrabromo-DiPhOBz, and its hydroxy- and methoxy-substituted analogues BDPB-402, HBDPB-401, and MOBDPB-401, respectively. Hydrogen atoms have been omitted for clarity.

4.3.2 *In vitro* binding assay procedure

A full and highly detailed account of the procedure, including reagent preparation, sample set up and separation, as well as data analysis are described in Chapter 2. The positive control used in this assay was 4-OH-BDE-49, which has been identified as having a higher affinity for both human and recombinant herring gull TTR and ALB over T3 and T4 using a similar assay (Ućan-Marin et al., 2010).

4.3.3 *In silico* methodology

Full details on the development of the *in silico* 3D homology model for gull TTR is described in Mortensen (2015). Homology modelling was performed with the internal coordinate mechanics (ICM) software v3.7 (www.molsoft.com; a well-established, efficient and user-friendly program), using the amino acid sequence for herring and glaucous gull determined by Ucán-Marín et al. (2009) and three selected template models in the protein data bank (PDB). Templates were selected based on preference for high resolution of the tetramer crystal structure, high homology to glaucous gull TTR sequence, and having a ligand bound to allow for definition of the binding pocket. Three models were applied herein, of which two were developed from mammalian templates (rat, *Rattus norvegicus*; PDB ID 1KGI and 1KGJ; Model 1 and 2) and one from a fish template (sea bream; *Sparus aurata*; PDB ID 1SNO; Model 3). Each model was

constructed by a) generating the backbone of the structurally conserved regions using the rigid body homology modelling method, b) assembling the non-conserved regions (i.e., loops), and c) placing the side chains. The models were then refined using energy minimization and Monte Carlo simulations, and evaluated by docking known binders and expected non-binders (decoys). The homology models were previously evaluated on their ability to separate ligands from decoys in a test set of 668 contaminants including suspected endocrine disrupting compounds (Mortensen, 2015). Receiver Operator Characteristics (ROC) curves were generated from the results, whereby ligands were labeled 1, decoys were labeled 0, and the scoring values from the docking exercise were plotted as the number of ligands predicted as binders (true positives) against the decoys predicted to bind (false positives). The area under the curve (AUC) was then calculated from each model's ROC curve, with a positive correlation between the AUC and accuracy of distinguishing between potential binders and non-binders. Resulting AUCs of 96, 95, and 88 % were generated for Models 1, 2, and 3, respectively (Mortensen, 2015).

During docking, the protein and binding pocket remained rigid while the ligand being docked was considered to be flexible. The ligand was put through random conformational changes to fit into the defined binding pocket, and the 10 ligand positions with the lowest energy conformation were selected, which returned a scoring value to evaluate the interaction between the ligand and the protein model. Docking was performed three times in each model, and the strongest scores (i.e., higher binding affinities) were selected in each case. In the previously completed model evaluation exercises, threshold values for each model were determined which distinguish between predicted binders and non-binders. The threshold values for Models 1, 2, and 3 were -18,

-17, and -16, respectively, whereby a value greater than the threshold indicates a non-binder and a lesser value indicates the compound is a potential ligand for gull TTR.

4.4 Results and discussion

4.4.1 *In vitro* interactions with human TTR and ALB

An IC50 of 91.5 nM was calculated for the natural ligand T4 and human TTR (Table 4.1), which is comparable to TTR-T4 IC50s published previously using similar assays (ranging from 50.9 to 88.3 nM) (Hamers et al., 2006; Lans et al., 1993; Meerts, 2000; Ucán-Marín et al., 2009; Weiss et al., 2009). The positive control 4-OH-BDE-49 was a potent competitor ligand as expected, with an IC50 of 12.2 nM for human TTR, which was lower than the natural ligand T4. The IC50 calculated for 4-OH-BDE-49 herein is similar to the IC50 of 10 nM reported in Cao et al. (2010) using a fluorescence assay with human TTR and T4. The *para*-OH-TB-DiPhOBz congener HBDPB-401 also exhibited concentration-dependent competition with T4 for human TTR binding, with an IC50 of 359 nM, which was higher and less potent than the natural ligand T4 (with an IC50 of 91.5 nM). Conversely, the *para*-MeO-TB-DiPhOBz and TB-DiPhOBz congeners, MOBDPB-401 and BDPB-402, did not show strong affinity for binding to human TTR, with maximum inhibition of T4-TTR binding of 15.4 and 1.46 %, respectively at concentrations *in vitro* up to 45,000 nM (Table 4.1, Figure 4-2). No significant reduction in T4-TTR binding was observed for BDPB-402. Significant reduction in T4-TTR binding by MOBDPB-401 was found at 512 and 45,000 nM, however results were not significant in between these study concentrations at 1,024 or 2,048 nM (Figure 4-2). The results for the TB-DiPhOBz compounds tested herein are

similar to those reported on some PBDE conjugates by Ućan-Marin et al. (2010) based on interactions with recombinant herring gull TTR and ALB and with T3 and T4. Ućan-Marin et al. (2010) identified lower binding potencies for the tetra-BDE BDE-49 and an associated 6-MeO-BDE-47 congener as compared to the 4-OH-BDE-49, where the calculated IC50s ranged from 89.1 to 529 nM, 42.2 to 234 nM, and 0.79 to 7.68 nM, respectively.

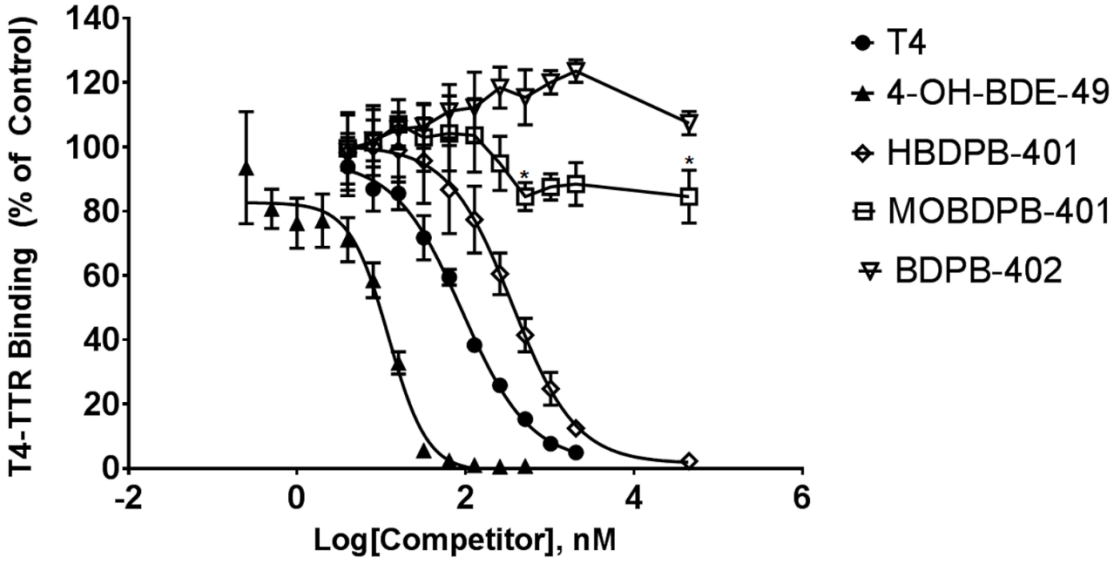


Figure 4-2 Competitive ligand binding curves generated in GraphPad Prism for thyroxine (T4) interactions with human transthyretin (TTR) with competitors including the natural ligand T4, the positive control and known potent ligand 4-OH-BDE-49, and the three PB-DiPhOBz congeners HBDPB-401, MOBDPB-401 and BDPB-402 (see Figure 4-1). Results are presented as means ± standard deviations, with six replicates for each concentration tested. Significant reductions in T4-TTR binding compared to controls are indicated by asterisks ($p<0.05$).

Based on the TB-DiPhOBz results with human TTR, the assays conducted with human ALB focused on interactions with HBDPB-401 only, which also showed competition for the T4-ALB binding in a concentration-dependent manner (Figure 4-3).

The IC50 value for T4-ALB binding inhibition by HBDPB-401 was 144 nM (Table 4.1), which was less potent relative to the natural ligand T4 with human ALB with an IC50 of 4.80 nM. A very similar T4-ALB IC50 for T4 of 7.1 nM was also reported by Ućan-Marin et al. (2010). Similar to T4-TTR competitive binding, the positive control 4-OH-BDE-49 was a more potent competitor for human ALB than the natural ligand T4 with an IC50 of 1.15 nM (Table 4.1).

Relative potencies were calculated by dividing the IC50 of T4 as a competitor ligand by the IC50 for each exogenous ligand for human TTR and ALB. For the positive control 4-OH-BDE-49 relative potencies of 7.49 and 4.18 were calculated for human TTR and ALB, respectively, indicating that this chemical is a more potent ligand than T4 for both proteins *in vitro* (Table 4.1). Relative potencies of 0.255 and 0.0333 were calculated for interactions of HBDPB-401 with human TTR and ALB against T4 (Table 4.1). The relative potencies of HBDPB-401 were much lower than those of 4-OH-BDE-49 and also less than one, indicating that HBDPB-401 is not a stronger competitor than T4 for either protein. Interestingly, relative potencies for both 4-OH-BDE-49 and HBDPB-401 were higher for human TTR than for ALB (Table 4.1). As such, these exogenous ligands are expected to be stronger competitors for T4 at the TH binding site on human TTR than ALB. Overall, the findings herein provide further evidence that the presence of a *para*-OH group on an exogenous halogenated phenyl ring-based chemical is an important contributor to binding potency on TH transport proteins. These results also indicate that the addition of a third phenyl ring to a potential ligand (4-OH-BDE-49 compared to HBDPB-401) does not hinder binding to human TTR or ALB (Figure 4-1, Table 4.1). Conversely, the *para*-MeO-TB-DiPhOBz and TB-DiPhOBz chemicals were

not found to be competitive ligands for T4 on human TTR at concentrations of up to 45,000 nM *in vitro*.

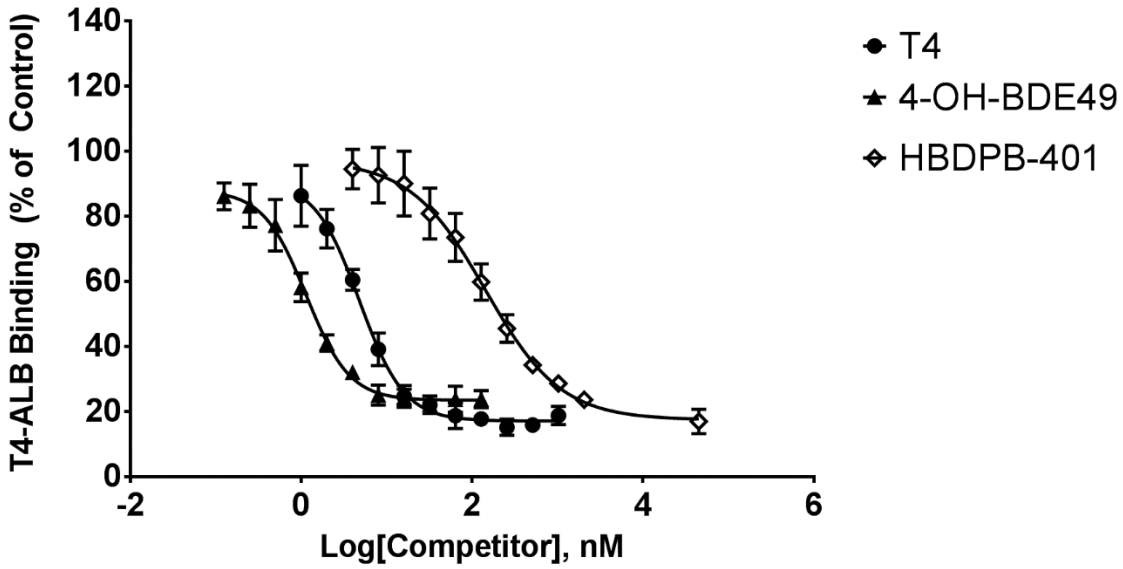


Figure 4-3 Competitive ligand binding curves generated in Graphpad Prism for thyroxine (T4) interactions with human albumin (ALB) with competitors including the natural ligand T4, the positive control and known potent ligand 4-OH-BDE-49, and the *para*-hydroxy PB-DiPhOBz congener HBDPB-401 (see Figure 4-1). Results are presented as means ± standard deviations, with six replicates for each concentration tested.

The K_i values calculated for each competitor are almost identical to the corresponding IC50 values, due to the low concentration of ^{125}I-T4 used in the assay (Table 4.1). Hill Slope values generated in GraphPad for the competitive ligand interaction with human TTR ranged from -1.08 for T4 to -1.89 for 4-OH-BDE-49. For ALB, Hill Slope values ranged from -0.984 for HBDPB-401 to -1.72 for 4-OH-BDE-49 (Table 4.1). A slope of -1 or steeper indicates that a ligand is binding to a single site on the substrate, whereas a slope shallower than -1 (i.e., closer to 0) indicates potential for binding to multiple sites. The results found herein thus suggest a single binding site for

T4 and other competitors on human TTR and ALB, which is consistent with previous findings of negative binding cooperativity (Chang et al., 1999; Vieira and Saraiva, 2014).

Table 4.1 IC50 values and relative potencies calculated for competitor interactions with human transthyretin (TTR) or albumin (ALB) and the natural ligand thyroxine (T4).

Competitor Compound	IC50 (nM)	R^2	Relative Potency[a]	Ki[b] (nM)	Hill Slope	Maximum % Competition[c]	Highest Tested Concentration (nM)
T4-human TTR Interactions							
T4	91.5	0.98	1	91.2	-1.08	95.1 ± 0.5	2,048
4-OH-BDE-49	12.2	0.95	7.49	12.2	-1.89	99.0 ± 0.4	128
HBDPB-401	359	0.94	0.255	358	-1.15	97.8 ± 0.3	45,000
MOBDPB-401	>45,000	ND	ND	ND	ND	15.4 ± 8.3	45,000
BDPB-402	>45,000	ND	ND	ND	ND	1.46 ± 5.74	45,000
T4-human ALB Interactions							
T4	4.80	0.97	1	4.52	-1.65	84.8 ± 2.5[d]	1,024
4-OH-BDE-49	1.15	0.98	4.18	1.08	-1.72	77.7 ± 1.0[e]	128
HBDPB-401	144	0.96	0.0333	135	-0.984	83.1 ± 3.6	45,000

ND = not determined; data not available due to lack of competition.
[a] Calculated by dividing T4 IC50 by competitor IC50.
[b] Calculated by dividing the Ki for T4 by the relative potency of the competitor.
[c] Highest concentration tested unless otherwise noted.
[d] Maximum % competition occurred at 256 nM; 81.2% competition at highest concentration tested.
[e] Maximum % competition occurred at 32 nM; 76.2% competition at highest concentration tested.

4.4.2 *In silico* interactions with gull TTR

All compounds included in the present *in silico* modelling exercise (HBDPB-401, MOBDPB-401, and BPDB-402; Figure 4-1) were predicted ligands in the three gull TTR models (Table 4.2). The scoring values for T3 and T4 were calculated to be -23.31 ± 6.45 and -22.83 ± 8.36, respectively (presented as mean ± standard deviation of the three models). A mean scoring value of -25.09 ± 0.163 was calculated for the known competitor 4-OH-BDE-49, and scoring values for the three PB-DiPhOBz compounds were calculated to be -29.39 ± 2.58 for HBDPB-401, -25.50 ± 4.18 for MOBDPB-401, and -26.15 ± 1.27 for BDPB-402 (Table 4.2). Standard deviations were lower for the exogenous ligands than the THs indicating less variability between model-predicted scoring values for the exogenous ligands than for the THs.

Table 4.2 Scoring values and relative scores calculated for competitor interactions with gull transthyretin (TTR) using *in silico* molecular homology modelling.

Parameter	Model No.	Competitor Compound					
		T3	**T4**	**4-OH-BDE-49**	**HBDPB-401**	**MOBDPB-401**	**BDPB-402**
Scoring value	1	-30.75	-32.44	-25.02	-26.52	-21.72	-25.65
	2	-19.28	-18.86	-25.28	-30.13	-24.79	-25.21
	3	-19.89	-17.19	-24.98	-31.51	-29.99	-27.60
	Mean ± SD	-23.31 ± 6.45	-22.83 ± 8.36	-25.09 ± 0.163	-29.39 ± 2.58	-25.50 ± 4.18	-26.15 ± 1.27
T3-relative score[a]	1	NA	1.05	0.814	0.862	0.706	0.834
	2	NA	0.978	1.31	1.56	1.29	1.31
	3	NA	0.864	1.26	1.58	1.51	1.39
	Mean	—	0.966	1.13	1.34	1.17	1.18
T4-relative score[b]	1	0.948	NA	0.771	0.818	0.670	0.791
	2	1.02	NA	1.34	1.60	1.31	1.34
	3	1.16	NA	1.45	1.83	1.74	1.61
	Mean	1.04	—	1.19	1.42	1.24	1.24

SD = standard deviation
NA = not applicable
[a] Calculated by dividing competitor scoring value by T3 scoring value.
[b] Calculated by dividing competitor scoring value by T4 scoring value.

The gull TTR models were designed to differentiate between binders and non-binders. In the docking exercise, the ligand is brought to the binding site without considering the entropy and enthalpy changes that may occur due to e.g., competition with water or a TH molecule at the binding site. Comparing scoring values of the various exogenous ligands to those of the natural ligands allows for an analysis of the relative ligand binding strength, however this does not necessarily represent a competitive advantage at the gull TTR TH binding pocket. Accordingly, TH-relative scores were calculated by dividing the competitor ligand scoring value to the scoring value for T3 or T4. The comparative scoring values between T3 and T4 were very similar, with a mean T4-relative scoring value of 1.04 indicating T3 is a slightly stronger ligand for gull TTR than T4. While the scoring values are not substantially different, this result is consistent with the literature as T3 is the major circulating TH in avian species (Ućan-Marin et al., 2010). The positive control 4-OH-BDE-49 as well as the three TB-DiPhOBz congeners were predicted to be slightly stronger ligands for gull TTR than both T3 and T4. Mean T3-relative scoring values of 1.13, 1.34, 1.17, and 1.18 were calculated for 4-OH-BDE-49, HBDPB-401, MOBDPB-401, and BDPB-402, respectively. Mean T4-relative scoring values for the same ligands were calculated to be 1.19, 1.42, 1.24, and 1.24 (Table 4.2).

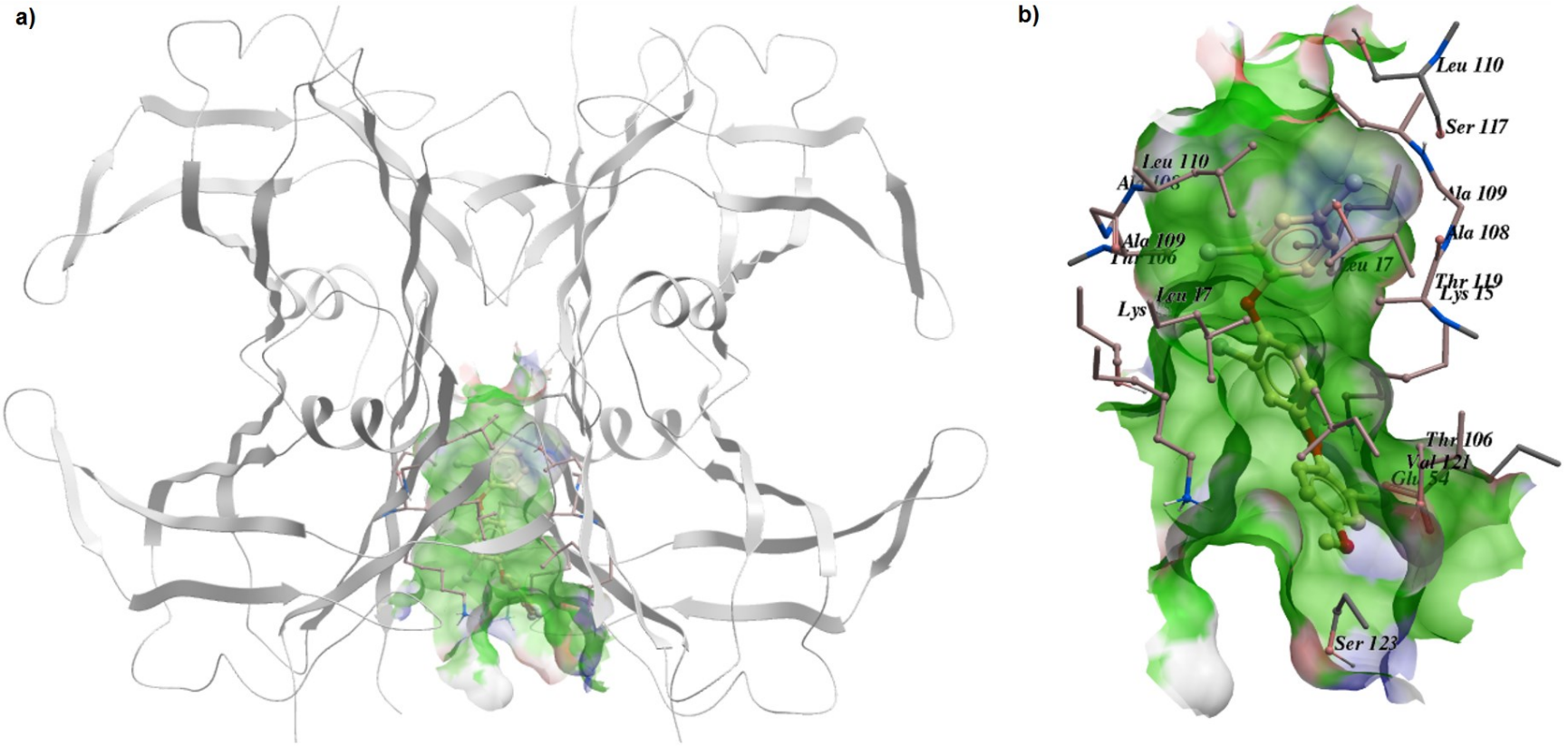


Figure 4-4 ***In silico*** **Model 3 depictions of the binding pocket of gull transthyretin (TTR), where green represents the hydrophobic surface, the hydrogen bond acceptor potential is displayed in blue, and hydrogen bond donor potential is displayed in red; with the exogenous ligands a) tetrabromo-diphenoxybenzene (TB-DiPhOBz) congener BDPB-402 and b) methoxy-TB-DiPhOBz congener MOBDPB-401. Interactions with specific amino acid residues are indicated in b), and labelled in accordance to human TTR sequence.**

In vertebrate species, TTR is a tetramer of two identical subunits with two TH binding pockets at the interface of these dimers. Negative binding cooperativity is commonly observed, whereby only one binding pocket is occupied at a time with a much lower TH binding affinity in the second pocket (Chang et al., 1999). As shown in the *in silico* Model 3 (Figure 4-4), the gull TTR binding pocket is very hydrophobic, with only certain amino acid residues offering hydrogen donor and acceptor potential. The natural ligands T3 and T4 are most likely to be oriented within the gull TTR binding pocket such that the hydroxyl group faces the inner pocket (forward orientation), however it may also bind with reverse-orientation (Weiss et al., 2009). In the forward orientation, the carboxyl group of T4 is predicted to form a hydrogen bond with Lys-15 near the outer edge of the binding site, whereas the hydroxyl group of T3 is predicted to form a hydrogen bond with Ser-117 at the inner binding site (Mortensen, 2015). Similar to T3, the hydroxyl group on HBDPB-401 is predicted to form a hydrogen bond between the oxygen atom and the polar side chain of the amino acid residue Ser-117 (Figure 4-5). Hydrogen bonding was not anticipated for MOBDPB-401 or BDPB-402 in the gull TTR binding pocket as there is no hydroxyl group on these ligands. Several other amino acid residues in the gull TTR binding pocket were predicted to form interactions with the TH and TB-DiPhOBz ligands, however, including: Met-13, Leu-17, Phe-52, Glu-54, Thr-106, Ala-108, Ala-109, Leu-110, Thr-118, Thr-119, and Val-121 (Figure 4-5; Appendix A). These residues align with many of those found to interact with ligands (e.g., OH-BDE congeners) in the human TTR binding pocket (Cao et al., 2010; Zhang et al., 2015).

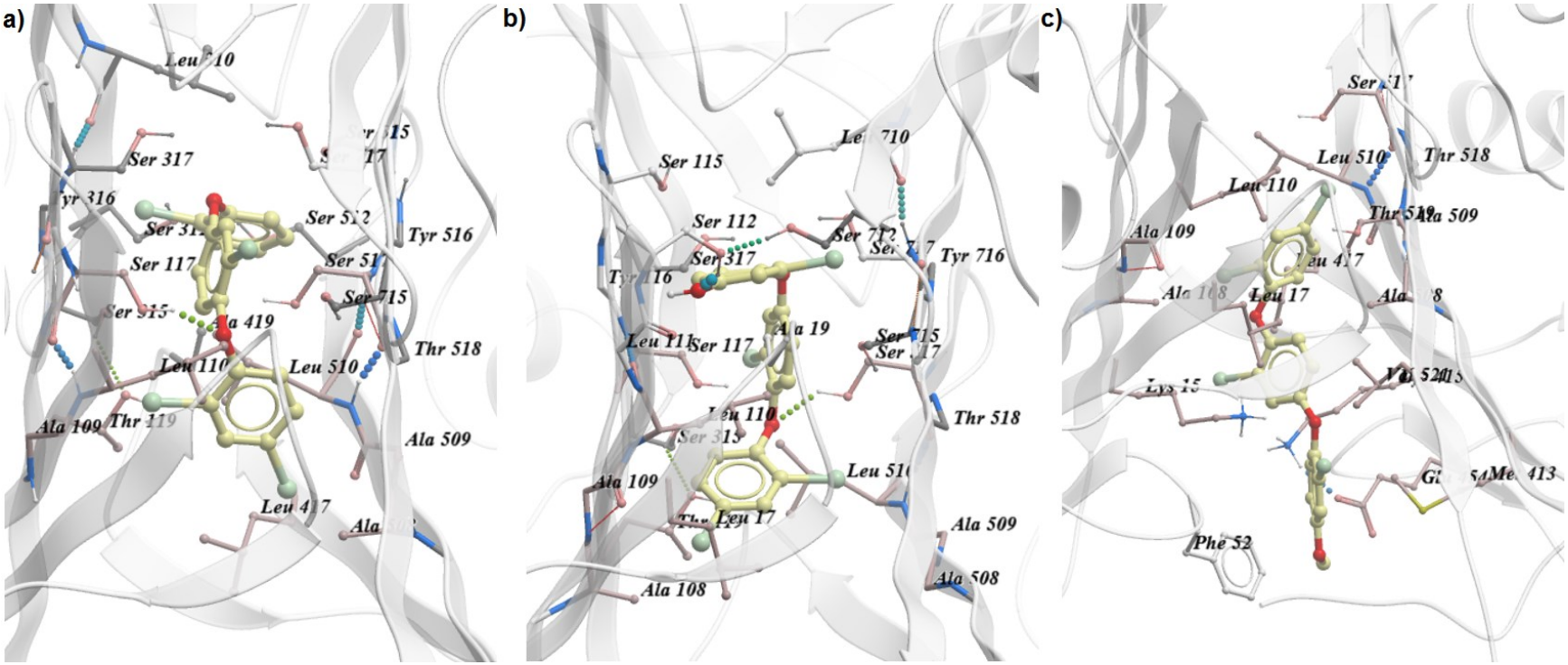


Figure 4-5 ***In silico*** **Model 1 depictions of interactions between exogenous ligands a) tetrabromo-diphenoxybenzene (TB-DiPhOBz) congener BDPB-402; b) hydroxy-TB-DiPhOBz congener HBDPB-401; and c) methoxy-TB-DiPhOBz congener MOBDPB-401 with amino acids in the gull transthyretin (TTR) binding pocket.**

4.4.3 Implications of *in vitro* and *in silico* results

TeDB-DiPhOBz is the suspected precursor of methoxylated Br_4- to Br_6-DiPhOBz contaminants reported in herring gulls from the Great Lakes of North America (Chen et al., 2012, 2011). To the best of our knowledge, these MeO-containing compounds are the only PB-DiPhOBz-based contaminants identified to-date in environmental samples in the Great Lakes and world-wide. A recent effort was made to characterize and quantify TeDB-DiPhOBz and MeO-PB-DiPhOBz contaminants in surficial bottom sediments collected in three sites of the Great Lakes (Lakes Huron and Erie), including sites in Saginaw Bay, which receives river in-flow from the Saginaw River and upon which is located a historical manufacturing site for TeDB-DiPhOBz flame retardant, but PB-DiPhOBzs were not detectable in any sediment sample (Trouborst et al., 2015). It is currently unknown whether TeDB-DiPhOBz and/or PB-DiPhOBz degradation products are present in sediments elsewhere in the Great Lakes, or in any other environmental compartments including other wildlife. The extent of exposure of humans to this flame retardant and potential degradation products is also currently unknown.

The present study provides *in vitro* and *in silico* evidence that known degradation products of TeDB-DiPhOBz are potential ligands for human and gull TH transport proteins. The compounds tested in the current study focused on three TB-DiPhOBz congeners with varying substitution, however it is hypothesized that several additional lower brominated (e.g., Br_4- to Br_6-) *para*-OH-DiPhOBz congeners are competitive TH ligands for TTR. Efforts by industry to reduce bioavailability of flame retardants by increasing molecular size, as is the case with TeDB-DiPhOBz compared to PBDEs, do not provide protection from these contaminants due to environmental and biological

degradation. It is imperative that chemicals introduced to commerce are assessed not only for the toxicological and physicochemical properties of the parent product, but also the degradation products that may be formed.

5 Chapter: General discussion and conclusions

5.1 Summary and implications of research findings

The overall goal of this thesis was to optimize and validate an *in vitro* competitive protein binding assay with human thyroid hormone (TH) transport proteins transthyretin (TTR) and albumin (ALB), and to apply this assay to investigate the potential thyroidogenicity of two classes of novel flame retardants. The chemicals studied included a suite of four organophosphate (OP) triesters and select metabolites of triphenyl phosphate (TPHP), as well as three recently identified tetrabrominated (TB-) degradation products of tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz), which is a suspected replacement flame retardant (FR) for decabromodiphenyl ether (deca-BDE).

The *in vitro* assay methodology was successfully optimized and refined for use with human TTR and ALB with thyroxine (T4). In order to ensure the necessary details are available to any future researcher interested in using this assay, a protocol manuscript was developed which has been submitted to the open-access journal *MethodsX*.

The OP triester and metabolite studies demonstrated a consistent and concentration-related increase in T4 bound to TTR for all compounds tested, suggesting a potential allosteric site of interaction of OP triester and diester compounds with TTR which may increase the ability of T4 to bind to the second TH binding site on TTR. While the results did not align with the predictions for this study, the findings suggest a novel mechanism of interaction of xenobiotic contaminants with TTR. The observed enhancement of T4 binding to TTR in the presence of OP triesters and metabolites *in vitro* indicates the potential for an increase in the levels of TH transported by TTR through the blood.

The studies on TB-DiPhOBz congeners including *para*-OH and *para*-MeO analogs demonstrated structure-related differences in binding potency to human TTR and ALB, with *p*-OH-TB-DiPhOBz exhibiting the highest potency. *In silico* analyses found all three TB-DiPhOBz to be potential ligands for herring gull TTR, and including the MeO-TB-DiPhOBz congener which has been identified as a contaminant in Great Lakes herring gull eggs. While three congeners were selected as representative analogs for these novel contaminants, it is likely that several additional lower brominated (e.g., Br_4 to Br_6-) *p*-OH-DiPhOBz congeners, which are known to be formed from *in vitro* microsomal studies, are competitive ligands for human and gull TH transport proteins. The results of the *in vitro* and *in silico* analyses support the existing evidence of the importance of hydroxylated, halogenated aromatics acting in a thyroidogenic manner, and indicate the potentially biologically active nature of these novel contaminants in both mammalian and avian species despite the presence of a third phenyl ring (compared to PBDEs). These findings also emphasize the importance of studying the environmental and biological degradation products of commercial chemicals, as the parent compound is biologically unavailable.

Protecting human populations from endocrine disrupting compounds is crucial to ensuring healthy reproduction and physiological development (WHO, 2012). Disruption of the level of circulating THs as a result of displacement from transport proteins by exogenous ligands is one mechanism of action that could potentially lead to alterations in processes driven by thyroid function, and particularly during development. Furthermore, a chemical that is found to interact competitively with the TH binding site on TTR or ALB may also act in a similar manner at other sites of thyroid activity, for example

thyroid hormone receptor (TR) at the site of gene transcription. Thus the *in vitro* competitive protein binding assay applied herein is a useful indicator of the potential thyroidogenicity of xenobiotic chemicals.

Overall, this thesis project presents interesting findings on two classes of FR contaminants of concern. Firstly, a novel mechanism of action has been identified whereby OP di- and triesters enhance the binding of T4 to human TTR *in vitro*. Secondly, *in vitro* and *in silico* analyses with human and gull TH transport proteins, respectively reveal structure-and species-related differences in binding potency for known degradation products of the novel brominated FR TeDB-DiPhOBz. Collectively, these results indicate that novel FRs with varying molecular properties, and especially some degradation products, are potential thyroid disrupting compounds in humans and wildlife.

5.2 Future directions

1) The interactions of xenobiotic ligands with human TH transport proteins are important for considering impacts to human health. While TTR and ALB are highly conserved across vertebrates, the slight differences that have recently occurred in the evolution of protein sequence and subsequent tertiary structure (and related alteration in TH binding affinity) indicate potential differences between species. As such the results of human TH transport protein interactions with xenobiotic contaminants are not necessarily reliable surrogates for assessing effects to wildlife species (Chang et al., 1999; Ucán-Marín et al., 2010). The *in silico* analyses conducted on TB-DiPhOBz congeners provide insight on potential interactions with herring gull (and avian) TH

transport proteins. It is important to investigate the variation of binding affinities for other classes of vertebrates to understand potential effects in various wildlife species.

2) Further research is needed to investigate the possibility of an allosteric site of interaction between OP esters and TTR, e.g., using *in silico* modelling, solution nuclear magnetic resonance spectroscopy, and/or x-ray crystallography, to increase the understanding of this potential mechanism of toxicity.

3) The TB-DiPhOBz results would be a useful addition to existing and/or future *in silico* datasets on human TTR and ALB binding to xenobiotic chemicals, due to the unique structure of these halogenated phenyl ether contaminants.

4) There is a paucity of data on the potential exposure of humans and wildlife to PB-DiPhOBz contaminants, apart from two recent studies identifying various MeO-PB-DiPhOBz congeners in Great Lakes herring gull eggs. Due to the potential thyroidogenic nature of *p*-OH-TB-DiPhOBz found in this study, future biomonitoring efforts to quantify exposure in various media would be of interest.

5) The results from this thesis would be informative in the development of an adverse outcome pathway (AOP) for thyroid hormone transport disruption. There is currently an AOP being developed on the interference with TTR and subsequent developmental toxicity in humans, which is in a draft stage as of July 2017 (https://aopwiki.org/aops/152). This effort is part of the "AOP-Wiki" collaborative

open access effort between the European Commission and US Environmental Protection Agency, as well as the Organization for Economic Co-operation and Development. The use of AOPs is an increasingly popular concept for advancing methods of risk assessment and moving away from animal testing.

Appendices

Appendix A

Table A.1 Model 1 predicted electrostatic interactions of natural ligands thyroxine (T4) and triiodothyronine (T3), as well as exogenous ligands tetrabromo-diphenoxybenzene (TB-DiPhOBz) BDPB-402, and hydroxy- and methoxy-TB-DiPhOBz congeners HBDPB-401 and MOBDPB-401, with gull transthyretin (TTR) in the thyroid hormone binding pocket.

Amino Acid No.	T3		T4		HBDPB-401		MOBDPB-401		BDPB-402	
	Subunit		Subunit		Subunit		Subunit		Subunit	
	1	2	1	2	1	2	1	2	1	2
Met13	X									X
Lys15	X	X	***X***	X	X	X	X	X	X	X
Leu17	X	X	X	X	X	X	X	X	X	X
Pro24										
Phe52									X	
Glu54					X					
Thr106	X		X	X	X			X		X
Ala108	X	X	X	X	X	X	X	X	X	X
Ala109	X	X	X	X		X	X		X	X
Leu110	X	X	X	X	X	X	X			X
Ser117	***X***	X	X		X	***X***	X	X		X
Thr118	X		X			X	X	X		X
Thr119	X	X	X	X	X	X	X	X		
Val121	X			X	X			X		X
Ser123										

Subunits 1 and 2 indicate which of the two dimers of gull TTR is interacting with the ligand.
"X" indicates predicted interaction between ligand and amino acid residue.
"*X*" indicates hydrogen bonds are predicted between ligand and amino acid residue.
Note: model configured at pH 7.4 to simulate blood.

Table A.2 Model 2 predicted electrostatic interactions of natural ligands thyroxine (T4) and triiodothyronine (T3), as well as exogenous ligands tetrabromo-diphenoxybenzene (TB-DiPhOBz) BDPB-402, and hydroxy- and methoxy-TB-DiPhOBz congeners HBDPB-401 and MOBDPB-401, with gull transthyretin (TTR) in the thyroid hormone binding pocket.

Amino Acid No.	T3		T4		HBDPB-401		MOBDPB-401		BDPB-402	
	Subunit		Subunit		Subunit		Subunit		Subunit	
	1	2	1	2	1	2	1	2	1	2
Met13						X		X		X
Lys15	X	***X***	X	***X***	X	X	X	X	X	X
Leu17	X	X	X	X	X	X	X	X	X	X
Pro24					X		X			
Phe52										
Glu54						X		X		
Thr106	X		X		X		X	X	X	X
Ala108	X	X	X	X	X	X	X	X	X	X
Ala109		X		X	X	X	X	X	X	X
Leu110	X	X	X	X	X	X	X	X	X	X
Ser117	***X***	***X***	***X***	***X***		X		X		X
Thr118	X		X			X		X		X
Thr119	X		X		X	***X***		X		X
Val121						X		X		X
Ser123										

Subunits 1 and 2 indicate which of the two dimers of gull TTR is interacting with the ligand.
"X" indicates predicted interaction between ligand and amino acid residue.
"*X*" indicates hydrogen bonds are predicted between ligand and amino acid residue.
Note: model configured at pH 7.4 to simulate blood.

Table A.3 Model 3 predicted electrostatic interactions of natural ligands thyroxine (T4) and triiodothyronine (T3), as well as exogenous ligands tetrabromo-diphenoxybenzene (TB-DiPhOBz) BDPB-402, and hydroxy- and methoxy-TB-DiPhOBz congeners HBDPB-401 and MOBDPB-401, with gull transthyretin (TTR) in the thyroid hormone binding pocket.

Amino Acid No.	T3		T4		HBDPB-401		MOBDPB-401		BDPB-402	
	Subunit		Subunit		Subunit		Subunit		Subunit	
	1	2	1	2	1	2	1	2	1	2
Met13					X					
Lys15	X	X	X	X	X	X	X	X	X	X
Leu17	X	X	X	X	X	X	X	X	X	X
Pro24										
Phe52										
Glu54		***X***		X	X			X		
Thr106	X	X	X	X	X	X	X	X		X
Ala108	X	X	X	X	X	X	X	X	X	X
Ala109	X		X	X	X	X	X	X	X	X
Leu110	X	X	X	X	X	X	X	X	X	X
Ser117					X			X		X
Thr118										
Thr119					***X***			X	X	X
Val121		X		X	X			X		
Ser123								X		

Subunits 1 and 2 indicate which of the two dimers of gull TTR is interacting with the ligand.
"X" indicates predicted interaction between ligand and amino acid residue.
"*X*" indicates hydrogen bonds are predicted between ligand and amino acid residue.
Note: model configured at pH 7.4 to simulate blood.

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