CHARACTERIZATION OF TOXICITIES, ENVIRONMENTAL CONCENTRATIONS, AND BIOACCESSIBILITIES OF NOVEL BROMINATED FLAME RETARDANTS

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Toxicology Graduate Program

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

By

David Saunders

© Copyright David Saunders, March 2017. All rights reserved.

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the Libraries of the University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purpose may be granted by the professor or professors who supervised this thesis work or, in their absence, by the Head of the Department or the Dean of the College in which this thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in this thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or parts should be addressed to:

Chair of the Toxicology Graduate Program
Toxicology Centre
University of Saskatchewan
44 Campus Drive
Saskatoon, Saskatchewan S7N 5B3

ABSTRACT

Brominated flame retardants (BFRs) are synthetic compounds which are added to consumer and industrial products to inhibit the propagation of fire. Several of the most predominantly used BFRs have been banned or phased out of use due to their toxicity, persistence in the environment, and potential to bioaccumulate. Novel brominated flame retardants (NBFRs) are replacement compounds of legacy BFRs and are generally designed to be less bioaccumulative and persistent in the environment. The NBFRs, bis(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (TBPH), 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB), and 1,2,5,6-tetrabromocyclooctane (TBCO) are components of several flame retardants mixtures including Firemaster® 550 and Saytex® BC-48 and are (potential) major replacements of legacy BFRs. These compounds have been detected in the outdoor and indoor environments, in tissues of wildlife, and serum/tissues of humans, though little information exists regarding potential toxicities and concentrations of these compounds in the indoor environment. Therefore, the aim of this research was to characterize toxicities of these compounds and investigate important parameters of exposure in early childhood environments (ECEs). Preliminary characterization of toxicities of TBPH, TBB, and TBCO focused on potential endocrine disrupting effects as these compounds were structurally similar to known endocrine disrupting compounds (EDCs). The screening level investigations of toxicity employed cellular assay systems to determine binding activities with hormone receptors and modulation of production of sex steroid hormones. Results obtained with these *in vitro* assays demonstrated potentials of NBFRs to modulate endocrine function through interactions with estrogen and androgen receptors and via alterations to the synthesis of 17-β-estradiol and testosterone. Therefore, further characterization of endocrine disrupting effects of these NBFRs was warranted. Short-term fish fecundity assays coupled to investigations of molecular mechanisms of effect along the hypothalamus-pituitary-gonadal-liver (HPGL) axis confirmed that TBPH, TBB, and TBCO affected normal endocrine functions. Exposure to a mixture of TBPH:TBB or TBCO reduced fecundity of Japanese medaka (*Oryzias latipes*) and caused alterations in transcript abundances of genes across the HPGL-axis. Though no distinct mechanisms of effects were determined, a pattern of down-regulation of genes across all tissues of the HPGL-axis was observed following exposure to the mixture of TBPH:TBB, while exposure to TBCO alone elicited organ-specific and dose-dependent alterations of expression of genes involved in steroidogenesis, metabolism of cholesterol, and estrogen signaling.

Concentrations of TBPH and TBB in dust from ECEs collected during summer and winter were determined to elucidate important factors of exposure of children. Novel hydroxylated isomers of TBPH and TBB were detected and characterized in dust from ECEs for the first time. Concentrations of TBPH, TBB, OH-TBPHs, and OH-TBBs in dust from ECEs from Saskatoon, SK, Canada were among the greatest reported globally though no seasonal differences in concentrations of compounds in dust were observed. Greater concentrations of these NBFRs were detected in microenvironments with greater numbers of children's toys which indicated that concentrations in dust might be related to increases in density of these consumer products. To further characterize exposure of children to NBFRs, bioaccessibilities of TBPH, TBB, OH-TBPHs, and OH-TBBs in dust from ECEs were assessed in an *in vitro* incubation assay system. TBPH and OH-TBPHs were minimally bioaccessible where TBB and OH-TBBs were moderately-highly bioaccessible, which indicated that TBPH and OH-TBPHs would not likely be readily bioavailable from dust in *in vivo* systems. The data generated in this thesis is important to inform accurate assessments of risk of these novel brominated flame retardants.

ACKNOWLEDGEMENTS

So many people have contributed directly to the work in this thesis, and indirectly by supporting me in my PhD, I hope I can thank them all appropriately, or at least an abbreviated version of 'all'. First, the obvious, I would like to thank my sources of funding: the NSERC CREATE HERA program and the NSERC Vanier Scholarship as well as the Toxicology Centre. This building has been my home for the past 5.67 years and I couldn't have asked for a better staff with whom to have worked. I would like to thank my committee, Dr. Markus Hecker, Dr. Paul Jones, Dr. Anas El-Aneed, and my chair, Dr. David Janz. Thank you all for the advice, guidance, and tutelage over the years. I would also like to extend my significant gratitude to Dr. Miriam Diamond for agreeing to participate as External Examiner – hopefully it goes well.

Thank you to my family Sue, Howard, Nicole, Johnny, Jakob and the new addition, Emma, who have been a source of constant support. Of course I'm referring to both the figurative support like unconditional love and patience, but also the more real and important support that you've given me, financial. I am a product of my upbringing and people seem to like me good enough, so thank you guys.

To the people I worked with, my friends, my compadres, my cohort, you've made this experience thoroughly acceptable. Thank you to the Giesy gang, Hattan, Garrett, and Abby. I could not have asked for a better group of scientists. You are intelligent people who do good science. You have constantly inspired me to work hard and diligently and have helped me to gain success in this program. I will miss you. I also need to thank my post-docs Dr. Peng Hui and Dr. Jianxian Sun, with whom I have spent many hours in the laboratory and an equal amount eating hot pot. Thank you for making me a better scientist.

I need to thank Dr. Steve Wiseman without whom I would not have had a comprehensive program of study. I could easily write two pages about how you were basically responsible for my PhD, or made me a better scientist, or how much I appreciated all our time talking about the world, culture and science. But, as you are firmly a man of few written words, I will just leave it at, Thank You.

Dr. John Giesy. I remember receiving my letter of acceptance to your group. It was one of the most exciting days of my life, and that feeling hasn't gone away. You've provided me

with this opportunity, helped to mold me into a professional and scientist, and shaped the person I am. You've paid for me to travel the world, present at conferences in foreign countries, and given me a graduate experience that was far beyond what I could have expected. One of the first things you told me was, 'if you love what you do, then you won't work a day of your life'. I feel like I've worked very few days at the Tox Centre. I owe this PhD and my future success to you.

Finally, to Keeley. Everything this is, the work, the sacrifice, the accomplishment, started with us. You & Me *vs*. the world.

TABLE OF CONTENTS

PERMISSION TO USE i
ABSTRACT ii
ACKNOWLEDGEMENTS iv
TABLE OF CONTENTS vi
LIST OF TABLES xi
LIST OF FIGURES xiii
LIST OF ABBREVIATIONS xx
NOTE TO READERS xxvii
1 CHAPTER 1: GENERAL INTRODUCTION 1
PREFACE 2
1.1 Flame retardants 3
1.2 Brominated flame retardants 4
1.3 Novel brominated flame retardants 7
1.4 Selection of novel brominated flame retardants 10
1.4.1 Detection of novel brominated flame retardants in the environment 12
1.4.2 Toxicities of TBPH, TBB and TBCO 13
1.5 Novel brominated flame retardants in the indoor environment 15
1.5.1 Dust as an important vector of exposure to brominated flame retardants 18
1.5.2 Exposure of children to brominated flame retardants 20
1.6 Conclusions 21
1.7 Objectives 22
2 CHAPTER 2: *IN VITRO* ENDOCRINE DISRUPTION AND TCDD-LIKE EFFECTS OF THREE NOVEL BROMINATED FLAME RETARDANTS: TBPH, TBB, & TBCO 27
PREFACE 28
2.1 Abstract 29
2.2 Introduction 30
2.3 Materials and methods 34
2.3.1 Chemicals 34
2.3.2 Cell viability 34
2.3.3 H4IIE-luc transactivation reporter gene assay 35

2.3.4 YES/YAS assays 35
2.3.5 H295R cell culture and exposure.. 36
2.3.6 17β-Estradiol and testosterone extraction and quantification by use of EIA 36
2.3.7 Statistics.. 36
2.4 Results 37
2.4.1 TCDD-like potencies of compounds 37
2.4.2 Receptor-mediated androgenic and estrogenic activities of compounds...................... 37
2.4.3 Androgen receptor mediated antiandrogenic activities of NBFRs 37
2.4.4 Estrogen receptor mediated antiestrogenic activities of compounds........................... 39
2.4.5 Effects of NBFRs on testosterone synthesis.. 39
2.4.6 Effects of NBFRs on E2 synthesis 42
2.5 Discussion 44
2.5.1 TCDD-like effects 44
2.5.2 (Anti) androgenic effects 44
2.5.3 (Anti) estrogenic effects 45
2.5.4 Effects on testosterone production in the H295R steroidogenesis assay...................... 46
2.5.5 Effects on estrogen production in the H295R steroidogenesis assay 47
3 CHAPTER 3: A MIXTURE OF THE NOVEL BROMINATED FLAME RETARDANTS TBPH AND TBB AFFECTS FECUNDITY AND TRANSCRIPT PROFILES OF THE HPGL-AXIS IN JAPANESE MEDAKA 48
PREFACE 49
3.1 Abstract 50
3.2 Introduction 51
3.3 Materials and methods 53
3.3.1 Chemicals and reagents 53
3.3.2 Animal care... 53
3.3.3 Exposure protocol.. 53
3.3.4 Chemical analysis 54
3.3.5 Gene selection and graphical model 55
3.3.6 Quantitative real-time PCR 55
3.3.7 Statistical analysis.. 56
3.4 Results 56
3.4.1 Concentrations of chemicals in food 56

3.4.2 Chemical-induced effects of fecundity of medaka 59
3.4.3 Gene expression profiles of TBPH/TBB exposures 61
3.5 Discussion 64
3.5.1 Fecundity 64
3.5.2 Abundances of transcripts 65
3.5.3 Conclusions 68
4 CHAPTER 4: EFFECTS OF THE BROMINATED FLAME RETARDANT TBCO ON FECUNDITY AND PROFILES OF TRANSCRIPTS OF THE HPGL-AXIS IN JAPANESE MEDAKA 70
PREFACE 71
4.1 Abstract 72
4.2 Introduction 73
4.3 Materials and methods 75
4.3.1 Chemicals and reagents 75
4.3.2 Animal care 75
4.3.3 Exposure protocol 75
4.3.4 Chemical analysis 76
4.3.5 Gene selection and graphical model 77
4.3.6 Quantitative real-time PCR 77
4.3.7 Statistical analysis 78
4.4 Results 78
4.4.1 Concentrations of chemicals in food 78
4.4.2 Chemical-induced effects on fecundity of medaka 80
4.4.3 Gene expression profiles 80
4.5 Discussion 86
4.5.1 Fecundity 86
4.5.2 Abundances of transcripts 87
4.5.3 Conclusions 89
5 CHAPTER 5: DETECTION, IDENTIFICATION, AND QUANTIFICATION OF HYDROXYLATED BIS(2-ETHYHEXYL)-TETRABROMOPHTHALATE ISOMERS IN HOUSE DUST 90
PREFACE 91
5.1 Abstract 93

5.2 Introduction 94
5.3 Materials and methods 95
5.3.1 Chemicals and reagents 95
5.3.2 Purification of OH-TBPH by HPLC fractionation 97
5.3.3 Collection of dust 97
5.3.4 Sample pretreatment and analysis 97
5.3.5 Instrumental analysis 98
5.3.6 Quality assurance/quality control 99
5.3.7 Data analysis 99
5.4 Results and discussion 100
5.4.1 Observation and chemical structure identification of OH-TBPH in TBPH standards 100
5.4.2 Development of analytical methods to measure TBPH and OH-TBPH in dust 105
5.4.3 Concentrations and profiles of TBPH and OH-TBPH in house dust 107
5.4.4 Implications 113
6 CHAPTER 6: CONCENTRATION, SEASONALITY AND BIOACCESSIBILITY OF NOVEL BROMINATED FLAME RETARDANTS IN DUST FROM CHILDCARE FACILITIES IN SASKATOON, SK, CANADA 114
PREFACE 115
6.1 Abstract 116
6.2 Introduction 117
6.3 Materials and methods 119
6.3.1 Chemicals and reagents 119
6.3.2 Collection of dust samples 120
6.3.3 Pretreatment of dust 120
6.3.4 Pretreatment of Tenax 121
6.3.5 Pretreatment of gastro-intestinal fluid 121
6.3.6 Instrumental analysis 121
6.3.7 Design of the Tenax bead incubation envelope 122
6.3.8 Tenax enhanced bioaccessible extraction 123
6.3.9 Quality control 123
6.3.10 Data analysis 124
6.4 Results and discussion 125

6.4.1 Concentrations of NBFRs and their hydroxylated isomers in dust from day care centers 125
6.4.2 Differences of concentrations of NBFRs and their hydroxylated isomers in dusts from specific microenvironments 128
6.4.3 Seasonal differences in concentrations of NBFRs and hydroxylated isomers in dust 133
6.4.4 Bioaccessibilities of NBFRs and their hydroxylated isomers in standard reference dust and dust collected from day care centers 134
7 CHAPTER 7: GENERAL DISCUSSION 141
7.1 History and project rationale 142
7.1.1 Regulations, the use of BFRs, and research regarding Firemaster® 550 142
7.1.2 History of research regarding TBCO 143
7.1.3 Project rationale 144
7.2 Toxicities of novel brominated flame retardants 144
7.2.1 Screening level *in vitro* assessments of endocrine disrupting effects of TBPH and TBB 144
7.2.2 *In vivo* assessments of endocrine disrupting effects of TBPH, TBB and TBCO 146
7.2.3 Epidemiological studies of legacy BFRs and potential for 'read-across' 149
7.3 Exposure to novel brominated flame retardants 150
7.3.1 Routes of exposure 150
7.3.2 Toxicokinetics and human exposure 153
7.4 Assessment of risk of TBPH and TBB 155
7.5 Future work 157
7.6 Final thoughts 159
REFERENCES 161
APPENDIX[1] 183

LIST OF TABLES

Table 1.1. Estimated global production volumes of total BFRs, TBBPA, ΣHBCDs, and ΣPBDE congeners for years 1992, 2000, and 2001……………………………………………………..6

Table 1.2. Median concentrations of TBPH, TBB, and TBCO reported in indoor dust (ng/g, dust)……………………………………………………………………………………17

Table 3.1. Concentrations of TBPH and TBB in three diets used in the 21-day fish fecundity assay. Concentrations of TBPH and TBB are presented as mean ± standard error (µg/g food). Three replicates were extracted and analyzed for each food type………………………………58

Table 3.2. Response profiles of genes of the hypothalamic-pituitary-gonadal-liver (HPGL) axis in Japanese medaka exposed to the greater dose of the TBPH/TBB mixture (1422:1474 µg/g food, w/w). Abundances of transcripts are expressed as fold change compared to corresponding solvent controls……………………………………………………………………………..62

Table 4.1. Concentrations of TBCO in three diets used in the 21-day fecundity assay. Concentrations of TBCO are presented as mean ± standard error (µg/g, wm food). Three replicates were extracted and analyzed for each food type……………………………………79

Table 4.2. Response profiles of genes of the hypothalamic-pituitary-gonadal-liver (HPGL) axis in Japanese medaka exposed to the greater (607 µg/g food) and lesser (58 µg/g food) concentrations of TBCO. Transcript responses are expressed as fold change compared to corresponding solvent controls………………………………………………………………..82

Table 5.1. Instrumental detection limits (IDLs, µg/L), method detection limits (MDLs, ng/g, dm) and recoveries (n=3) of OH-TBPH isomers and TBPH of different methods………………….106

Table 5.2. Concentrations of OH-TBPH and TBPH (ng/g, dm) in samples of house dust from Saskatoon, Saskatchewan, Canada…………………………………………………………..109

Table 6.1. Concentrations of TBPH, OH-TBPH, TBB, and ΣOH-TBB (ng/g, dm) in samples of dust collected from daycare centers in summer or winter of 2012 and 2013, respectively…….127

Table C2.S1 Physical-chemical properties of 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB), *Bis*(2-ethylhexyl)-2,3,4,5-tetrabromophtalate (TBPH), and 1,2,5,6-tetrabromocyclooctane (TBCO)……………………………………………………………………………………184

Table C3.S1. Target gene, accession number, primer sequence, efficiency, and annealing temperatures of 35 genes across the HPGL axis of Japanese medaka………………………….187

Table C3.S2. Toxicant-induced effects on medaka gonadal-somatic index (GSI) and hepatic-somatic index (HSI). GSI and HSI are presented as mean ± standard error……………………188

Table C4.S1 Target gene, accession number, primer sequence, efficiency, and annealing temperatures of 35 genes across the HPGL axis of Japanese medaka………………………….191

Table C4.S2. Toxicant-induced effects on medaka gonadal-somatic index (GSI) and hepatic-somatic index (HSI). GSI and HIS are presented as mean ± standard error……………………192

Table C6.S1. Ionization sources, ions, and instrumental detection limits for the analysis of TBPH, TBB, and their OH-isomers……………………………………………………………….201

Table C6.S2. Measurements of bioaccessibility for TBPH, TBB and their OH-isomers in dust samples (DS) (n = 14)…………………………………………………………………………..205

LIST OF FIGURES

Figure 1.1. Chemical structures of the major BFRs, TBBPA, HBCD, and Deca-BDE…………5

Figure 1.2. Chemical structures of major replacement NBFRs, BTBPE, DBDPE, TBBPA-DBPE, TBBPA-DHEE, and TBBPA-DAE……………………………………………………9

Figure 1.3. Chemical structures of selected NBFRs, TBB, TBPH, and TBCO included in subsequent studies…………………………………………………………………………..11

Figure 2.1. Chemical structures of 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB), bis(2-ethylhexyl)-3,4,5,6-tetrabromo-phthalate (TBPH), and 1,2,5,6-tetrabromocyclooctane (TBCO)……………………………………………………………………………………..33

Figure 2.2. The antiandrogenic activity of (A) TBB at seven exposure concentrations, (B) TBPH at eight exposure concentrations, and (C) TBCO at eight exposure concentrations, in mg/L measured by the yeast androgen screen. Antiandrogenic activity is presented as the reduction in signal intensity (mean ± SE) compared to DHT activated control cells (CTRL). Hydroxyflutamide (HF) acted as a positive control. Each assay contained four wells per NBFR exposure concentration and a total of four assays were used for analysis. Exposure concentrations that resulted in effects that were significantly different than activated controls are indicated by asterisks (*$p<0.05$)……………………………………………………………38

Figure 2.3. The antiestrogenic activity of (A) TBB at seven exposure concentrations, (B) TBPH at seven exposure concentrations, and (C) TBCO at six exposure concentrations in mg/L measured by the yeast estrogen screen. Antiestrogenic activity is presented as the reduction in signal intensity (mean ± SE) compared to E2 activated control cells (CTRL). 4-Hydroxytamoxifen (HT) acted as a positive control. Each assay contained four wells per NBFR exposure concentration and a total of four assays were used for analysis. Exposure concentrations that resulted in effects that were significantly different than activated controls are indicated by asterisks (*$p<0.05$)……………………………………………………………40

Figure 2.4. The effects of (A) TBPH and (B) TBCO exposures on relative testosterone hormone concentrations measured in the H295R cell assay. Four concentrations (mg/L) of TBPH and TBCO were tested and data are given as relative fold change in hormone production (mean ± SE)

compared to solvent controls (DMSO). Each assay contained four wells per NBFR exposure concentration and a total of four assays were used for analysis. Exposure concentrations that resulted in effects that were significantly different than solvent controls are indicated by asterisks (*p<0.05)……………………………………………………………………………………41

Figure 2.5. The effects of (A) TBB, (B) TBPH, and (C) TBCO exposures on relative 17-β-estradiol hormone concentrations measured in the H295R cell assay. Four concentrations (mg/L) of each NBFR were tested and data are given as relative fold change in hormone production (mean ± SE) compared to solvent controls (DMSO). Each assay contained four wells per NBFR exposure concentration and a total of four assays were used for analysis. Exposure concentrations that resulted in effects that were significantly different than solvent controls are indicated by asterisks (*p<0.05)………………………………………………………………43

Figure 3.1. Cumulative production of eggs (fecundity) by medaka exposed to the high dose of the TBPH/TBB mixture (1422:1474 µg/ g food, w/w), the low dose of the TBPH/TBB mixture (138:144 µg/g food, w/w) and solvent control. The values represent the mean cumulative number of eggs per female over a 21-day period. The experiment included 4 replicate tanks, and each contained 8 female/male medaka. Asterisks (*) indicate a significant difference ($p < 0.05$) when compared to the control group……………………………………………………………….60

Figure 3.2. Graphical representation of the transcript response profile of the HPGL-axis in Japanese medaka exposed to the greater dose of the TBPH/TBB mixture (1422:1474 µg/ g food, w/w). Gene expression data are represented as striped colour sets with notches denoting sex of medaka. Eight colours were used to represent different fold-change thresholds. Criteria not met denotes a lack of statistical difference ($p < 0.05$) or lack of physiological relevance (< ±2-fold change). E2, 17β-estradiol; T, testosterone; KT, 11-ketotestosterone; FSH, follicle stimulating hormone; LH, luteinizing hormone; HDL, high-density lipoprotein; LDL, low density lipoprotein……………………………………………………………………………………..63

Figure 4.1. Cumulative production of eggs (fecundity) by medaka exposed to the greater concentration of the TBCO (607 µg/ g food, w/w), the lesser concentration of TBCO (58 µg/g food, w/w) and solvent control. The values represent the mean cumulative number of eggs per female over a 21-day period. The experiment included 4 replicate tanks, and each contained 8

female/male medaka. Asterisks indicate a significant difference ($p < 0.05$) when compared to the control group……………………………………………………………………………………..81

Figure 4.2. Graphical representation of the transcript response profile of the HPGL-axis in Japanese medaka exposed to the lesser concentration of TBCO (58 µg/ g food). Gene expression data are represented as striped colour sets with notches denoting sex of fish. Eight colours were used to represent different fold-change thresholds. Criteria not met denotes a lack of statistical difference ($p < 0.05$) or lack of physiological relevance (< ±2-fold change). E2, 17β-estradiol; T, testosterone; KT, 11-ketotestosterone; FSH, follicle stimulating hormone; LH, luteinizing hormone; HDL, high-density lipoprotein; LDL, low density lipoprotein………………………84

Figure 4.3. Graphical representation of the transcript response profile of the HPGL-axis in Japanese medaka exposed to the greater concentration of TBCO (607 µg/ g food). Gene expression data are represented as striped colour sets with notches denoting sex of fish. Eight colours were used to represent different fold-change thresholds. Criteria not met denotes a lack of statistical difference ($p < 0.05$) or lack of physiological relevance (< ±2-fold change). E2, 17β-estradiol; T, testosterone; KT, 11-ketotestosterone; FSH, follicle stimulating hormone; LH, luteinizing hormone; HDL, high-density lipoprotein; LDL, low density lipoprotein…………...85

Figure 5.1. Chemical structures of TBPH and two identified OH-TBPH isomers……………...96

Figure 5.2. Chromatogram of extracted ions with *m/z* 640.9946 (10 ppm window) for (A) commercial standard (B) FM-550 technical product (C) BZ-54 technical product using Q Exactive in negative ion mode. (D) Mass spectra of OH-TBPH. (E) Product ion mass spectra of ion at *m/z* 640.9946. (a) OH-TBPH1, (b) OH-TBPH2, (c) TBPH……………………………...101

Figure 5.3. Chromatogram of extracted ions with *m/z* 640.9946 and *m/z* 723.9486 (10 ppm window) for (A) FM-550 technical product (B) house dust using Q Exactive (SIM) in both negative ion mode and positive ion mode. (C) Mass spectra of TBPH in positive ion mode. (D) Product ion mass spectra of ion at *m/z* 723.9486 in positive ion mode……..……………..…...104

Figure 5.4. Concentrations of TBPH (A), OH-TBPH1 (B) and OH-TBPH2 (C) in 23 dust samples from 8 houses. Dotted lines were used to separate house dust samples among different houses. The samples between the two red dotted lines were from a house built in 2004, which had greater concentrations of TBPH and OH-TBPH isomers…………………………………110

Figure 5.5. (A) Log−linear regression between concentrations of TBPH and OH-TBPH in 23 dust samples. (B) Comparison of relative contributions of TBPH and OH-TBPH isomers in dust samples, commercial standard, BZ-54 technical product, and FM-550 technical product. The *y* axis indicates the log-transformed percentages of TBPH and OH-TBPHs in different samples. Colors differentiate dust samples or standard. Chinastd indicates the commercial TBPH standard produced in China. A *t*-test was used to evaluate statistical difference. ***$p < 0.001$............112

Figure 6.1. Mean concentrations of TBPH (A), OH-TBPH1 (B), and OH-TBPH2 (C) in dust from daycares across Saskatoon, SK, Canada (n=10). Dust was collected from higher traffic-higher toy environments (HT-HT), lower traffic-lower toy environments (LT-LT), and higher traffic-lower toy environments (HT-LT). Samples were collected in summer of 2013 and winter of 2014 (n=10, per room type/season). Error bars represent standard deviation, lower case letters represent statistically significant differences, $p < 0.05$............131

Figure 6.2. Mean concentrations of TBB (A) and ΣOH-TBBs (B) in dust from daycares across Saskatoon, SK, Canada (n=10). Dust was collected from higher traffic-higher toy environments (HT-HT), lower traffic-lower toy environments (LT-LT), and higher traffic-lower toy environments (HT-LT). Samples were collected in summer of 2013 and winter of 2014 (n=10, per room type/season). Error bars represent standard deviation, lower case letters represent statistically significant differences, $p < 0.05$............132

Figure 6.3. Bioaccessibilities of TBPH, TBB and their OH-isomers (ΣOH-TBPH1/2 and ΣOH-TBBs) in reference dust (n=4). Bioaccessibilities were tested with and without Tenax enhancement and compared to data from a previous *in vitro* study[177]. Error bars represent standard deviation, lower case letters represent statistically significant differences, $p < 0.05$............137

Figure 6.4. Bioaccessibilities of TBPH, TBB and their OH-isomers (ΣOH-TBPH1/2 and ΣOH-TBBs) in dust (n=14) collected in the summer of 2013 and winter of 2014 from daycares in Saskatoon, SK, Canada. Bioaccessibilities of TBPH and TBB were compared to data from a previous *in vitro* study[177]. Error bars represent standard deviation, lower case letters represent statistically significant differences, $p < 0.05$............138

Figure C2.S1. The control for recovery of signal activity of (A) TBB at $5x10^{-01}$ mg/L, (B) TBPH at 1000 mg/L, and (C) TBCO at 300 mg/L measured by the yeast androgen screen (YAS).

A baseline agonist (DHT) concentration of $1.45 x 10^{-3}$ mg/L was added to each well with increasing concentrations added to demonstrate the recovery of signal activity. Activity is presented as mean± SE. Each assay contained four wells per NBFR exposure concentration. Exposures that resulted in effects that were significantly different than inhibition controls (agonist + NBFR) are indicated by asterisks (*p<0.05)……………………………………..185

Figure C2.S2. The control for recovery of signal activity of (A) TBB at $5x10^{-01}$ mg/L, (B) TBPH at 0.03 mg/L, and (C) TBCO at 30 mg/L measured by the yeast estrogen screen (YES). A baseline agonist (E2) concentration of $8.17x 10^{-4}$ mg/L was added to each well with increasing concentrations added to demonstrate the recovery of signal activity. Activity is presented as mean± SE. Each assay contained four wells per NBFR exposure concentration. Exposures that resulted in effects that were significantly different than inhibition controls (agonist + NBFR) are indicated by asterisks (*p<0.05)…………………………………………………………..186

Figure C3.S1. Profile analysis of daily fecundity of (A) solvent control vs. the greatest dose of the TBPH/TBB mixture and (B) solvent control vs. the low dose of the TBPH/TBB mixture. The experiment included 4 replicate tanks, and each contained 8 female fish. The profile (parallelism) of TBPH/TBB high was statistically different than solvent control. Significant differences of parallelism were set at $p < 0.05$…………………………………………………189

Figure C3.S2. Within-group repeated measures analysis of variance of (A) daily egg production and (B) pooled time-points of fish exposed to the greatest dose of the TBPH/TBB mixture. Time-points were pooled to preserve significant differences after Bonferroni adjustments. Asterisks indicate significant differences ($p < 0.05$) when compared to 100% fecundity (group 1). Significant within-group main effects were also observed in daily egg production……………190

Figure C4.S1 Profile analysis of daily fecundity of (A) solvent control vs. the high dose of TBCO and (B) solvent control vs. the low dose of TBCO. The experiment included 4 replicate tanks, and each contained 8 female fish. The profile (parallelism) of TBCO low was statistically different than solvent control. Significant differences of parallelism were set at $p < 0.05$……193

Figure C4.S2. Within-group repeated measures analysis of variance of (A) daily deposition of eggs and (B) pooled time-points of fish exposed to the lesser concentration of TBCO. Time-points were pooled to preserve significant differences after Bonferroni adjustments. Asterisks

indicate significant differences ($p < 0.05$) when compared to 100% fecundity (group 1). Significant within-group main effects were also observed in daily egg production……………194

Figure C5.S1. Chromatogram of extracted ions with *m/z* 640.9946 (10 ppm window) in negative ion mode for commercial standard using pure methanol as mobile phase……………………..195

Figure C5.S2. Chromatogram of extracted ions with *m/z* 640.9946 (10 ppm window) in negative ion mode for highly purified standard (AccuStandard, Connecticut, U.S.)…………………….196

Figure C5.S3 (A) Chromatogram of extracted ions with *m/z* 666.9861 (10 ppm window) in positive ion mode for BZ-54 standard. (B) Mass spectra of OH-TBPH in positive ion mode with mass error of 0.75 ppm to sodium adduct……………………………………………………197

Figure C5.S4. Ultra-High Resolution LC/mass spectrometry (above) and ^{1}H NMR (bottom) analysis of purified OH-TBPH standards. The impurity of TBPH was 100-fold lower than OH-TBPH2 in purified standard…………………………………………………………………….198

Figure C5.S5. (A) TBPH was eluted in the first fraction from Florisil cartridges using DCM; (B) TBPH isomers were eluted in the third fraction from Florisil cartridges using a mixture of methanol:DCM (v/v, 1:1)……………………………………………………………………….199

Figure C5.S6. Comparison of the SIM mode and full scan mode for OH-TBPH analysis in dust samples. (A) OH-TBPH isomers could not be detected under full scan mode when ions were extracted in a 10 ppm window. (B) Two OH-TBPH isomers were successfully detected using SIM mode when ions were extracted in a 10 ppm window. (C) TBPH was observed in full scan mode. (D) The total ion intensity in negative ion mode was much greater than those of OH-TBPH at the similar elution time. (E) Total ion intensity in positive ion mode and comparison to TBPH intensity……………………………………………………………………………200

Figure C6.S1. Schematic depicting (A) a pre-loaded Tenax incubation envelope, and (B) Tenax loaded (sealed) incubation envelopes…………………………………………………………..202

Figure C6.S2. Recovery of Tenax and dust (NIST) following incubation in CE-PBET (n=6). Error bars represent standard deviation…………………………………………………………...203

Figure C6.S3. Distribution of TBPH, TBB or their OH-isomers in gastro-intestinal fluid, Tenax, colon fluid, and dust…………………………………………………………………………204

Figure C6.S4. Log transformed concentration of TBPH in higher traffic-higher toy environments (HT-HT), lower traffic-lower toy environments (LT-LT), and higher traffic-lower toy environments (HT-LT). Dust was collected from each of these environments in ten daycares across Saskatoon, SK, Canada in summer (A), and winter (B)……………………………...206

Figure C6.S5. Log transformed concentration of TBB in higher traffic-higher toy environments (HT-HT), lower traffic-lower toy environments (LT-LT), and higher traffic-lower toy environments (HT-LT). Dust was collected from each of these environments in ten daycares across Saskatoon, SK, Canada in summer (A), and winter (B)……………………………...207

Figure C6.S6. Log transformed concentration of OH-TBPH1 (A,B) and OH-TBPH2 (C,D) in higher traffic-higher toy environments (HT-HT), lower traffic-lower toy environments(LT-LT), and higher traffic-lower toy environments (HT-LT). Dust was collected from each of these environments in ten daycares across Saskatoon, SK, Canada in summer (A,C), and winter (B,D)……………………………………………………………………………………208

Figure C6.S7. Log transformed concentration of and ΣOH-TBB1/2/3 in higher traffic-higher toy environments (HT-HT), lower traffic-lower toy environments (LT-LT), and higher traffic-lower toy environments (HT-LT). Dust was collected from each of these environments in ten daycares across Saskatoon, SK, Canada in summer (A), and winter (B)………………………………209

LIST OF ABBREVIATIONS

< d.l	below limit of detection
°C	degree celsius
16S	16S rRNA
20β-HSD	20-beta-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
Activin BA	activin beta A chain
Activin BB	activin beta B chain
ADD_{pot}	potential average daily dose
AF	bioaccessibility factor
AhR	aryl hydrocarbon receptor
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
AR	androgen receptor
ARα	androgen receptor alpha
AT	average time
ATRF	Aquatic Toxicology Research Facility
BEH-TEBP or TBPH	bis(2-ethylhexyl)-3,4,5,6-tetrabromophthalate
BFRs	brominated flame retardants
BTBPE	1,2-bis-(2,4,6-tribromophenoxy)ethane
BZ-54	Firemaster® BZ-54
C	concentration of contaminant
C18	carbon chain with length of 18-carbons
cDNA	complementary DNA
CE-PBET	colon-extended physiologically based extraction test
cGnRH-II	chicken-type gonadotropin-releasing hormone II
CID	collision induced dissociation
cm	centimeter
CPRG	chlorophenol red-β-D-galactopyranoside
CYP	cytochrome P450

CYP11A	cytochrome P450 11A (desmolase)
CYP11B	cytochrome P450 11B
CYP17	cytochrome P450 17A1
CYP19A	cytochrome P450 19A
CYP19B	cytochrome P450 19B
CYP21	cytochrome P450 21 (steroid 21-hydroxylase)
CYP3A	cytochrome P450 3A
d	day(s)
DBDPE	decabromodiphenyl ethane
DCM	dichloromethane
DecaBDEs	deca-polybrominated diphenyl ethers
DEHP	bis(2-ethylhexyl)-phthalate
DfE	design for the environment
DHT	dihydrotestosterone
dm	dry mass
DNA	deoxyribonucleic acid
E2	17-β-estradiol
EC50	the concentration at which half-maximal response is observed
ECE	early childhood environment
ED	exposure duration
EDC	endocrine disrupting compound
EDSP	endocrine disruptor screening program
EC	environmental concentrations
EF	exposure factor
EHB	2-ethylhexyl benzoate
EH-TBB or TBB	2-ethylhexyl-2,3,4,5-tetrabromobenzoate
EI	electron impact
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EPS	expandable polystyrene

ER	estrogen receptor
EREs	estrogen response elements
ERα	estrogen receptor alpha
Erβ	estrogen receptor beta
ESI	eletrospray ionization
EtOH	ethanol
EU	European Union
eV	electron volt
F	frequency of exposure
F-BDE-47	fluorinated polybrominated diphenyl ether-47
FM-550	Firemaster® 550
FRs	flame retardants
FSHR	follicle stimulating hormone receptor
g	gram
g	gravity
GAPS	Global Atmospheric Sampling Network
GC	gas chromatography
GM	geometric mean
GnRH RI	gonadotropin receptor type I
GnRH RII	gonadotropin receptor type II
GnRH RIII	gonadotropin receptor type III
GSD	geometric standard deviation
GSI	gonadal somatic index
GTHa	glycoprotein hormone alpha chain
H295R	human adrenocortical cell line
H4IIE	rat hepatoma cell reporter assay
hAR	human androgen receptor
HBCD	hexabromocyclodecane
HCD	high-energy collisional dissociation
HDLR	high density lipoprotein receptor

hER	human estrogen receptor
HF	hydroxyflutamide
HSI	hepatic somatic index
HMGR	hydroxymethylglutaryl CoA reductase
HPLC	high pressure liquid chromatography
HPV	high production volume
HQ	hazard quotient
hr or hrs	hour(s)
HT	4-hydroxytamoxifen
IADN	Integrated Atmospheric Deposition Network
IC50	concentration at which 50% of a response is inhibited
IDL	instrumental detection limit
Inhibin A	inhibin alpha chain precursor
IR	intake rate
ITPs	isopropylated triphenylphosphate
iTPs	isopropylated triaryl phosphates
K_{ow}	octanol-water partition coefficient
kV	kilovolt
L	litre
LC/MS	liquid chromatography/mass spectrometry
LC50	the concentration which is lethal to 50% of the population
LC-UHRMS	ultra-high resolution liquid chromatography/mass spectrometry
LDLR	low density lipoprotein receptor
LHR	luteinizing hormone receptor
LH-β	luteinizing hormone, beta polypeptide
LOAEL	lowest observed adverse effect level
LRAT	long-range atmospheric transport
lw	lipid weight
M	molar
m/z	mass to charge ratio

MANOVA	multivariate ANOVA
MDL	method detection limit
MEHP	mono-(2-ethylhexyl) tetrabromophthalate
mfGnRH	medaka-type gonadotropin-releasing hormone
mg	milligram
min or mins	minute(s)
mL	millilitre
mm	millimeter
MNGs	multinucleated germ cells
mRNA	messenger ribonucleic acid
MS	mass spectrometry
ms	millisecond
MW	molecular weight
n	sample size
NBFRs	novel brominated flame retardants
ND	non-detect
NeuropepY	neuropeptide Y
ng	nanogram
NGO	non-governmental organization
NIST	National Institute of Standards and Technology
nm	nanometer
NMR	nuclear magnetic resonance
NOAEL	no-observed-adverse-effect-level
NOAEL	no observed adverse effect level
OctaBDE	octa-polybrominated diphenyl ethers
OECD	Organization for Economic Co-operation and Development
OPFR	organophosphate flame retardants
PBDE	polybrominated diphenyl ethers
PBT	persistence, bioaccumulation, toxicity
PCR	polymerase chain reaction

PentaBDE	penta-polybrominated diphenyl ethers
pmol	picomole
POP	persistent organic pollutant
PPARα	peroxisome proliferator activated receptor alpha
ppm	parts per million
psi	pounds per square inch
PUF	polyurethane foam
PXR	pregnane X receptor
q PCR	real-time quantitative polymerase chain reaction
QSAR	quantitative structure activity relationship
R	resolution
REACH	Registration, Evaluation, Authorisation, and Restriction of Chemicals
RPL-7	ribosomal protein L7
s	second(s)
SE	standard error
SEM	standard error of the mean
sGnRH	salmon-type gonadotropin-releasing hormone
SIM	selective ion monitoring
SPE	solid phase extraction
SRM	standard reference material
StAR	steroidogenic acute regulatory protein
T	testosterone
T3	triiodothyronine
TA	Tenax
TB 117	Technical Bulletin 117
TBBA	tetrabromobenzoic acid
TBBPA	tetrabromobisphenol-A
TBBPA-DAE	TBBPA-bis (allyl ether)
TBBPA-DBPE	TBBPA-2,3-dibromopropyl ether
TBBPA-DHEE	TBBPA-dihydroxyethyl ether

TBCO	1,2,5,6-tetrabromocyclooctane
TBMEHP	mono-(2-ethylhexyl) tetrabromophthalate
TCDD	2,3,7,8-tetrachlorodibenzodioxin
TDCPP	tris(1,3-dichloro-2-propyl)phosphate
TPP	triphenyl phosphate
TR	thyroid receptor
U.S.	United States of America
U.S. EPA	United States Environmental Protection Agency
UHR	ultra-high resolution
UK	United Kingdom
v/v	volume/volume
VTG	vitellogenin
VTG I	vitellogenin I
VTG II	vitellogenin II
wk	week
wm	wet mass
ww	wet weight
XPS	extruded polystyrene
YAS	yeast androgen screen
YES	yeast estrogen screen
yr or yrs	year(s)
µg	microgram
µL	microliter
µm	micrometer

NOTE TO READERS

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate Studies and Research guidelines for a manuscript-style thesis. Chapter 1 is a general introduction and literature review, including project goals and objectives. Chapter 7 contains a general discussion and overall conclusion. Chapters 2, 3, 4, 5, and 6 of this thesis are organized as manuscripts for publication in peer-reviewed scientific journals. Chapter 2 was published in the journal, Toxicology Letters, Chapters 3 and 4 were published in Aquatic Toxicology, Chapter 5 was published in Environmental Science & Technology, and Chapter 6 is in preparation for submission for publication. Full citations for the research papers and a description of author contributions are provided following the preface of each chapter. As a result of the manuscript-style format, there is some repetition of material in the introduction and material and methods sections of the thesis. The tables, figures, supporting information, and references cited in each chapter have been reformatted here to a consistent thesis style. References cited in each chapter are combined and listed in the 'References' section of the thesis. Supporting information associated with research chapters are presented in the 'Appendix' section at the end of this tehsis as Cx.Sy format, where 'Cx' indicates chapter number and 'Sy' indicates figure or table number.

1 CHAPTER 1: GENERAL INTRODUCTION

PREFACE

Chapter 1 is a general introduction and literature review regarding the topics of flame retardants, novel brominated flame retardants, their toxicities and prevalence in the indoor and outdoor environments, and relevant characteristics of exposure. Chapter 1 also includes the overall goals and objectives of the project and each study in particular, and includes null hypotheses.

1.1 Flame retardants

Uncontrolled fires are major sources of damage to property and loss of life. In 2007 in the United States alone, uncontrolled fires resulted in $14 billion in damages and over 3,000 deaths[1]. Many of these fires were likely due to the use of greatly flammable materials, which included synthetic polymers and electronics that were incorporated into consumer and industrial products. In efforts to limit uncontrolled fires and their subsequent damage, industries within several countries, which included Canada and the U.S., developed strict standards of fire retardancy that required the addition of flame retardant chemicals to consumer and industrial materials. In 1975, the California State government proposed Technical Bulletin 117 (TB 117), which required upholstered furniture and children's products to withstand a small open flame for 12 seconds, a feat that was generally achieved through the addition of flame retardant compounds. Due to the scale of the Californian economy, several manufacturers have applied the standards of TB 117 to all products destined for North American markets. There were several classes of flame retardants which included a variety of inorganic compounds, most notably metal oxides and aluminum trihydrate, that accounted for 50% of the global annual production of FRs, phosphorous and nitrogen flame retardants which together accounted for 25%, and halogenated flame retardants which accounted for 25%[2]. Of the halogenated flame retardants, brominated flame retardants (BFRs) had the greatest magnitude of total production volume and were most frequently added to consumer and industrial materials[3].

Halogenated flame retardants inhibit the propagation of fire via the halogen atom's interaction with free radicals. These free radicals are formed during the combustion process and act as oxidizing agents. Halogens are effective at trapping free radicals, thereby reducing the capability of the fire to propagate. All four halogens can effectively interact with free radicals, but bromine's properties which include a greater trapping efficiency than chlorine and fluorine and a greater decomposing temperature than iodine, are the best suited to the requirements of flame retardants. Brominated flame retardants can be divided into three categories: additive, reactive, and polymeric, designations which depend on their mode of incorporation into the polymer[1]. Additive BFRs are mixed with the components of a polymer and tend to leach over time whereas reactive and polymeric BFRs are chemically bonded or incorporated directly into the backbone of molecules and are more resistant to release[2]. Due to their tendency of leaching

into the environment and potential effects on health of humans and ecosystems, additive BFRs are the focus of this program of study.

1.2 Brominated flame retardants

Brominated flame retardants are added to numerous products that range from home electronics, furniture, polyurethane foam, and children's toys to industrial cables, plastics, and textiles[4]. There are over 75 brominated compounds that are listed as flame retardants, which include the current major use BFRs tetrabromobisphenol-A (TBBPA), hexabromocyclodecane (HBCD), and deca-polybrominated diphenyl ethers (DecaBDEs) (Figure 1.1)[4]. From 1992 to 2000 total annual global production of BFRs increased by 207% (Table 1.1). In the same period, production of TBBPA increased from 33% of total BFRs to 68% of total annual production, which makes this compound the greatest volume produced globally. Based on total production of BFRs in 2001 and market estimates of HBCD production in 2000, HBCD comprised roughly 5.4% of annual total BFR production and is the second greatest volume BFR used in Europe[5].

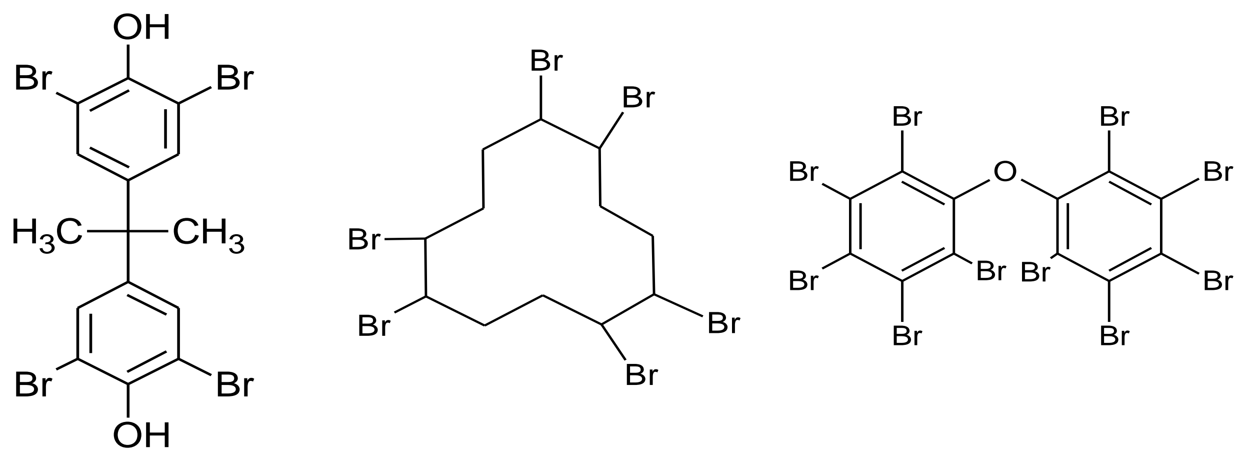

Figure 1.1. Chemical structures of the major BFRs, TBBPA, HBCD, and Deca-BDE.

Table 1.1. Estimated global production volumes of total BFRs, TBBPA, ΣHBCDs, and ΣPBDE congeners for years 1992, 2000, and 2001.

Compound	Volume (tonnes/yr)	Year	Reference
ΣBFRs	150 000	1992	[4]
	>310 000	2000	[2]
TBBPA	50 000	1992	[2, 4]
	210 000	2000	
ΣHBCD	16 700	2001	[6]
ΣPBDEs	16 700	2001	[7]

Several high production volume BFRs including HBCD, and the Penta-, Octa-, and Deca- formulations of polybrominated diphenyl ethers (PBDEs) are ubiquitous in the environment and accumulate in wildlife and humans[8]. PBDEs and HBCD have been detected air, sediment, soil, and sewage sludge in Asia[7, 9], North America[10-14], and Europe[8, 15-19] and in fish[15], fish eating birds[19, 20], marine mammals[19-21], and adipose tissues, serum, and mother's milk of humans[4, 22-25]. Among the major-use BFRs, PBDEs and HBCD were of particular interest because of their larger volumes of production, ubiquity in the environment, and toxic potencies. Potential and known effects of PBDEs which include, endocrine and thyroid modulation, abnormal development, and neurotoxicity[25], have led to global actions imposed on PBDE mixtures. Polybrominated diphenyl ethers were the most widely produced and distributed BFRs until 2004 when manufacturing of two of three technical mixtures was discontinued in the U.S.; in 2009, these mixtures were subsequently added to the list of Persistent Organic Pollutants (POPs) under the international Stockholm Convention[1]. PentaBDE and OctaBDE technical mixtures were phased out of production and importation to North America and Europe. The remaining technical mixture, DecaBDE, has been banned in electrical equipment in the EU and was phased out of production and importation to the U.S. by 2013[26]. HBCD was also considered bioaccumulative, persistent, and was shown to cause harmful reproductive and developmental effects, as such, the EU's REACH program mandated the phase-out of HBCD from Europe by 2015[26]. Though many countries, which included Canada and the U.S., have phased out the use of several formulations of PBDEs, global demand for BFRs has continued to rise, with a 5% annual increase in production in 2005[9]. Consequently, the production and consumption of replacement brominated flame retardants might increase drastically.

1.3 Novel brominated flame retardants

Withdrawal of PBDEs from North American markets led to increased production of non-PBDE BFRs which include novel brominated flame retardants (NBFRs)[27]. Recent investigations show that many replacement NBFRs have similar potential for long-range atmospheric transport (LRAT)[27], environmental persistence, and bioaccumulation[28], however, environmental fates of these replacement compounds remain unclear. Many NBFRs are derivatives of existing BFR chemical structures. Some NBFRs are designed to have greater molecular weights, molecular sizes, and log K_{ows} (Figure 1.2), which has implications for their bioavailabilities and presence in

aquatic systems. These large NBFRs have theoretical log K_{ows} of 8-12 which, due to bulkiness and extreme hydrophobicity, might limit the molecules' bioavailability and bioaccumulation, but increase their persistence in the environment. In spite of these physical-chemical characteristics, several NBFRs have been discovered in biotic and abiotic samples[1], though few toxicological data and environmental measurements yet exist. More information is required to understand the toxicological profiles, transportation mechanisms, and fate of these NBFRs. To date, the most intensively studied emerging NBFRs are: 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB or TBB), bis(2-ethylhexyl)-3,4,5,6-tetrabromophthalate (BEH-TEBP or TBPH), 1,2-bis-(2,4,6-tribromophenoxy)ethane (BTBPE), decabromodiphenyl ethane (DBDPE), and the tetrabromobisphenol A derivatives:TBBPA-2,3-dibromopropyl ether (TBBPA-DBPE), TBBPA-dihydroxyethyl ether (TBBPA-DHEE), and TBBPA-bis (allyl ether) (TBBPA-DAE) (Figure 1.2).

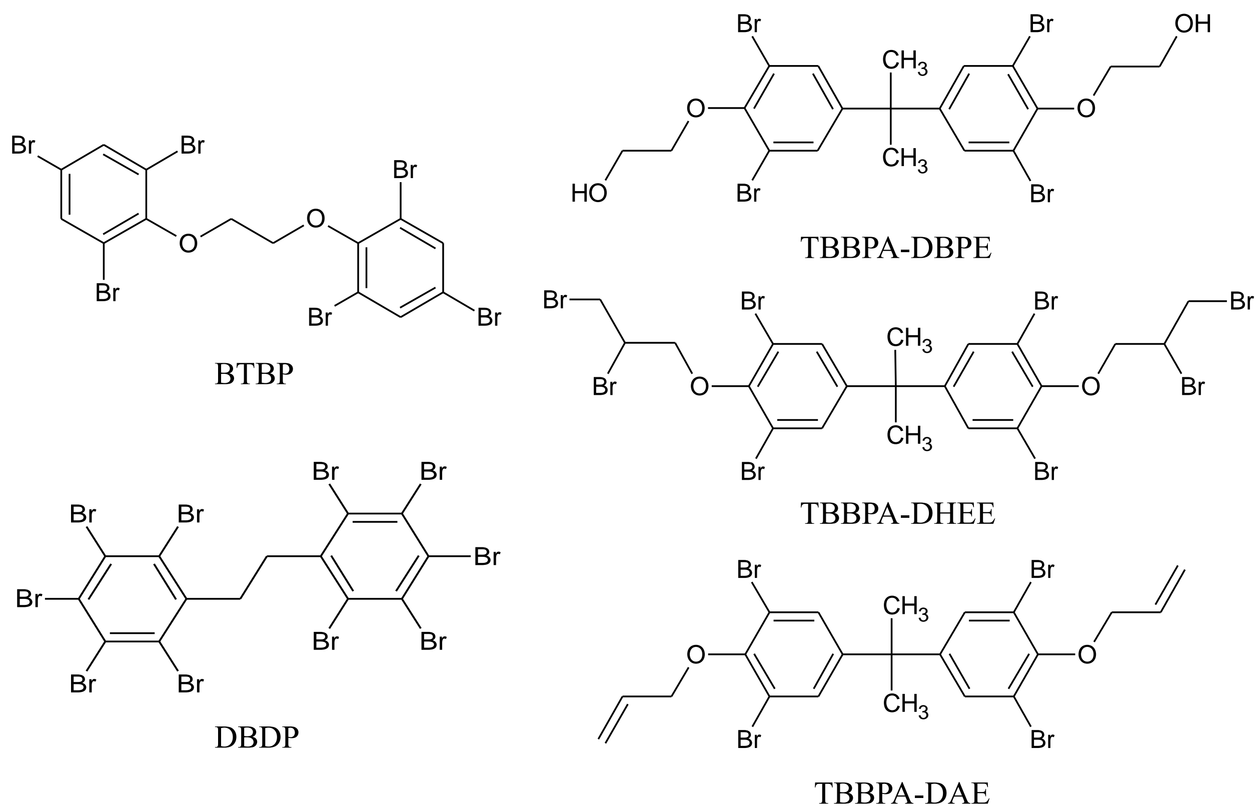

Figure 1.2. Chemical structures of major replacement NBFRs, BTBPE, DBDPE, TBBPA-DBPE, TBBPA-DHEE, and TBBPA-DAE.

1.4 Selection of novel brominated flame retardants

Criteria for the selection of NBFRs to include in the current program of study were as follows: The compound should have, (a) moderate to high production volumes. The production volumes are defined by use of EU definitions of high production volume (HPV), chemicals produced above 1000 tonnes/yr; (b) indications of potential persistence, bioaccumulation, or toxicities from studies of analogous compounds or via modeling software (i.e. PBT profiler, EpiWeb 4.1). Concurrently the NBFRs must have few toxicological data which represents a relevant gap in knowledge; and (c) the compounds should be detected in abiotic/biotic environmental samples. Three NBFRs adequately fit these simple criteria: TBPH, TBB, and tetrabromocyclooctane (TBCO) (Figure 1.3).

TBB and TBPH are additive flame retardants and are components of the technical mixtures Firemaster® 550 (35% TBB, 15% TBPH), Firemaster® BZ-54 (70% TBB, 30% TBPH), and DP-45 (TBPH only), marketed by the Chemtura Corporation[29, 30]. TBCO is an additive flame retardant and is a component of Saytex® BC-48, marketed by the Albermarle Corporation[31]. Firemaster® 550 is used as a replacement for PentaBDE mixtures in polyurethane foams, PVC, and neoprene and TBPH has been used as a plasticizer and listed as a high production volume chemical by the U.S. EPA[30]. From 1990 to 2006, TBPH had a U.S. production volume of 450 – 4,500 metric tons/yr[31] but there is little data on the production volumes of TBB. TBCO is mainly employed as an additive flame retardant in textiles, paints, and plastics[32], and there is currently no information regarding production volumes. TBCO is on the Canadian Environmental Protection Act's non-domestic substances list with as much as 10 tons/yr currently imported into Canada and is a potential replacement compound for HBCD[32, 33].

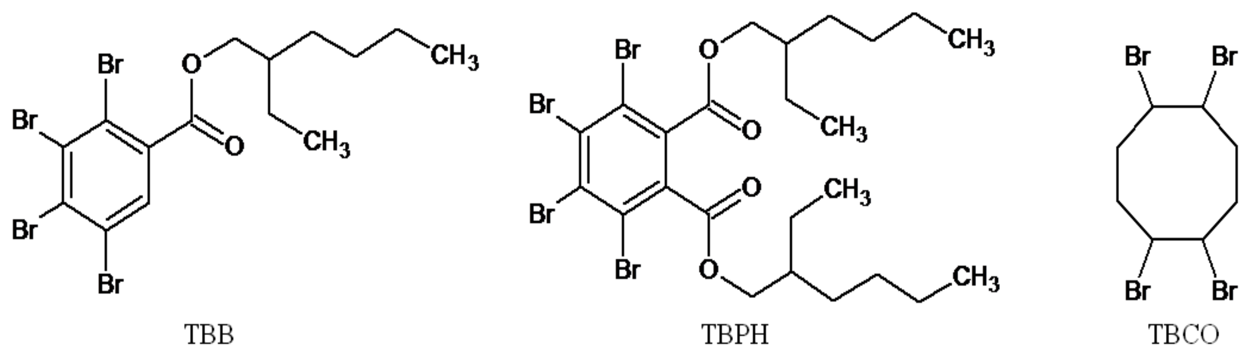

Figure 1.3. Chemical structures of selected NBFRs, TBB, TBPH, and TBCO included in subsequent studies.

1.4.1 Detection of novel brominated flame retardants in the environment

Certain NBFRs, which include TBPH, TBB and TBCO, have similar potentials for bioaccumulation, persistence, and long-range atmospheric transport as PBDEs and HBCD[30, 34, 35]. For example TBPH and TBB have both been detected in several environmental matrices, which include dust, air, and biota and have been listed as NBFRs relevant for further investigation and monitoring in the Norwegian environment[36]. From 2008 to 2010, as part of the Integrated Atmospheric Deposition Network (IADN), TBPH and TBB were detected in the particle-phase at six locations near the North American Great Lakes, and in urban areas from Chicago and Cleveland[30]. The study showed that concentrations of both TBPH and TBB in the atmosphere increased rapidly during the two year sampling period, which indicated that use and/or accumulation of these NBFRs was increasing. As of 2011, the two compounds have also been detected in samples from the Global Atmospheric Sampling (GAPS) Network[37], in house dust in the U.S.[38], and indoor dust in New Zealand[39]. TBPH and TBB have been detected in polyurethane foam in retail baby products in the U.S. as the second most abundant BFRs[40], and were detected in couch foam at 4.2% by weight of flame retardant[41]. Both compounds were detected in sewage sludge from wastewater treatment plants in San Francisco, California[31], and TBPH alone was detected in environmental samples from the high arctic[34]. TBB and TBPH have been discovered in biota, which included blubber from humpback dolphins (mean: <0.04 ng/g, lw; 0.51 ± 1.3 ng/g, lw) and finless porpoises (mean: 5.6 ± 17 ng/g, lw; 342 ± 883 ng/g, lw) from Hong-Kong, South China[42], in filter feeding bivalves (2220 ng/g, lw; 1370 ng/g, lw) , and grazing gastropods (1740 ng/g, lw; 380 ng/g, lw) collected downstream from a textile manufacturing outfall[26]. TBPH has also been detected in 89% of sampled livers from ring-billed gull collected from an industrialized section of the St. Lawrence River downstream from Montreal, Canada[35]. The ring-billed gull samples from the St. Lawrence site boast both the greatest detection frequency of TBPH and the greatest concentration in any avian species (17.6 ng/g, ww). TBCO was detected in herring gull eggs in the North American Great Lakes, though it could not be quantified[27]. TBCO was classified as a potential aquatic hazard and a very persistent and bioaccumulative substance. As such, it was surprising that few data had been collected regarding its occurrence in environmental and biotic matrices. Though data which exhibited the deposition and concentrations of TBPH, TBB, and TBCO in biotic and abiotic environments have been collected, there have been few investigations of their potential toxicities.

1.4.2 Toxicities of TBPH, TBB and TBCO

There are limited data regarding sub-lethal toxicities of TBPH, TBB, and TBCO. TBPH and TBB are brominated analogues of bis(2-ethylhexyl)-phthalate (DEHP) and 2-ethylhexyl benzoate (EHB) respectively. DEHP, is a known toxicant and endocrine disrupting compound (EDC) and is a controlled substance in Canada. Both TBPH and TBB have been observed to undergo sequential debromination in photodegradation experiments[43]. Total debromination of TBPH which leads to the formation of DEHP is possible, and requires further investigation due to DEHP's noted biological effects. DEHP and its active metabolites have several sub-lethal toxicological effects which include, endocrine disruption[44], reproductive dysfunction[45, 46], activation of the aryl hydrocarbon receptor[44, 47], and peroxisome proliferation[48]. The potential endocrine disruption and ability to affect functions of biological pathways of reproduction of DEHP has been tested in several fish species. A Zebrafish (*Danio rerio*) *in vitro* hepatocyte assay system has been used to measure reproductive dysfunction caused by DEHP. Researchers measured modulation of transcript abundances of the estrogen receptor (ER) and production of vitellogenin (VTG). DEHP exposures resulted in significant increases of VTG in male/female hepatocytes, though no definitive pattern was observed regarding modulation of ER[48]. DEHP also affected reproduction of fishes by altering sexual behaviours, egg production, circulating hormone concentrations, and VTG synthesis, a marker of exposure to estrogen-like compounds. In a recent experiment, exposure of Chinese rare minnow (*Gobiocypris rarus*) to DEHP, resulted in greater circulating concentrations of testosterone (T) and 17-β-estradiol (E2) with increased abundances of transcripts of Cyp17 and Cyp19a in female fish and male gonads[49]. Transcription of VTG was also increased in liver of both male and female fish. Exposures of Japanese medaka (*Oryzias latipes*) to DEHP caused decreases in gonadal-somatic indices, decreases in concentrations of VTG in blood, and a reduction in the percentage of females with mature oocytes in ovaries[50]. The anti-estrogenic potential of DEHP might arise from competition with endogenous compounds for interaction with the ER, while the perturbation of oocyte growth and maturation signals have been proposed as mechanism of a decreased ratio of mature oocytes in female ovaries[51]. DEHP is a non-brominated structural analog to TBPH and the NBFR might elicit similar toxic effects, as such, it is surprising there are limited data regarding potential toxicities of TBPH.

While few investigations of toxic effects of TBPH and TBB exist, those studies which have tested sub-lethal endpoints generally exposed test system to the technical mixtures, Firemaster® 550 and Firemaster® BZ-54. Though TBPH and TBB are principle components of these technical mixture, due to the proprietary nature of these formulations, there is little information regarding total components of the mixture. Recent studies have identified at least four components of Firemaster® 550: triphenyl phosphate (TPP), mixtures of isopropylated triphenylphosphate isomers (ITPs), TBPH, and TBB[38, 40]. These previously unidentified flame retardants, TPP and mixtures of ITPs, have associated toxicological properties. Thus exposure to technical mixtures represent the mixed toxicities of all components. These mixed effects might be additive, synergistic, or antagonistic, and cannot represent single component toxicities. Indeed due to differences in the physical-chemical properties of these four components, the compounds would likely enact differing toxicities, differ in partitioning, and differ in their bioavailabilities and bioaccumulative properties. Though exposures to technical mixtures are useful, they are limited in that they cannot identify mechanisms of toxic effect due to potential interactions of the components and alterations to toxicities. Therefore, interpretation and use of toxicological data produced from exposure to technical mixtures requires caution.

Recent studies of the toxic effects of TBPH and TBB have demonstrated potential endocrine disrupting properties of these compounds. Rats exposed to environmentally relevant concentrations of the Firemaster® 550 mixture (1000 μg/day) have shown a 65% increase in concentrations of serum thyroxin, advanced female pubertal onset, and weight gain[52]. This study was one of the first to observe endocrine disrupting effects in terrestrial mammals following exposure to Firemaster® 550 at concentrations less than the no-observed-adverse-effect-level (NOAEL) previously reported by the manufacturer. Due to the toxicities of DEHP, researchers isolated TBPH for further toxicological investigations. A yeast *in vitro* assay system was used to determine potential agonism or antagonism of TBPH to the estrogen and androgen receptors (ER/AR)[53]. The yeast assay demonstrated no agonistic or antagonistic effects to either receptor at all concentrations of TBPH. Because the yeast assay is a receptor mediated system, these results have implications regarding the mode of endocrine disruption of TBPH. The major metabolite of TBPH, mono-(2-ethylhexyl) tetrabromophthalate (TBMEHP) was also tested in *in vivo* systems for potential toxicities[54]. Pregnant rats were exposed to TBMEHP for two days which resulted in hepatotoxicity and maternal hypothyroidism with decreased triiodothyronine

(T3) serum concentrations. Similar to DEHP, the bioactivation and subsequent metabolites of TBPH might have greater toxicological implications than the parent compound. A single investigation which exposed fish to TBPH was conducted; fathead minnow (*Pimephales promelas*) were exposed to the Firemaster® 550 and Firemaster® BZ-54 at 1 mg fish/day[29]. DNA damage, specifically DNA strand breaks, were detected in liver cells during exposure to both Firemaster® formulations, while the effect was lost during subsequent depuration. Both technical mixtures adversely affected DNA integrity in fish, though any of the noted components of the mixtures might have caused the observed effects. These investigations represent the breadth of information regarding toxic effects of TBPH and TBB and have successfully demonstrated potential toxicities of these compounds as well as a current gap in toxicological knowledge. Based on EU criteria TBCO is a potential aquatic hazardous substance and is characterized as a potentially persistent and bioaccumulative compound[55]. Though TBCO is a potential aquatic hazard, there exists no sub-lethal data regarding the toxicological profile or potency of this compound.

1.5 Novel brominated flame retardants in the indoor environment

TBPH, TBB, and TBCO are found in consumer products which include paint, insulation, textiles, polyurethane foams, and adhesives[3, 30, 31] and have been detected in several environmental matrices[30, 36, 37]. Though these NBFRs have been detected in the outdoor environment, BFRs are distinguished from other POPs, such as PCBs, as exposures are principally from indoor sources. BFRs likely migrate from consumer products and partition to dust and air via several processes which include, chemical (volatilization – adsorption) and mechanical (abrasion or direct contact). The processes by which these compounds migrate to dust have implications for distribution within the indoor environment, seasonal changes in concentrations, and bioavailability. Though we are aware of these emission processes, little is known about indoor partitioning, distribution, and exposures to NBFRs and few studies have investigated the presence and concentrations of TBPH, TBB, and TBCO in house dust[38, 39, 56]. Concentrations of these NBFRs in dust are important to exposure characterization and subsequent assessments of risk as dust is considered a relevant vector of exposure; pharmacokinetic models have suggested up to 82% of total BFR exposure in children might be of dust origin[57]. Due to recent global restrictions on legacy BFRs, production of these NBFRs is expected to increase, thus investigations are required to report

concentrations of these compounds in indoor dust and monitor potential changes. As of 2011, there are few data regarding concentrations of TBPH, TBB, or TBCO in house or office dust, and to our knowledge, there exists no data regarding concentrations in dust at Canadian sites (Table 1.2). Additionally, little data exists regarding concentrations of these NBFRs in dust from early childhood environments (ECEs), such as childcare centers or schools. Those data that do exist for homes or offices show that TBPH and TBB are detected at concentrations roughly one order of magnitude lower than PBDEs[38]. This data paired with time course monitoring experiments indicate these compounds might not have yet reached peak production volumes. Indeed, a recent investigation of house dust documented an approximate 2-fold increase in concentrations of TBPH and TBB from 2006 to 2011 (Table 1.2)[56]. Dust is an important vector of exposure for BFRs, thus, investigations into current concentrations and documentation of potential shifts with increases in production of NBFRs are required.

Table 1.2. Median concentrations of TBPH, TBB, and TBCO reported in indoor dust (ng/g, dust)

Country	Location	TBPH	TBB	TBCO	Reference
U.S.	Homes	142	133	-	[38]
U.S.	Homes	140	48	<2	[56]
U.S.	Homes	260	100	<d.l	[56]
UK	School	96	25	-	[58]
Belgium	Homes	13	1	-	[58]
Belgium	Offices	64	7	-	[58]
New Zealand	Homes	12	2	-	[39]
Pakistan	Homes	3.5	0.03	-	[59]

'<d.l' – below limit of detection

1.5.1 Dust as an important vector of exposure to brominated flame retardants

Initial investigations into sources and human exposure pathways of BFRs used studies of organochlorines as reference models. Humans are exposed to dioxins primarily through outdoor and dietary sources[60]. BFRs challenge this paradigm as the primary route of exposure is likely from dietary and indoor sources such as, electronics, furniture and other consumer products. Indications of this paradigm shift arose from the discrepancies discovered between food intake of PBDEs and concentrations found in serum[57, 61]. Scientists noted that food could not account for total body burdens of PBDEs, and concluded there were likely other sources of exposure. For example, concentrations of PBDEs in food and differences in consumption rates could not explain differences in serum concentrations of PBDEs between North Americans and Europeans[62]. Concentrations of PBDEs in dust and serum were compared in California and Massachusetts[63]. Median concentrations of PBDEs in Californian house dust were 4 to 10 times greater than previously reported in the U.S., and serum concentrations of Californian residents were nearly 2-fold greater than residents of Massachusetts. These elevated concentrations of PBDEs in dust from California were likely due to TB 117.

Despite growing evidence of indoor dust as a relevant vector of exposure of BFRs, attempts to correlate concentrations between dust and serum have been hindered. Error in attaining correlative significance was likely due to the inherent variability and challenges of dust collection and variability in type and quantity of FRs added to consumer products. For example, many preliminary investigations sampled dust by collection of vacuum bags from participants[62]. This method was cost-effective, simple, and enhanced participation from the public as it did not require researchers to enter the home. However, samples collected from vacuum bags did not accurately reflect exposure scenarios, because dust from numerous microenvironments was integrated into a single sample[60]. This integration might have reduced the accuracy of exposure assessments if there were varying durations spent in each room or if concentrations of BFRs differed between rooms. Such issues in dust sampling techniques introduced measurement error that might have obscured potential relationships between concentrations of BFRs in dust and serum. Though many challenges were encountered in sample collection, several studies have observed significant correlations between concentrations of BFRs in dust and serum. For example, Swedish researchers have reported significant differences in serum concentrations of

PBDEs in the population, though they were not able to link the differences to occupational or dietary factors. In an investigation of different households and dust, researchers reported a positive linear relationship between concentrations of ΣPBDEs in dust and plasma[64]. However, the relationship was significantly dependent on one observation. In a Belgian study of adults with duplicate diets, concentrations of HBCD in dust, but not diet, were significantly, positively correlated with those in serum[65]. A Danish study of 51 pregnant women from Copenhagen determined concentrations of PBDEs in maternal and umbilical cord plasma and house dust. Positive correlations were found for ΣPBDEs in maternal and umbilical cord plasma and house dust[66]. One of the only associations with PBDE body burdens and dust in North America was conducted in the Greater Boston Area of Massachusetts[67]. Breast milk from 46 first time mothers and a subset of house dust was collected and analyzed for PBDEs. The researchers found a significant positive association between concentrations of PBDEs, excluding BDE 209, between the two sample groups. Another European study of Danish participants observed a significant positive correlation between concentrations of BDE-47 in dust and placental tissue, though the correlation did not exist for any other congener[68].

Significant positive correlations between concentrations of BFRs in dust and serum support dust as an important vector of exposure. As of yet, there are no standard methods for dust collection, which might affect future investigations of concentrations in house dust and assessments of risk[60]. Given the benefits and disadvantages of each sampling method, and uncertainty regarding their relevance, there is yet insufficient information to develop a standard method of sample collection. Additionally, in any assessment of exposure, the predicted ingestion rates of dust are generally conservative (protective). The daily intake for dust is estimated at 60-100 mg/day for small children (1-4 yr) and 50 mg/day for adults[69]. The estimated dust ingestion rate used in most risk assessments of dust is based on a small number of primary studies designed to derive estimates of soil ingestion. These intake estimates generally do not account for different densities and organic content of the matrix or differences in time spent indoors or outdoors. These latter parameters are important for Canadian populations as in winter months, time spent indoors can increase to > 90%[61]. These predicted ingestion rates might skew exposure estimates for at-risk populations which include children.

1.5.2 Exposure of children to brominated flame retardants

Young children are a susceptible population and are at greater risk of exposure to BFRs than adults. There has recently been greater attention regarding BFRs in dust at ECEs (childcare centers and schools)[70]. It has been noted by several researchers that children generally have greater body burdens of BFRs than adults[71-73]. For example, measurements of PBDE congeners in serum from 2-5 yr children in California discovered concentrations that were 2 to 10 times greater than in most adults in the U.S.[74]. Increased body burdens in children might be partially explained by increased exposures to dust. Young children exhibit greater exploratory behaviours which include hand-to-mouth actions and other activities that place them in direct contact with contaminated surfaces. Young children generally have greater associations with floors/surfaces, and have poor hygienic practices. Children also have smaller body masses relative to adults, breathe more air, and eat more food per unit of body mass[75]. In addition, small children are susceptible to the adverse effects of BFRs because they are still developing and have not matured immunologically and physiologically.

In North America, young children spend a great amount of time in ECEs. Many young children spend as much as ten hours per day, five days per week in child care and preschool centers. In California alone there are over 49 000 licensed childcare facilities with 80% listed as family run centers located in homes[75]. By kindergarten over 50% of all children attend some licensed childcare facility. Recent studies indicate that childcare facilities might be sources of contaminants that are hazardous to children's health[75, 76]. Greater exposure of children to BFRs in care facilities might be due to the relatively greater amounts of children's products and toys within. A research group from North Carolina recently detected great concentrations of BFRs in 78% of all children's products tested[40] with detection frequencies of TBPH and TBB at roughly 17%. TBPH and TBB are replacement compounds for Penta-BDE mixtures, and as such, are added to polyurethane foam products. Due to the clumsy nature of children, many products including furniture and toys contain polyurethane foam. It has also been discovered that in California some baby products are considered juvenile furniture, and as such, must comply with the stringent fire-safety standards of TB 117[40]. The amount of time spent in childcare facilities coupled with increased densities of children's products that generally contain great quantities of BFRs likely result in increased exposures to children, and might explain heightened

concentrations of BFRs in children's serum. Children represent a relevant demographic for characterization of exposure to BFRs. But due to ethical and practical purposes there are limited studies that have attempted to correlate dust and serum concentrations of these compounds in children. Some researchers have used organisms that mimic environmental exposures and behaviour patterns of children. In California, serum congener profiles of PBDEs in house cats correlated significantly with congener profiles in house dust, but did not correlate with dietary congener patterns, which indicates a non-dietary source of PBDE exposure[77]. Though the study did not mention differential metabolism of PBDEs between organisms or differences in time spent indoors/outdoors, it represented new research that attempted to address relevant gaps in current knowledge of BFR exposures.

1.6 Conclusions

Due to recent global regulations which have banned all congener formulations of PBDEs, and increased scrutiny of HPV compounds such as HBCD, there have been increases in production of several NBFRs. The increased production of these compounds was accompanied by increases in frequencies of detection, and concentrations detected in biotic and abiotic environmental samples. *In silico* modeling predicted similar physical-chemical characteristics of many NBFRs and legacy BFRs which would have implications for a NBFR's persistence, bioaccumulation, and toxicological profiles. The NBFRs, TBPH, TBB, and TBCO were selected as candidate compounds for this program of study due to their high production volumes, presence in environmental samples, and potential toxicities. These compounds were of concern to regulatory entities which include Environment and Climate Change Canada, the U.S. EPA, and the Norwegian Pollution Control Authority and were targets in several active monitoring programs which include the IADN and GAPS. There existed few toxicological data regarding these compounds, yet initial screening data generated from *in silico* and *in vitro* experiments indicated these NBFRs were potential EDCs. Further explorations into mechanisms of toxic effects, whole-organism effects, and concentrations in the indoor environment are required to generate data to more accurately characterize toxicological profiles and potential exposures to TBPH, TBB, and TBCO.

1.7 Objectives

The overall objective of this research program was to produce data which described the potential toxicities and exposures of TBPH, TBB, and TBCO to humans in the indoor environment. Two distinct but connected phases of research were used to characterize the hazards and risks to human health associated with these NBFRs. The first phase of this research program focused on the characterization of toxicity of TBPH, TBB, and TBCO. Specific goals of this phase were reviewed in objectives 1 and 2 (chapters 2,3,4). The second phase of this research program focused on the characterization of exposure to these NBFRs from the indoor environment. Specific goals of this phase were reviewed in objectives 3 and 4 (chapters 5 and 6). Though these two phases of research were distinct, together they constituted a comprehensive program of research which described the toxicities and exposures of these NBFRs.

Objective 1. Generate screening level data regarding endocrine disruption and TCDD-like effects for TBPH, TBB, and TBCO by use of *in vitro* bioassays (Chapter 2).

Little was known about the potential endocrine modulating and TCDD-like effects of TBPH, TBB, or TBCO. DEHP, the non-brominated analogue of TBPH is a controlled substance with endocrine disrupting effects which can lead to changes in fertility and fecundity and has been shown to interact with- and activate the AhR[44, 47]. Experiments of *in vitro* metabolism have also shown that TBPH is metabolized to mono(2-ethylhexyl) tetrabromophthalate (TBMEHP)[78], a brominated analogue of MEHP which itself was shown to affect concentrations of steroid hormones including estradiol and testosterone in rat ovarian follicles[79, 80]. Due to similarities of these NBFRs with known EDCs, screening level experiments to characterize potential endocrine modulating effects were necessary. Therefore, the specific objectives and associated null hypotheses were:

1) To determine receptor mediated endocrine disrupting effects of TBPH, TBB, and TBCO by use of the yeast estrogen screen (YES) and yeast androgen screen (YAS) assay systems and non-receptor mediated steroidogenic effects via the mammalian H295R cell model.

   $H_0$1: There are no statistically significant differences in activity of β-galactosidase in the YES or YAS assay between control cells and cells exposed to TBPH, TBB, or TBCO

$H_0$2: There are no statistically significant differences in activity of β-galactosidase in the YES or YAS assay between control cells activated by E2 or DHT respectively, and activated cells co-exposed to TBPH, TBB or TBCO.

$H_0$3: There are no statistically significant differences in concentrations of T in H295R conditioned media between control cells and cells exposed to TBPH, TBB, or TBCO.

$H_0$4: There are no statistically significant differences in concentrations of E2 in H295R conditioned media between control cells and cells exposed to TBPH, TBB, or TBCO.

2) To determine aryl hydrocarbon receptor (AhR) binding activities of TBPH, TBB, and TBCO by use of the H4IIE rat hepatoma cell reporter assay.

$H_0$1: There are no statistically significant differences in AhR activity in the H4IIE assay between control cells and cells exposed to TBPH, TBB, or TBCO

Objective 2. Identify potential endocrine disrupting effects of a mixture of TBPH and TBB or TBCO via fecundity of Japanese medaka (*Oryzias latipes*) and investigate potential mechanisms of action via expression of genes across the HPGL-axis (Chapters 3,4).

TBPH, TBB and TBCO elicited endocrine disrupting effects in *in vitro* assessment of ER and AR activity, and via modulation of concentrations of hormones (Chapter 2). Positive results from these screening level assessments necessitated further characterization of the endocrine disrupting effects of these compounds. Small fish models were appropriate test organisms to further test EDC like effects due to the significant conservation of the HPGL axis across vertebrates, which allowed for extrapolation of results from fish tests to predict mechanisms of action in other vertebrates (mammals). Therefore these studies determined the potential modulation of fish fecundity and reproductive success in Japanese medaka (*Oryzias latipes*) following exposures to a mixture of TBPH and TBB or TBCO. It further investigated potential mechanisms of action via expression of genes along the HPGL-axis. Therefore, the specific objectives and associated null hypotheses were:

1) To determine if exposure to the mixture of TBPH and TBB or TBCO alters fecundity of Japanese medaka (*Oryzias latipes*).

$H_0 1$: There is no statistically significant difference in daily egg production between fish exposed to the mixture of TBPH and TBB and freshwater/solvent control fish.

$H_0 2$: There is no statistically significant difference in daily egg production between fish exposed to TBCO and freshwater/solvent control fish.

$H_0 3$: There is no statistically significant difference in transcript abundance of the 36 genes along the HPGL axis between fish exposed to the mixture of TBPH and TBB and freshwater/solvent control fish.

$H_0 4$: There is no statistically significant difference in transcript abundance of the 36 genes along the HPGL axis between fish exposed to TBCO and freshwater/solvent control fish.

Objective 3. Detect, identify and quantify TBPH and the hydroxylated contaminants, OH-TBPH1 and OH-TBPH2 in, analytical standards, the technical mixtures Firemaster® 550 and BZ-54, and environmental samples (Chapter 5).

In an effort to quantify TBPH in dust from ECEs, a new analytical method which used ultra-high resolution LC/MS was developed. Due to the high resolution of the instrument, peaks which represented two novel compounds were observed in chromatograms from analytical standards of TBPH. Further examination of the technical mixtures, Firemaster® 550 and BZ-54, confirmed the presence of these unknown peaks. Therefore, this study determined their chemical formula and structures and attempted to detect and quantify these compounds in samples of indoor dust. The specific objectives and associated null hypotheses were:

1) To determine the precise chemical formulae of the two compounds and discover the molecular structures via fragmentation (MS^2) analysis and confirm via H^1 NMR.

$H_0 1$: There is no difference between predicted chemical formulae and actual chemical formulae derived from molecular structures of these two OH-compounds and TBPH.

2) To determine the presence and concentrations of these compounds in environmental samples

$H_0 1$: There are no statistical differences between concentrations of the novel compounds in dust and procedural or laboratory blanks.

3) To determine differences in relative contributions of TBPH and the two novel compounds in technical formulations and in environmental samples.

$H_0 1$: There are no statistical differences between relative contributions of TBPH and the two novel compounds in technical formulations and environmental samples.

Objective 4. Quantify TBPH, OH-TBPH1, OH-TBPH2, TBB and ΣOH-TBBs in samples of dust from ECEs to determine seasonal differences in concentrations and microenvironment specific influences. Further characterize exposure to children by determining bioaccessibilities of these compounds (Chapter 6).

TBPH and TBB are endocrine disrupting compounds (Chapters 2,3) which have been detected in environmental samples, though few studies have attempted to quantify these compounds in dust from ECEs. Further, ingestion of dust is an important exposure pathway of BFRs, particularly for children, though there have been few studies regarding bioaccessibilities of these compounds. To more accurately evaluate exposure of children to NBFRs via ingestion of dust, the oral bioaccessibility of NBFRs associated with dust were investigated. Studies have shown that concentrations of legacy BFRs in indoor dust can differ between summer and winter and between microenvironments with varying amounts and types of consumer products. Therefore, this study attempted to determine concentrations of these compounds in ECEs and characterize seasonal differences or microenvironment specific influences on concentrations. It further characterized the bioaccessibilities of these compounds in the dust matrix. The specific objectives and associated null hypotheses were:

1) To determine concentrations of TBPH, OH-TBPH1, OH-TBPH2, TBB and ΣOH-TBBs in samples of dust from ECEs in summer and winter seasons.

$H_0 1$: There are no statistical differences between concentrations of TBPH, OH-TBPH1, OH-TBPH2, TBB or ΣOH-TBBs in dust from ECEs in summer and winter.

2) To determine differences in concentrations of TBPH, OH-TBPH1, OH-TBPH2, TBB or ΣOH-TBBs in three microenvironments in ECEs.

$H_0$1: There are no statistical differences between concentrations of TBPH, OH-TBPH1, OH-TBPH2, TBB or ΣOH-TBBs in high traffic/high toy and low traffic/low toy microenvironments.

$H_0$2: There are no statistical differences between concentrations of TBPH, OH-TBPH1, OH-TBPH2, TBB or ΣOH-TBBs in high traffic/high toy and high traffic/low toy microenvironments.

$H_0$3: There are no statistical differences between concentrations of TBPH, OH-TBPH1, OH-TBPH2, TBB or ΣOH-TBBs in low traffic/low toy and high traffic/low toy microenvironments.

3) To determine differences in bioaccessibilities of TBPH and OH-TBPHs via the Tenax enhanced colon-extended, physiologically based extraction method (CE-PBET).

$H_0$1: There is no statistical difference between bioaccessibilities of TBPH and OH-TBPHs in the CE-PBET model system.

4) To determine differences in bioaccessibilities of TBB and OH-TBBs via the Tenax enhanced colon-extended, physiologically based extraction method (CE-PBET).

$H_0$1: There is no statistical difference between bioaccessibilities of TBB and OH-TBBs in the CE-PBET model system.

2 CHAPTER 2: *IN VITRO* ENDOCRINE DISRUPTION AND TCDD-LIKE EFFECTS OF THREE NOVEL BROMINATED FLAME RETARDANTS: TBPH, TBB, & TBCO

PREFACE

Little was known about the potential endocrine disrupting- and TCDD-like effects of TBPH, TBB, and TBCO. For example, DEHP, the non-brominated analogue of TBPH is a known endocrine disrupting compound and has been shown to interact with the AhR, though no studies have investigated these effects for TBPH. The aim of Chapter 2 was to utilize *in vitro* screening level assessment tools, similar to procedures used by the U.S. EPA Endocrine Disruptor Screening Program (EDSP), to determine if these NBFRs elicited endocrine disrupting, or TCDD-like effects. Initial screening level assessments allowed for rapid determinations of potential endocrine disrupting effects and were necessary to ensure the appropriate use of further *in vivo* experimentation. This chapter was included in the first phase of this research program, the characterization of potential toxicities of TBPH, TBB, and TBCO.

The content of Chapter 2 was reprinted (adapted) from *Toxicology Letters*, (10.1016/j.toxlet.2013.09.009) D.M.V. Saunders, E.B. Higley, M. Hecker, R. Mankidy, J.P. Giesy, "*In vitro* endocrine disruption and TCDD-like effects of three novel brominated flame retardants: TBPH, TBB, & TBCO" 223, 252-259. Copyright 2013, with permission from Elsevier.

Author Contributions:

David M.V. Saunders (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript

Eric B. Higley (University of Saskatchewan) provided laboratory assistance with the *in vitro* assay systems and subsequent analysis.

Drs. Rishikesh Mankidy, Markus Hecker and John P. Giesy (all at University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

2.1 Abstract

The novel brominated flame retardants (NBFRs), 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB), Bis(2-ethylhexyl)-2,3,4,5-tetrabromophtalate (TBPH), and 1,2,5,6-tetrabromocyclooctane (TBCO) are components of flame retardant mixtures including Firemaster® 550 and Saytex® BC-48. Despite the detection of these NBFRs in environmental and biotic matrices, studies regarding their toxicological effects are poorly represented in the literature. The present study examined endocrine disruption by these three NBFRs using the yeast YES/YAS reporter assay and the mammalian H295R steroidogenesis assay. Activation of the aryl hydrocarbon receptor (AhR) was also assessed using the H4IIE reporter assay. The NBFRs produced no TCDD-like effects in the H4IIE assay or agonistic effects in the YES/YAS assays. TBB produced a maximal antiestrogenic effect of 62% at 0.5 mg/L in the YES assay while TBPH and TBCO produced maximal antiandrogenic effects of 74% and 59% at 300 mg/L and 1500 mg/L, respectively, in the YAS assay. Significant effects were also observed in the H295R assay. At 0.05, 15, and 15 mg/L TBB, TBPH, and TBCO exposures, respectively resulted in a 2.8-fold, 5.4-fold, and 3.3-fold increase in concentrations of E2. This is one of the first studies to demonstrate the *in vitro* endocrine disrupting potentials of TBB, TBPH, and TBCO.

2.2 Introduction

Brominated flame retardants (BFRs) are added to materials such as electronics, textiles, polyurethane foams, and plastics to increase their fire resistance. There are at least 175 brominated compounds that are listed as flame retardants[2] including hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) which had the largest worldwide production volumes at 22,000 tons/yr in 2003[27] and 170,000 tons/yr in 2004, respectively. Polybrominated diphenyl ethers (PBDEs) were once the most widely used BFRs, but several of the technical mixtures were phased-out of use in Europe, followed by several U.S. states. In an agreement between the U.S. EPA and chemical manufacturers, the PentaBDE and OctaBDE technical mixtures were voluntarily phased out of production. The two PBDE formulations were eventually added to the list of Persistent Organic Pollutants (POPs) under the international Stockholm Convention[30] while the remaining technical mixture of PBDE, DecaBDE, will be phased out of production and importation to the U.S. by 2013.

Withdrawal of PBDEs from North American markets has led to increased production of non-PBDE BFRs including novel BFRs (NBFRs)[27]. Though some of these replacement NBFRs have potential for long-range atmospheric transport, environmental persistence, and bioaccumulation, their environmental concentrations and toxicological effects are poorly represented in the literature[31]. Examples of NBFRs are 2-ethylhexyl tetrabromobenzoate (TBB), bis-(2-ethylhexyl) tetrabromophthalate (TBPH), and 1,2,5,6-tetrabromocyclooctane (TBCO). TBB and TBPH are components of the technical mixtures, Firemaster® 550 (35% TBB, 15% TBPH), Firemaster® BZ-54 (70% TBB, 30% TBPH), and DP-45 (TBPH only) marketed by Chemtura Corporation[29, 30] and TBCO is a component of Saytex® BC-48 marketed by Albermarle Corporation[31]. Firemaster® 550, which is a technical mixture of TBPH and TBB, was used as a replacement for PentaBDE mixtures in polyurethane foams, and both compounds have been listed as high production volume chemicals by the U.S. EPA[30]. From 1990 to 2006, TBPH had a U.S production volume of 450–4,500 metric tons/yr[31], but there is little data on production volumes of TBB or TBCO.

Certain NBFRs including TBB, TBPH, and TBCO have similar potentials for bioaccumulation, persistence, and long-range atmospheric transport as PBDEs and HBCDs[30, 34, 35]. For example, TBB and TBPH have both been detected in several environmental matrices

including dust, air, and biota and have been listed as NBFRs requiring further investigation and monitoring in the Norwegian environment[36]. From 2008 to 2010, as part of the Integrated Atmospheric Deposition Network, TBB and TBPH had been detected in the particle-phase at six locations near the North American Great Lakes, and in urban areas from Chicago and Cleveland[30]. The study showed that atmospheric concentrations of both TBB and TBPH increased rapidly during the two-year sampling period possibly indicating that the use and/or accumulation of these NBFRs was increasing. The two compounds have also been detected in samples from the Global Atmospheric Sampling (GAPS) Network[37], in house dust in the U.S.[38], and indoor dust in New Zealand[39]. TBPH and TBB have been detected in polyurethane foam in retail baby products in the United States as the second most abundant BFRs[40], and were detected in couch foam at 4.2% by weight of total flame retardants[41]. Both compounds were detected in sewage sludge from wastewater treatment plants in San Francisco, California[31], and TBPH alone was detected in environmental samples from the high arctic[9]. TBB and TBPH have been detected in biota, including blubber from hump-back dolphins (mean: <0.04 ng/g, lw; 0.51 ± 1.3 ng/g, lw) and finless porpoises (mean: 5.6 ± 17 ng/g, lw; 342 ± 883 ng/g, lw) from Hong-Kong, South China (Lam et al., 2009), in filter feeding bivalves (2220 ng/g, lw; 1370 ng/g, lw), and grazing gastropods (1740 ng/g, lw; 380 ng/g, lw) collected downstream from a textile manufacturing outfall[26]. TBPH has also recently been detected in 89% of sampled ring-billed gull livers collected from an industrialized section of the St. Lawrence River downstream from Montreal, Canada[35]. The ring-billed gull livers from the St. Lawrence site exhibit the greatest frequency of detection of TBPH and the greatest concentrations in any bird (17.6 ng g−1ww). TBCO has been detected but was not quantifiable in herring gull eggs in the North American Great Lakes[27], but overall few data have been collected regarding the occurrence of TBCO in environmental and biotic matrices.

Based on screening-level assessments using EU criteria, TBCO is a potential aquatic hazardous substance and is characterized as a potentially persistent and bioaccumulative compound[55]. TBCO is also included on the Canadian non-domestic Substances List with as much as 10 tons/yr being imported into Canada[27]. Though TBCO is a potential aquatic hazard, few data on mode of action or toxic potency are available. There are limited data regarding sub-lethal toxicological studies for either TBPH or TBB; Fathead Minnow exposed to the technical mixtures, Firemaster® 550 and Firemaster® BZ-54 (1 mg fish/d), exhibited acute genotoxicity

with DNA damage observed in liver cells[29]. In a recent investigation, rats exposed to Firemaster® 550 (1000 ug/day) exhibited a 65% increase in total concentrations of thyroxine in serum and a significantly advanced pubertal onset[52]. TBPH and TBB which are derived from bis(2-ethylhexyl)-phthalate (DEHP) and 2-ethylhexyl benzoate (EHB),respectively have been observed to undergo sequential debromination in photodegradation experiments[43]. Total debromination of TBPH leading to the formation of di-(2-ethylhexyl) phthalate (DEHP) is possible, and requires further investigation due to DEHP's possible biological effects.

The purpose of this investigation was to generate toxicological data for TBB, TBPH, and TBCO by use of *in vitro* bioassays. The *in vitro* bioassay endpoints were based on the toxicities of structural analogs of the compounds (Figure 2.1). Recent *in vitro* metabolism experiments have shown that TBPH is metabolized to mono(2-ethylhexyl) tetrabromophthalate (TBMEHP)[78], a brominated analog of MEHP which itself has been shown to affect concentrations of steroid hormones including estradiol and testosterone in rat ovarian follicles[79, 80].

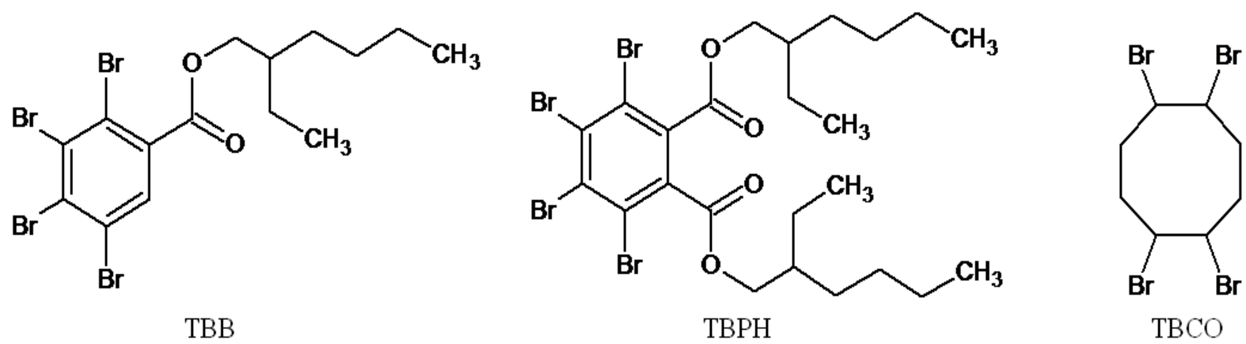

Figure 2.1. Chemical structures of 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB), bis(2-ethylhexyl)-3,4,5,6-tetrabromo-phthalate (TBPH), and 1,2,5,6-tetrabromocyclooctane (TBCO).

In this study, the capabilities of three NBFRs to disrupt normal endocrine functions were investigated. Potential as receptor agonists or antagonists were measured by use of the yeast estrogen screen (YES) and yeast androgen screen (YAS) reporter assays while non-receptor mediated steroidogenic effects were investigated by use of the mammalian cell model, the H295R steroidogenesis assay. Following reports of aryl hydrocarbon receptor (AhR) activity by DEHP[44, 47], the three NBFRs were tested for AhR binding activities by use of the H4IIE rat hepatoma cell reporter assay. To our knowledge this report presents the first data regarding these potential sub-lethal effects of TBB and TBCO.

2.3 Materials and methods

2.3.1 Chemicals

2-Ethylhexyl tetrabromobenzoate (TBB) was obtained from Wellington Laboratories (Ontario, Canada), bis-(2-ethylhexyl) tetrabromophthalate (TBPH) was obtained from Waterstone Technology (Indiana, U.S.), and 1,2,5,6-tetrabromocyclooctane (TBCO) was obtained from Specs (Delft, Netherlands). All single compounds were reported to be >95% pure by the manufacturer. All solvents, DMSO, EtOH, ethylacetate, and hexane, were of analytical grade and obtained from Sigma–Aldrich (Ontario, Canada).

2.3.2 Cell viability

Cytotoxic effects of the three NBFRs to the H4IIE and H295R cells were evaluated by use of the WST-1 assay (Roche Applied Science, Indiana, U.S.). Cells were propagated as mentioned below. Cytotoxicities were determined after 48 hr incubation with individual NBFRs. WST-1 reagent was used to determine metabolically active cells at the end of the incubation period according to the manufacturer's recommendations.

In the YES/YAS assays, cytotoxic effects were measured by use of optical density (690 nm)[81]. After 48 hr incubation, each well was assayed for turbidity and compared to solvent control values. Cellular cytotoxicity was defined as ≥30% reduction in cell density from solvent controls.

2.3.3 H4IIE-luc transactivation reporter gene assay

The H4IIE-luc cellular assay is derived from rat hepatoma cells which have been stably transfected with a luciferase gene under control of a dioxin-responsive element[82-84]. H4IIE-luc cells were propagated as previously described[85]. Cells were incubated for 24 hr prior to dosing. Test and control wells were dosed with 1% per well volume of the individual NBFRs prepared in DMSO. Luciferase activity was measured by use of the SteadylitePlus Kit (Perkin Elmer, MA, U.S.). The following concentrations of the test compounds were used: (TBB) 5×10^{-5}, 5×10^{-4}, 5×10^{-3}, 5×10^{-2} mg/L, (TBPH) 0.75, 1.5, 3, 15, 30, 150 mg/L, and (TBCO) 0.3, 1.5, 3, 15, 30 mg/L. A TCDD standard curve was included in each plate to control for inter-plate variability.

2.3.4 YES/YAS assays

Estrogenic and androgenic activities of the three NBFRs: TBB, TBPH, and TBCO were measured via production of β-galactosidase and the subsequent metabolism of chlorophenol red-β-D-galactopyranoside (CPRG). All media and procedures used for the YES/YAS assays were prepared according to the original protocol[81]. 17 β-estradiol (E2) and dihydrotestosterone (DHT) standards were included with each plate to control for inter-plate variability. Activity was measured at 570 nm and 690 nm by use of Eq. (1). The corrected value represents the test response corrected for potential toxicity to cells.

Corrected value = A570 nm – A690 nm……………………………………………………(2.1)

Anti-estrogenic (YES) and anti-androgenic (YAS) activities of the three NBFRs were measured by reduction in activity of β-galactosidase in yeast cells in the presence of 8.17×10^{-4}mg/L E2 (YES), and 1.45×10^{-3} mg/L DHT (YAS). 4-Hydroxytamoxifen (3.88×10^{-9} mg/L), and hydroxyflutamide (2.92×10^{-8} mg/L) were used as E2 and DHT antagonist controls for the YES and YAS assays, respectively. Concentrations of the three NBFRs which elicited the greatest inhibition (YES: 5×10^{-01}, 0.03, 30 mg/L; YAS: 5×10^{-01}, 1000, 300 mg/L; TBB, TBPH and TBCO, respectively) were used to test for recovery of activation signals of the cellular assay systems. This control was employed to test for inhibitory effects due to non-receptor mediated mechanisms. To elicit an inhibitory response, each NBFR was combined with a specific receptor agonist, E2 or DHT, then incubated with an additional volume of agonist at three different concentrations. Recoveries of activation signals were tested by use of three concentrations of E2:

2.72×10^{-4}, 8.17×10^{-4}, and 2.72×10^{-3} mg/L (YES) and three concentrations of DHT: 2.90×10^{-4}, 1.45×10^{-3}, and 2.90×10^{-3} mg/L (YAS) (Figures C2.S1., C2.S2). All procedures for the anti-estrogenic and anti-androgenic assays were the same as those for the YES/YAS agonist assays described above.

2.3.5 H295R cell culture and exposure

The H295R human adrenocarcinoma cell line was cultured according to the standardized H295R assay protocol approved by the OECD[86]. H295R cells were dosed with the following concentrations of the test compounds: (TBB) ranging from $5x10^{-5}$ to $5x10^{-2}$ mg/L, (TBPH) ranging from 1.5 to 30 mg/L, and (TBCO) ranging from 0.3 to 15 mg/L. Forskolin (4.11 mg/L), a strong inducer of both E2 and T production, and prochloraz (1.13 mg/L), a strong inhibitor of both E2 and T production, were used as controls in the H295R steroidogenesis assay. The final concentration of the solvent carriers did not exceed 0.1%. Conditioned media was collected following 48 hr of exposure and assayed for [E2] and [T] by use of ELISA.

2.3.6 17β-Estradiol and testosterone extraction and quantification by use of EIA

Extraction of E2 and T from media was performed according to established protocol[87]. Concentrations of E2 and T were determined by competitive EIA according to the manufacturer's recommended method (Caymen Chemical Company, MI, U.S.).

2.3.7 Statistics

Statistical analysis for all cellular assays was completed by use of IBM SPSS Statistics software (V.20). Data was initially tested for normality by use of the Shapiro-Wilk's test and homogeneity of variance by use of Levene's test ($p>0.05$). If assumptions of normality and homogeneity of variance were met a one-way ANOVA was used to evaluate differences between sample treatment and solvent controls. Differences were considered significant at a p-value < 0.05. In those cases where the basic assumptions for parametric statistics were not met, distribution-free tests such as Kruskal-Wallace followed by Mann-Whitney U tests were employed. All data is reported as mean ± SE.

2.4 Results

2.4.1 TCDD-like potencies of compounds

The three NBFRs, TBB, TBPH, and TBCO caused no TCDD-like activities in the H4IIE-luc bioassay. A TCDD standard curve [2.25×10^{-7} mg/L to 4.83×10^{-5} mg/L] was used to calculate TCDD equivalents. The three NBFRs had no cytotoxic effects at the tested concentrations.

2.4.2 Receptor-mediated androgenic and estrogenic activities of compounds

The three NBFRs, TBB, TBPH, and TBCO caused no estrogen-like or androgen-like activities in the YES/YAS bioassays. A six point E2 standard curve [2.72×10^{-6} mg/L to 2.72×10^{-3} mg/L] (YES), and a seven point DHT standard curve [2.90×10^{-6} mg/L to 8.71×10^{-3} mg/L] (YAS) were used to calculate E2 and androgen equivalents. The three NBFRs had no cytotoxic effects at the tested concentrations.

2.4.3 Androgen receptor mediated antiandrogenic activities of NBFRs

The three NBFRs, TBB, TBPH, and TBCO were screened for antiandrogenic activities by use of the YAS assay. The signal from cells activated by a 1.45×10^{-3} mg/L DHT control was set at 100%. Cells co-treated with androgen antagonist control hydroxyflutamide [2.92×10^{-8} mg/L] exhibited a 52% reduction in β-galactosidase signal. The following concentrations of the test compounds were used in the YAS assay: (TBB) 5×10^{-10}, 5×10^{-8}, 5×10^{-6}, 5×10^{-4}, 5×10^{-3}, 5×10^{-2}, 5×10^{-01} mg/L, (TBPH) 3×10^{-2}, 0.3, 3, 15, 30, 150, 300, 1500 mg/L, and (TBCO) 3×10^{-3}, 3×10^{-2}, 0.3, 3, 15, 150, 300 mg/L. Each NBFR tested resulted in statistically significant inhibition of receptor mediated β-galactosidase production. At 0.5 mg/L TBB exposures resulted in a maximal antiandrogenic response of 31% inhibition of β-galactosidase production compared to the DHT control (Figure 2.2A). TBPH, the brominated structural analogue of the phthalate DEHP, demonstrated dose-dependent inhibition of β-galactosidase production. At 1500 mg/L TBPH exposures resulted in a maximal antiandrogenic response of 59% compared to the DHT control (Figure 2.2B). TBCO responded in a dose-dependent manner and produced the greatest inhibition of β-galactosidase production of all tested compounds. At 300 mg/L TBCO exposures resulted in a maximal antiandrogenic response of 74% compared to the DHT control (Figure 2.2C).

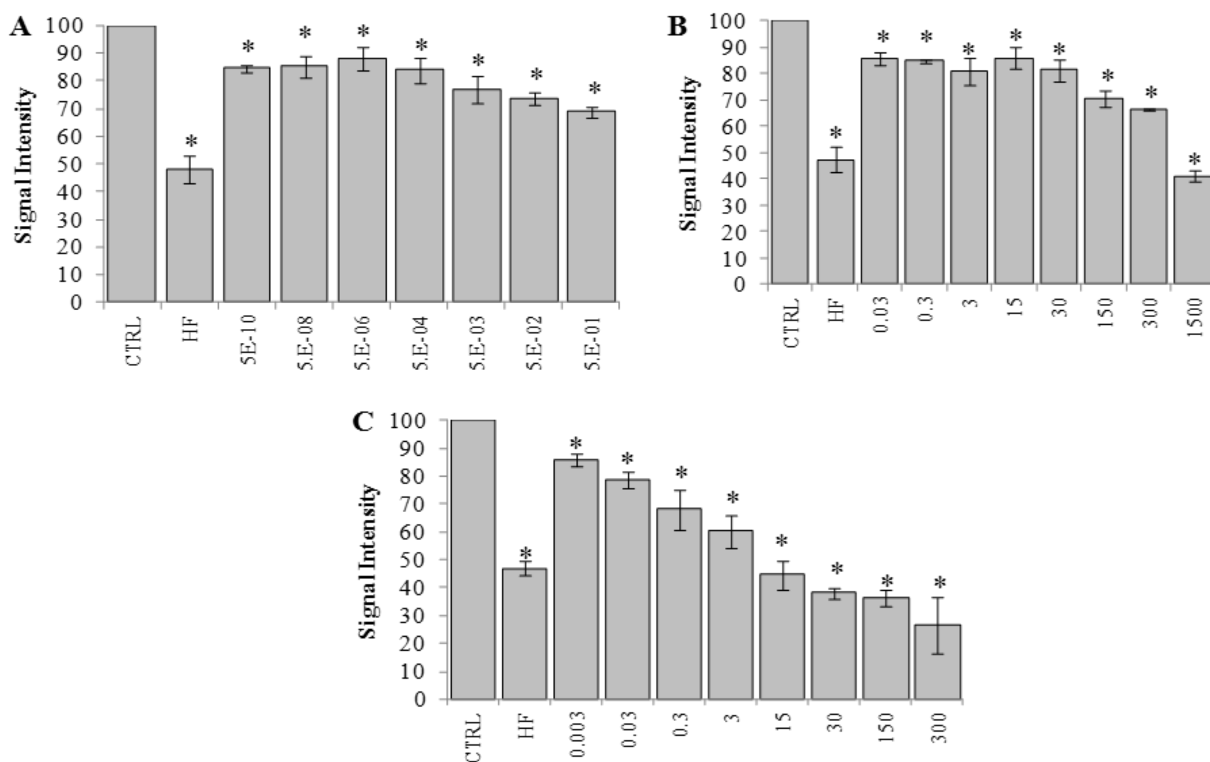

Figure 2.2. The antiandrogenic activity of (A) TBB at seven exposure concentrations, (B) TBPH at eight exposure concentrations, and (C) TBCO at eight exposure concentrations, in mg/L measured by the yeast androgen screen. Antiandrogenic activity is presented as the reduction in signal intensity (mean ± SE) compared to DHT activated control cells (CTRL). Hydroxyflutamide (HF) acted as a positive control. Each assay contained four wells per NBFR exposure concentration and a total of four assays were used for analysis. Exposure concentrations that resulted in effects that were significantly different than activated controls are indicated by asterisks (*p<0.05).

2.4.4 Estrogen receptor mediated antiestrogenic activities of compounds

The three NBFRs were screened for antiestrogenic activities by use of the YES assay. The signal from cells activated by 8.17×10^{-4} mg/L E2 controls was set at 100%. Cells co-treated with hydroxytamoxifen [3.88×10^{-9} mg/L] exhibited a 71% reduction in β-galactosidase signal. The following concentrations of the test compounds were used in the YES assay: (TBB) 5×10^{-10}, 5×10^{-8}, 5×10^{-6}, 5×10^{-4}, 5×10^{-3}, 5×10^{-2}, 5×10^{-01} mg/L, (TBPH) 3×10^{-3}, 3×10^{-2}, 0.3, 3, 15, 30, 150 mg/L, and (TBCO) 3×10^{-3}, 3×10^{-2}, 0.3, 3, 15, 30 mg/L. Each NBFR resulted in statistically significant inhibition of receptor mediated β-galactosidase production. Of the three NBFRs, TBB resulted in the greatest reduction of β-galactosidase production while responding in a dose-dependent manner. At 0.5 mg/L TBB exposures resulted in a maximal antiestrogenic response of 62% compared to the E2 control (Figure 2.3A). TBPH and TBCO exposures resulted in maximal antiestrogenic responses of 21% and 46% at concentrations of 3×10^{-2} mg/L and 30 mg/L, respectively compared to E2 controls (Figures 2.3B, 2.3C). TBPH exposures resulted in a reverse dose response trend where the lesser exposure concentrations resulted in the greatest inhibition.

2.4.5 Effects of NBFRs on testosterone synthesis

Only two of three NBFRs, TBPH and TBCO significantly affected the production of testosterone in conditioned media compared to solvent controls in the H295R cellular assay. The maximal exposure concentration of TBPH, 30 mg/L, resulted in a moderate1.96 fold increase in concentrations of T compared to controls (Figure 2.4A). Across four exposure concentrations TBPH exposures produced a range of 1.17 to 1.96 indicating limited dose-responsive behaviour. At doses of 3 mg/L and 15 mg/L TBCO exposures resulted in slightly lesser concentrations of T compared to solvent controls. At 15 mg/L TBCO, the concentration of T was 0.79 fold lesser compared to solvent controls (Figure 2.4B), while exposures of 0.3 and 1.5 mg/L produced no significant differences from solvent controls.

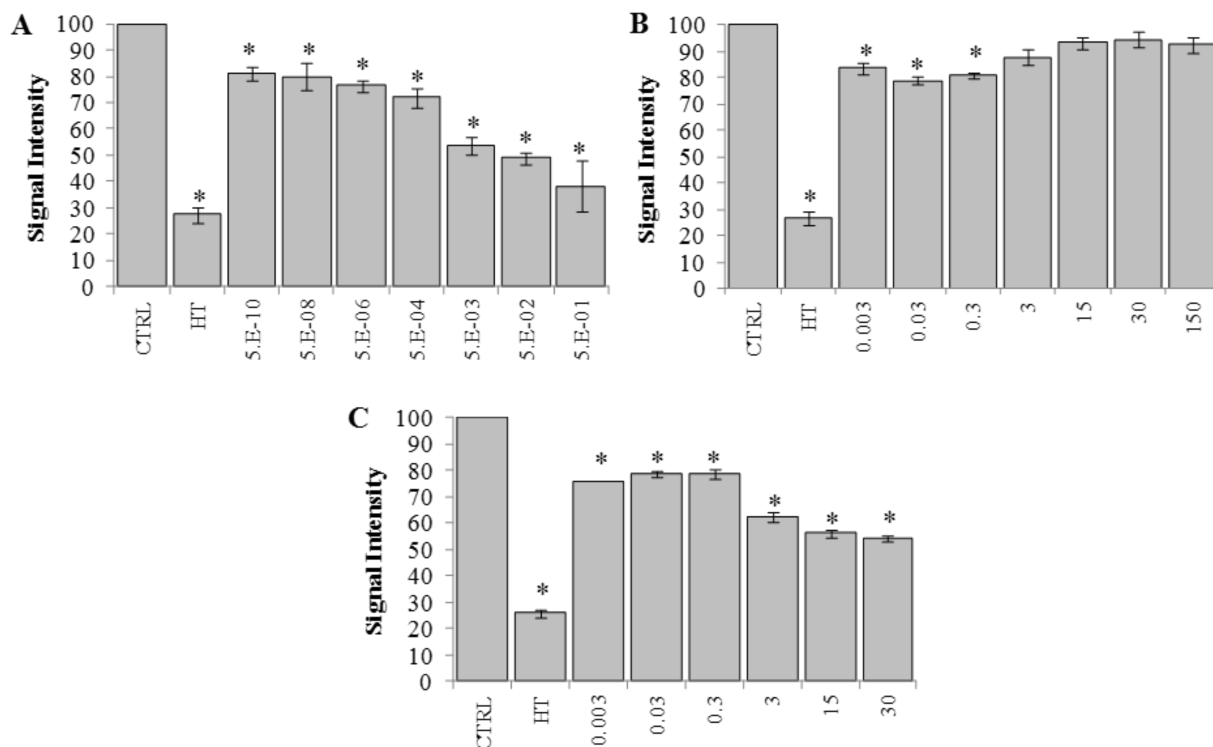

Figure 2.3. The antiestrogenic activity of (A) TBB at seven exposure concentrations, (B) TBPH at seven exposure concentrations, and (C) TBCO at six exposure concentrations in mg/L measured by the yeast estrogen screen. Antiestrogenic activity is presented as the reduction in signal intensity (mean ± SE) compared to E2 activated control cells (CTRL). 4-Hydroxytamoxifen (HT) acted as a positive control. Each assay contained four wells per NBFR exposure concentration and a total of four assays were used for analysis. Exposure concentrations that resulted in effects that were significantly different than activated controls are indicated by asterisks (*p<0.05).

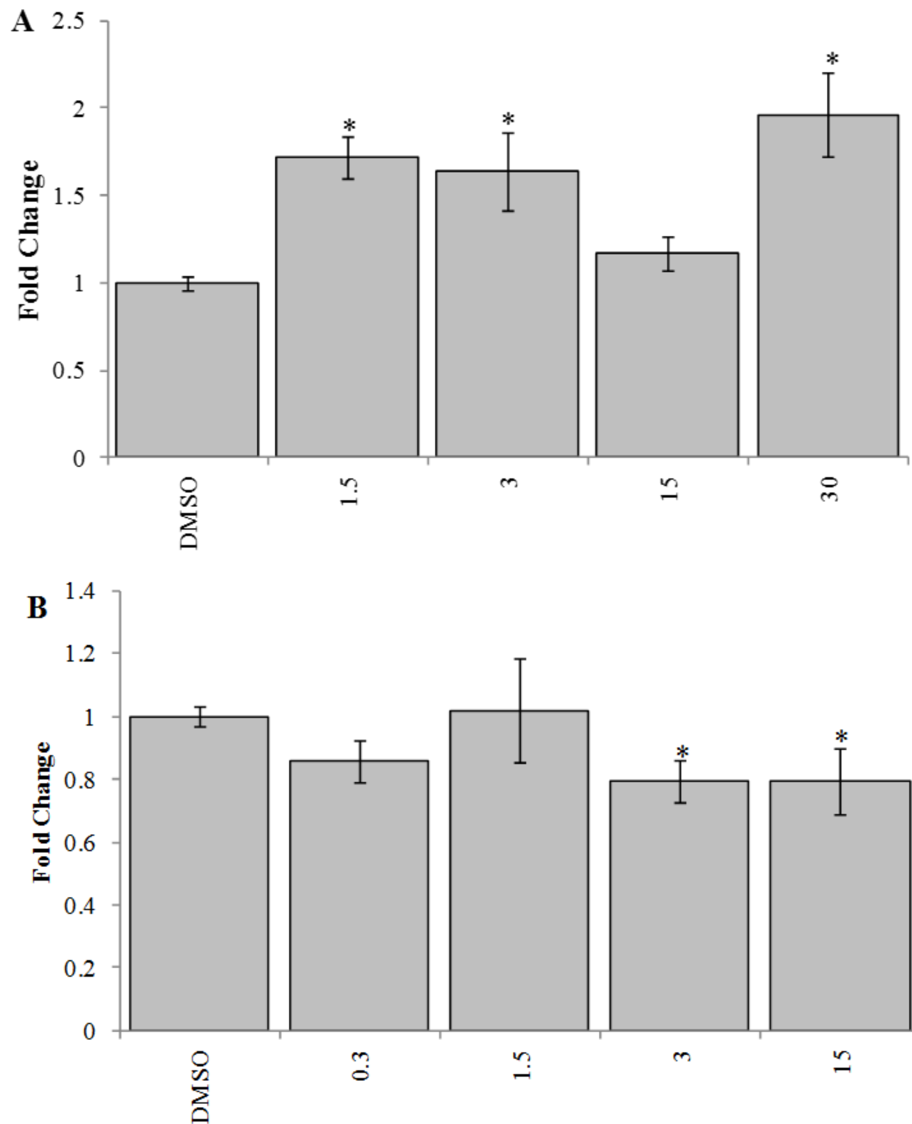

Figure 2.4. The effects of (A) TBPH and (B) TBCO exposures on relative testosterone hormone concentrations measured in the H295R cell assay. Four concentrations (mg/L) of TBPH and TBCO were tested and data are given as relative fold change in hormone production (mean ± SE) compared to solvent controls (DMSO). Each assay contained four wells per NBFR exposure concentration and a total of four assays were used for analysis. Exposure concentrations that resulted in effects that were significantly different than solvent controls are indicated by asterisks (*p<0.05).

2.4.6 Effects of NBFRs on E2 synthesis

At all exposure doses, the three NBFRs elicited significant increases in concentrations of E2 in conditioned media compared to solvent controls. TBB exposed cells responded at a maximum of 2.82 fold change compared to solvent controls (Figure 2.5A). TBPH exposure resulted in the greatest increase of concentrations of E2 eliciting a maximal response of 5.29 fold change compared to solvent controls (Figure 2.5B). At 15 mg/L, TBCO elicited a maximal response of 3.29 fold change compared to solvent controls (Figure 2.5C).

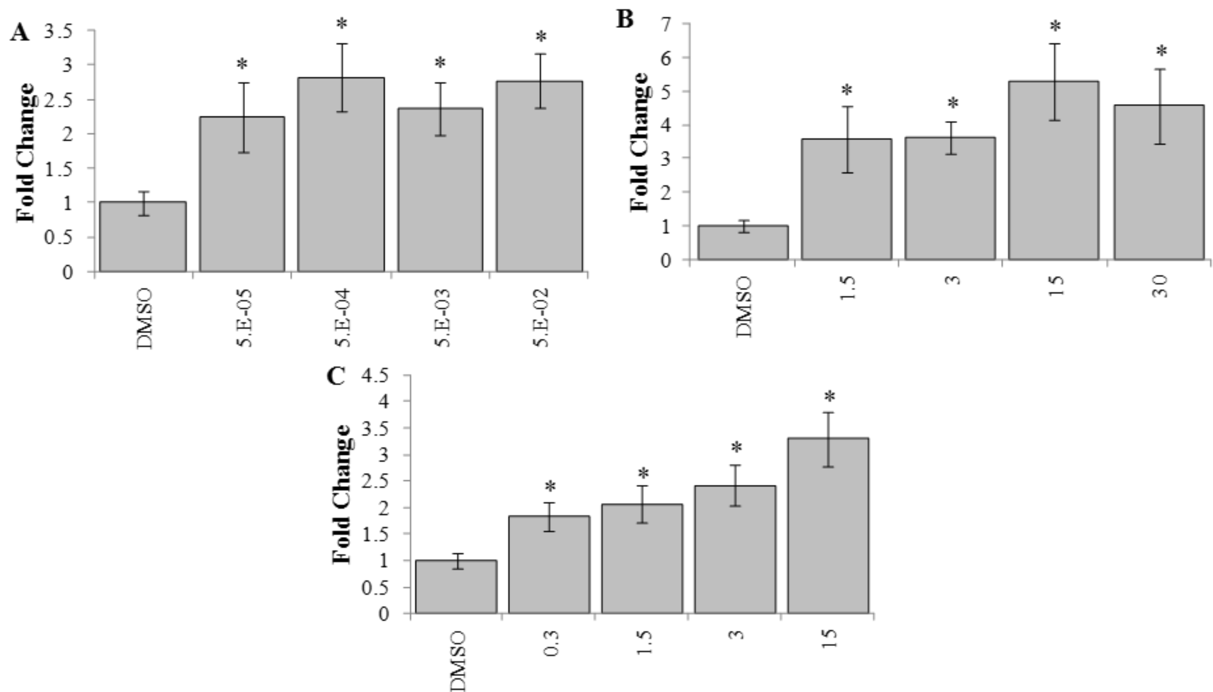

Figure 2.5. The effects of (A) TBB, (B) TBPH, and (C) TBCO exposures on relative 17-β-estradiol hormone concentrations measured in the H295R cell assay. Four concentrations (mg/L) of each NBFR were tested and data are given as relative fold change in hormone production (mean ± SE) compared to solvent controls (DMSO). Each assay contained four wells per NBFR exposure concentration and a total of four assays were used for analysis. Exposure concentrations that resulted in effects that were significantly different than solvent controls are indicated by asterisks (*$p<0.05$).

2.5 Discussion

The three NBFRs TBB, TBPH, and TBCO are components of several flame retardant technical mixtures and have been discovered in numerous environmental and biotic samples. TBPH is a brominated analogue of the phthalate plasticizer DEHP, which has several associated toxicities including endocrine disruption, AhR agonism, and developmental and reproductive toxicities[88, 89]. There are yet few published reports of toxicities of TBB and TBCO. Our investigation elucidated the potential biological effects with respect to the endocrine disrupting and TCDD-like properties of the three NBFRs.

Dosing concentrations of the three NBFRs were based on pilot data regarding cytotoxicity and solubility in media, which was previously generated by the authors. In this study, antagonism was defined as a dose dependent inhibitory effect that was comparable in magnitude to the inhibitory controls, hydroxyflutamide or 4-hydroxytamoxifen. Compounds that did not meet these criteria but demonstrated significant inhibitory effects were deemed potential weak antagonists. Controls for recovery of activation signals with exposures to TBB, TBPH, and TBCO showed recoveries of activation responses with the addition of three concentrations of DHT: $2.90x10^{-4}$, $1.45x10^{-3}$, and $2.90x10^{-3}$ mg/L (YAS) and E2: $2.72x10^{-4}$, $8.17x10^{-4}$, and $2.72x10^{-3}$ mg/L (YES) (Figures C2.S1, C2.S2).

2.5.1 TCDD-like effects

The three NBFRs TBB, TBPH, and TBCO did not result in any TCDD-like effects at tested concentrations. DEHP has previously elicited weak agonistic AhR activity[44, 47]. Discrepancies between the TCDD-like activities of TBPH and its structural analogue DEHP are likely due to the bromine atoms at the 2, 3, 4, 5 positions. The bromine atoms increase steric hindrance and change the physical-chemical characteristics of the compound resulting in differential interaction with the AhR. To our knowledge this is the first investigation of the TCDD-like effects of TBB, TBPH, or TBCO.

2.5.2 (Anti) androgenic effects

DEHP is a known endocrine disruptor with several toxic effects that can act via antiandrogenic mechanisms[89]. It has been previously detailed that the antiandrogenic toxicity of DEHP is moderated through its mono ester metabolite MEHP[80]. Several studies have shown that MEHP

exerts little affinity for the androgen receptor and does not produce androgen receptor mediated effects[90]. MEHP likely exerts its antiandrogenic effects by blocking activities of enzymes of the steroidogenic pathway and through the inhibition of cholesterol transportation[90]. Unlike its mono ester metabolite, *in vitro* androgenic screening of DEHP has demonstrated that the un-metabolized phthalate might bind to the androgen and estrogen receptors[90, 91]. TBPH demonstrated no agonistic effects in the YAS assay (data not shown), but produced significant antiandrogenic effects. Contrary to previous studies[53] TBPH produced antagonistic effects greater than hydroxyflutamide, and responded in a dose dependant trend (Figure 2.2B). Differences from previous *in vitro* investigations can be attributed to differences in exposure doses in the yeast system. Previous investigations which have used mammalian cellular assays have demonstrated the inability of DEHP to bind with the androgen receptor, though this might be due to rapid biotransformation of DEHP to its metabolite MEHP. Yeast cells might have different mechanisms and/or rates of metabolism of DEHP than mammalian cells, which might help to explain the observed antagonistic effects of DEHPs brominated analog, TBPH.

The results presented here represent some of the first data regarding potential androgenic effects of TBB and TBCO. The weak antagonistic response of TBB might be due to limitations in dosing concentrations which were restricted by the concentrations of the stock solutions and cytotoxicity. TBCO can be characterized as an androgen receptor antagonist; the compound responded in a dose-dependent manner and had a significantly greater antagonistic response than hydroxyflutamide at 2.92×10^{-8} mg/L (Figure 2.2C). Further exposure and investigations of mechanisms are required to confirm the potential antiandrogenic effects of TBCO.

2.5.3 (Anti) estrogenic effects

By use of the aforementioned characteristics of an antagonist, the three NBFRs can be classified as weak estrogen antagonists. The antagonistic effects of the three compounds indicated weak antagonism, while only TBB and TBPH responded in dose-dependent trends (Figures 2.3A–C). Contrary to previous *in vitro* investigations in which no antagonistic effects were observed[53], TBPH exposures resulted in weak antagonistic effects. The discrepancies between these data and previous investigations might be due to differences in exposure concentrations. The rationale of the reverse TBPH dose-response is unknown, though initial cytotoxicity experiments showed no

significant increase in cellular cytotoxicity at greater concentrations. The weak antagonistic effects of TBPH indicate that it differs from DEHP in its interaction with the estrogen receptor.

The results of this investigation are the first to indicate the potential for antagonism of TBB and TBCO with the ER. Further *in vitro* investigations and *in vivo* assays are required to elucidate any mechanisms of toxicity and gauge potential organismal effects.

2.5.4 Effects on testosterone production in the H295R steroidogenesis assay

TBB did not demonstrate statistically significant changes in concentrations of T (data not shown) at any of the tested concentrations. TBPH is a structural analog to the plasticizer DEHP which is ubiquitously found in the environment and causes several toxic effects including male reproductive abnormalities in animal models[89]. It is hypothesized that several of the toxic effects of DEHP are mediated through interactions and disruption of endocrine homeostasis[89, 90]. The results of TBPH exposures, though significantly different than controls, represent a weak increase in concentrations of T (Figure 2.4A). These results are contrary to existing data for DEHP[44, 90, 92] and might be attributed to the bromine atoms attached to the phthalate moiety, differences in exposure concentrations, or differences in the cellular physiology of the assay system. For example the observed reductions in concentrations of testosterone in DEHP exposed cells are partially moderated through the activation of the PPARα (peroxisome proliferator activated receptor) nuclear receptors. Activation of PPARα via exposure to DEHP has been linked to decreases in concentrations of T. Experimentation with PPARα null mice has resulted in lesser reductions of concentrations of testosterone than in their wild-type counterparts[93, 94]. Though PPARα affects the concentration of T *in vivo* and *in vitro*, PPARs in general have differential tissue and species specific expression patterns[95]. For example, DEHP has demonstrated limited effects on the liver in humans, due to the limited expression, and/or truncated or mutant variations of PPARα[95, 96]. These differences between *in vitro* experimentation and cellular physiologies might account for differences in results.

Exposure of H295R cells to TBCO resulted in a statistically significant decrease in concentrations of T at the two greatest concentrations (Figure 2.4B). Similar to the TBPH exposures, TBCO elicited a weak response in the H295R system. This is the first data regarding

the potential androgen disrupting effects of TBCO. From this preliminary data, further investigations into TBCO's endocrine disrupting potentials are warranted.

2.5.5 Effects on estrogen production in the H295R steroidogenesis assay

The three NBFRs significantly increased synthesis of E2 in the H295R system. TBPH exposures resulted in the greatest increase of concentrations of E2 (Figure 2.5B), though of the three compounds only TBCO responded in a dose-dependent fashion (Figure 2.5C). These results for TBPH exposures are in accordance with previous *in vitro* exposures of DEHP which demonstrated the compounds potential endocrine disrupting effects[44, 97].

A greater understanding of the effects/mechanisms of the three NBFRs can be achieved in the comparison of the two specific assay systems, the YES and H295R. The YES system represent a receptor mediated endpoint that is relegated to those elements that have been transfected into the cells, specifically the human estrogen receptor (hER)[81], while the H295R cellular system inherently expresses the complete biosynthetic pathway of E2. The data from the YES assay shows that the NBFRs do not interact with the estrogen receptor in an agonistic fashion; a hypothesis for TBPH that is supported by investigations into toxicities of DEHP[90]. While data from the H295R assays suggest that the three compounds target the biosynthetic pathway of E2. Indeed MEHP, the metabolite of DEHP is known to affect aromatase, a major enzyme in E2 synthesis[97]. Due to the analogous structures of TBPH and DEHP, many of the limited toxicological investigations currently focus on potential androgenic disruption. To our knowledge the results from the YES and H295R assays represent some of the first data regarding potential estrogen specific mechanisms of endocrine disruption of TBB, TBPH, and TBCO in an *in vitro* system.

3 CHAPTER 3: A MIXTURE OF THE NOVEL BROMINATED FLAME RETARDANTS TBPH AND TBB AFFECTS FECUNDITY AND TRANSCRIPT PROFILES OF THE HPGL-AXIS IN JAPANESE MEDAKA

PREFACE

Chapter 2 demonstrated that TBPH and TBB did not activate the AhR, but each compound elicited effects in the EDC screening assays. TBB produced antiestrogenic effects in the YES assay while TBPH produced antiandrogenic effects in the YAS assay system. TBPH and TBB also altered concentrations of the steroid hormone E2 in the H295R assay. Following positive responses of TBPH, TBB in the *in vitro* screening level assessment, the goal of Chapter 3 was to characterize whole-organism endocrine-related adverse effects and mechanisms of action. In-depth characterization of adverse effects and mechanisms of action was critical to increase knowledge regarding profiles of toxicity of these compounds to inform accurate assessments of risk. This chapter was included in the first phase of this research program, the characterization of potential toxicities of TBPH, TBB, and TBCO.

The content of Chapter 3 was reprinted (adapted) from *Aquatic Toxicology*, (10.1016/j.aquatox.2014.10.019) D.M.V. Saunders, M. Podaima, G. Codling, J.P. Giesy, Steve Wiseman "A mixture of the novel brominated flame retardants TBPH and TBB affects fecundity and transcript profiles of the HPGL-axis in Japanese medaka" 158, 14-21. Copyright 2015, with permission from Elsevier.

Author Contributions:

David M.V. Saunders (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Michelle Podaima (University of Saskatchewan) provided laboratory assistance with fish culture, maintenance and *in vivo* exposure.

Drs. Gary Codling (University of Saskatchewan) provided laboratory assistance with the analytical instrumentation.

Drs. John P. Giesy and Steve Wiseman (both at University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

3.1 Abstract

The novel brominated flame retardants (NBFRs), bis(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (TBPH) and 2-ethylhexyl-2,3,4,5 tetrabromobenzoate (TBB) are components of the flame retardant mixture Firemaster® 550 and both TBPH and TBB have recently been listed as high production volume chemicals by the U.S. EPA. These NBFRs have been detected in several environmental matrices but very little is known about their toxic effects or potencies. Results of *in vitro* assays demonstrated potentials of these NBFRs to modulate endocrine function through interactions with estrogen (ER) and androgen receptors (AR) and via alterations to synthesis of 17-β-estradiol (E2) and testosterone T, but *in vivo* effects of these chemicals on organisms are not known. Therefore a 21-day short term fish fecundity assay with Japanese medaka (*Oryzias latipes*) was conducted to investigate if these NBFRs affect endocrine function *in vivo*. Medaka were fed a diet containing either 1422 TBPH:1474 TBB or 138:144 µg/g food, wet weight (w/w). Cumulative production of eggs was used as a measure of fecundity and abundances of transcripts of 34 genes along the HPGL-axis were quantified to determine mechanisms of observed effects. Cumulative fecundity was impaired by 32% in medaka exposed to the greatest dose of the mixture of TBPH/TBB. A pattern of global down-regulation of gene transcription at all levels of the HPGL axis was observed, but effects were sex-specific. In female medaka the abundance of transcripts of ERβ was lesser in livers, while abundances of transcripts of VTG II and CHG H were greater. In male medaka, abundances of transcripts of ERα, ERβ, and ARα were lesser in gonads and abundances of transcripts of ERβ and ARα were lesser in brain. Abundances of transcripts of genes encoding proteins for synthesis of cholesterol (HMGR), transport of cholesterol (HDLR), and sex hormone steroidogenesis (CYP 17 and 3β-HSD) were significantly lesser in male medaka, which might have implications for concentrations of sex hormones. The results of this study demonstrate that exposure to components of the flame retardant mixture Firemaster® 550 has the potential to impair the reproductive axis of fishes.

3.2 Introduction

Brominated flame retardants (BFRs) are synthetic compounds that are added to consumer and industrial products to inhibit propagation of fire. Polybrominated diphenyl ethers (PBDEs), which have three technical mixtures (PentaBDE, OctaBDE, and DecaBDE) have historically been the most widely used BFRs worldwide, but due to their ubiquity in the environment and potential toxic effects, PBDEs have been increasingly scrutinized and two of three technical mixtures (PentaBDE and OctaBDE) have been phased-out of production from North American and global markets. Though PBDEs have been phased out of global use, legislation in North America and other countries requires that consumer and industrial products adhere to specific standards of fire retardation. Additionally, demand for BFRs has continued to grow with a 5% increase in production in 2005 alone[9]. Consequently there has been an increase in production of novel brominated flame retardants (NBFRs). Many NBFRs are replacement compounds for PBDE formulations though in several instances their PBT (persistence, bioaccumulation, toxicity) profiles are similar to the legacy BFRs they have replaced[27, 31].

The two NBFRs *bis*(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (TBPH or BEHTBP) and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB or EHTBB) are components of several mixtures of additive flame retardants including, Firemaster® 550 (35% TBB, 15% TBPH), Firemaster® BZ-54 (70% TBB, 30% TBPH), and DP-45 (TBPH only)[29, 30]. Firemaster® 550 is a replacement for PentaBDE technical mixtures used in polyurethane foams, PVC, and neoprene. Both TBPH and TBB have been listed as high production volume chemicals by the U.S. EPA[30], and due to the phase-out of legacy BFRs, production of these two compounds is hypothesized to be increasing. In partial confirmation of this hypothesis, these compounds have been detected in a variety of abiotic and biotic matrices. TBPH and TBB have been detected in air by the Global Atmospheric Sampling Network[37], in air collected in the great lakes area of North America by the Integrated Atmospheric Deposition Network[30] and in dust in North America[38] and New Zealand[39]. TBPH and TBB have also been detected in blubber from humpback dolphins and finless porpoises in South China[42], and Ring-Billed Gulls in the St. Lawrence River downstream of Montréal, Canada[35].

TBPH and TBB have been detected in several environmental matrices but due to their novelty there is little information regarding toxic effects or potencies. TBPH and TBB are

brominated analogues of di(2-ethylhexyl)-phthalate (DEHP), a controlled substance in Canada and the EU, and 2-ethylhexyl benzoate (EHB), respectively, and due to similarities in structure, might have comparable toxicities. DEHP and its active metabolites are known to exert adverse effects which include hepatic carcinogenicity[98], endocrine disruption[44], and impairment of reproduction[45, 46]. For example, exposure of the Chinese rare minnow (*Gobiocypris rarus*) to DEHP resulted in greater concentrations of testosterone (T) and 17-β-estradiol (E2) in blood plasma and greater abundances of transcripts of vitellogenin (VTG) in livers of male and female minnow[49]. In another study Japanese medaka (*Oryzias latipes*) exposed to DEHP had lesser concentrations of VTG in blood plasma and the percentage of female medaka with mature oocytes in their ovaries was lesser[50]. Due to the endocrine disrupting effects of DEHP and its metabolites, there is concern that organisms exposed to TBPH and TBB might experience similar impacts.

Few studies have investigated endocrine disrupting effects of TBPH and TBB. By use of the yeast estrogen/androgen screening assays (YES/YAS) it was demonstrated that TBPH and TBB at 1500 mg/L and 0.5 mg/L, respectively, interact antagonistically with the human estrogen/androgen receptors (hERα/hARα)[99]. In the same study, concentrations of E2 increased 2.8- fold and 5.4-fold in H295R cells exposed to 15 mg/L of TBPH and 0.05 mg/L of TBB, respectively. Greater synthesis of T and E2, possibly because of greater expression of enzymes of steroidogenesis such as Cyp19A, was also detected in porcine primary testicular cells exposed to 0.15 mg/mL of TBPH[100]. It is of particular interest that effects elicited in these *in vitro* studies were similar to effects of DEHP on Chinese rare minnow[49]. There are yet few assessments of potential endocrine disrupting effects *in vivo*. In one study Wistar rats exposed to the technical mixture Firemaster® 550 (1000 µg/day) exhibited greater concentrations of thyroxine in serum and a significantly advanced pubertal onset[52].

Additional studies are required to verify and augment the understanding of potential endocrine disrupting effects of TBPH and TBB *in vivo*. Therefore, the purpose of this study was to investigate the endocrine disrupting potentials of TBPH and TBB by use of the OECD, 21-day short-term fecundity assay[101] with Japanese medaka (*O. latipes*). Male and female medaka were exposed to a mixture of these chemicals via their diet and cumulative fecundity, which is an integrated and holistic measure of endocrine disruption and can represent population-level

biological effects, was assessed. In addition, abundances of transcripts of 34 genes along the hypothalamic–pituitary–gonadal–liver (HPGL) axis were quantified by use of a PCR array[102-104].

3.3 Materials and methods

3.3.1 Chemicals and reagents

Bis(2-ethylhexyl)tetrabromophthalate (TBPH), bis(2-ethylhexyl-d_{17})-tetrabromo[$^{13}C_6$]phthalate (`TBPH), 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB), and 2-ethylhexyl-$d_{17}$-tetrabromo[$^{13}C_6$]benzoate (`TBB) were obtained from Wellington Laboratories (Ontario, Canada). All solvents including acetone, toluene, hexane, and dichloromethane (DCM) were of analytical grade and obtained from Fisher Scientific (Ontario, Canada).

3.3.2 Animal care

Embryos of medaka were obtained from the aquatic culture unit at the U.S. Environmental Protection Agency Mid-Continent Ecology Division (Minnesota, U.S.). Medaka were maintained in 30 L tanks under static-renewal conditions (27°C, 16:8 light/dark) and fed to satiation with flaked food and *artemia* 4-times daily. Culturing of medaka and exposures were performed in accordance with protocols approved by the University of Saskatchewan Committee on Animal Care and Supply and Animal Research Ethics Board (# 200090108).

3.3.3 Exposure protocol

Food was prepared according to methods described previously[105]. Briefly, commercial flaked food (Nutrafin Basix Staple Food) was ground with a mortar and pestle and spiked with a 150 mL solution of 1.4×10^{-2} M:1.8×10^{-2} M or 1.4×10^{-3} M:1.8×10^{-3} M, TBPH:TBB to attain 1500:1500 µg TBPH:TBB /g food or 150:150 µg TBPH:TBB/g food.

Flasks containing spiked food were shaken for 30 min to ensure thorough mixing of food and chemicals and subsequently air dried in a dark fume hood for 7 hr. An identical protocol was used to prepare food spiked with acetone for use as a control diet. Concentrations of TBPH and TBB were selected from a previous study where exposure to these chemicals via their diet caused DNA damage in fathead minnows (*Pimephales promelas*)[29].

Exposure protocols were adapted from the *Fish Short Term Reproductive Assay*, OECD test 229[101]. Japanese medaka (14-wk-old) which ranged in mass from 0.3 to 0.6 g were

randomly assigned to 10 L tanks to which dechlorinated, City of Saskatoon municipal water was supplied under flow-through conditions. Eight females and eight males were placed into each tank and acclimated at 25±2 °C with a 16:8 light/dark cycle and fed to satiation with flaked food for 7-d prior to initiation of experiments. There were no mortalities during the acclimation period, after which, medaka were exposed to either dose (greater/lesser) of the mixture of TBPH/TBB or the vehicle control (acetone prepared food) for 21-d. Each treatment was replicated in quadruplicate. Medaka were fed approximately 6% of body mass per day, and to ensure all food was consumed it was provided in two feeding events (morning and afternoon). At each 24 hr interval, eggs from female medaka in each tank were collected and enumerated, and the total number of eggs collected in each tank normalized to number of females per tank. A single mortality was observed in the solvent control treatment during the exposure period. At the end of the 21-d experiment medaka were euthanized by cervical dislocation and total mass of each individual was recorded. Masses of livers and gonads were recorded to determine hepatic somatic index (HSI) and gonadal somatic index (GSI). Livers, brains (including pituitary), and gonads from each medaka were immediately frozen in liquid nitrogen and stored at -80 °C for quantification of abundances of transcripts by real-time PCR (qPCR).

3.3.4 Chemical analysis

Three replicates of each food type were homogenized with clean sodium sulphate and a mortar and pestle. Stainless steel extraction cells (33 mL) were packed with an in-cell absorbent (activated alumina) to remove lipids (20:1, absorbent:lipid ratio) and 0.5 g of food[106], spiked with an internal standard - `TBB and extracted by use of a pressurized liquid extraction (ASE 200, Dionex, California, U.S.). Cells were extracted with a 1:1 solution of hexane and DCM at a temperature of 100 °C and 1500 psi for 10 min. The resulting extract was reduced in volume to 500 µL under a gentle stream of nitrogen and 100 ng of `TBPH was added. Three laboratory blanks and matrix spikes (spiked with $1.0 \times 10^5$ ng of TBB, TBPH, and 100 ng of `TBB) were extracted for quality assurance purposes.

Extracts were analyzed for TBPH and TBB by use of an Agilent (California, U.S.) 7890A gas chromatograph (GC) system coupled to an Agilent 5975C mass spectrometer (MS) operating in the electron impact ionization mode (EI). Two (2) µL samples were injected at an injection port temperature of 280 °C in the splitless mode. Chromatographic separation was achieved with

a 15-m x 250-µm i.d. Rtx-1614 fused silica capillary GC column, which had a 0.1-µm film thickness (Restek Corporation, Pennsylvania, U.S.). The carrier gas was helium at a constant flow of 1.5 mL/min. The following GC oven temperature program was used: 80 °C for 2 min, 25 °C/min to 250 °C, 3 °C/min to 270 °C, 25 °C/min to 300 °C, and 300 °C for 6 min[30]. Selected ion monitoring of *m/z* 467/465 and 421/419 was used for TBPH and TBB quantification/confirmation, respectively. TBPH and TBB were quantified by use of the internal standard method using `TBB. Recovery of `TBB was measured as 89.1±6.3%. TBPH and TBB were not detected in the laboratory blanks. The mean and standard error of the mean for TBB and TBPH recovery in the matrix spikes were 91.2±7.3 and 94.3±9.5%, respectively.

3.3.5 Gene selection and graphical model

A total of 34 genes which represent key signaling pathways, genes in steroidogenesis, and biomarkers of exposure to estrogens in the HPGL axis of Japanese medaka were selected for study based on results of previous research [102-104, 107]. Primers not mined from previous experiments were designed by use of NCBI Primer-Blast software and were based on sequences available in the NCBI GeneBank database. Sequences of nucleotide primers and efficiencies of reactions with these primers are given in the appendix (Table C3.S1). Graphical models depicting abundances of transcripts of 34 genes across the HPGL axis were produced by use of GenMapp 2.0 (Gladstone Institutes, U.S.) and were constructed and maintained by Dr. Xiaowei Zhang (Nanjing University, China). Two criteria were required for inclusion in the graphical model (a) statistically significant changes in abundances of transcripts and (b) ≥ 2-fold change in abundances of transcripts to represent physiological relevance (Figure 3.2).

3.3.6 Quantitative real-time PCR

Total RNA was extracted from livers, brains, and gonads by use of the RNeasy Plus Mini Kit (Qiagen, Ontario, Canada) according to the protocol provided by the manufacturer. Concentrations of RNA were determined by use of a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Delaware, U.S.) and stored at -80°C. First strand cDNA was synthesized from 1 µg RNA and was performed by use of the QuantiTect Reverse Transcription Kit (Qiagen) according to the protocol provided by the manufacturers. Real-time quantitative PCR (qPCR) was performed in 96-well plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems, California City, U.S.). A 50 µL reaction mixture of 25 µL of 2x

concentrated Power SYBR Green master mix (Qiagen), an optimized concentration of cDNA, 10 pmol of gene-specific primers, and nuclease free water was prepared for each combination of cDNA sample and primer. Reactions were conducted in duplicate with 20 µl reaction volumes per well. The reaction mixture for PCR was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile consisted of denaturing at 95 °C for 10 s and extension for 1 min at 60 °C for a total of 40 PCR cycles. Amplification of a single product from PCR was confirmed by melt curve analysis and target gene transcript abundance was quantified by use of the $2^{-\Delta\Delta Ct}$ method by normalizing to expression of the RPL-7 housekeeping gene [108, 109].

3.3.7 Statistical analysis

Statistical analyses were conducted by use of SPSS statistics software (V.20). Normality of each dataset was determined by use of the Kolmogorov-Smirnov Test and homogeneity of variance was determined by use of Levene's Test. Unless otherwise noted, data were analyzed by use of analysis of variance (ANOVA) or Kruskal-Wallis test, followed by Tukey's Test or Mann-Whitney U Test, respectively. Differences in daily production of eggs within groups were determined by use of repeated-measures ANOVA. If sphericity, an assumption of repeated measures ANOVA was violated a Greenhouse-Geisser correction was applied. Further post hoc tests were corrected by use of a Bonferroni adjustment. Due to the conservative nature of the Bonferroni adjustment, data points were pooled according to statistical difference which was assessed by pairwise comparison. All post hoc comparisons were made to group 1 which represented initial egg deposition numbers in the experiment. Profile analyses to test parallelism between control and exposed groups were completed by use of multivariate ANOVA (MANOVA) tests. A probability level of $p \leq 0.05$ was considered significant except in cases of Bonferroni adjustments. All data are shown as mean ± standard error of mean (S.E.M.).

3.4 Results

3.4.1 Concentrations of chemicals in food

Measured concentrations of TBPH and TBB in three food types did not significantly differ from nominal concentrations (Table 3.1). The measured concentration of TBPH was 95% and 92% of the desired nominal concentration in both types of spiked food, while the measured concentration

of TBB accounted for 98% and 96%, respectively. Concentrations of TBPH and TBB in food spiked with clean acetone were below the method limits of detection.

Table 3.1. Concentrations of TBPH and TBB in three diets used in the 21-day fish fecundity assay. Concentrations of TBPH and TBB are presented as mean ± standard error (µg/g food). Three replicates were extracted and analyzed for each food type

Feed	**TBPH**	**TBB**
Control	ND	ND
1500:1500 µg/g food	1422±156.4	1474±265.9
150:150 µg/g food	138±22.1	144±28.8

ND: below limit of detection

3.4.2 Chemical-induced effects of fecundity of medaka

Neither the HSI nor GSI of medaka fed either concentration of the mixture of TBPH/TBB was significantly different from the HSI or GSI of medaka exposed to the control diet (Table C3.S2). The proportion of eggs that were fertilized was determined on days 7, 14, and 21. There were no differences between groups of medaka exposed to the control diet and medaka exposed to either concentration of the mixture of TBPH/TBB.

Exposure to the mixture of TBPH/TBB affected fecundity of Japanese medaka (Figure 3.1). There were no significant differences in cumulative production of eggs between medaka exposed to solvent controls and the lesser concentration of TBPH/TBB (150:150 μg/g food). However, statistically significant differences in cumulative production of eggs between medaka exposed to solvent controls and the greatest concentration of TBPH/TBB (1500:1500 μg/g food) were observed. Numbers of eggs produced relative to solvent control were 68% and 94% by female medaka exposed to the greater and lesser concentrations of the mixture of TBPH/TBB, respectively. Profiles of daily production of eggs were significantly different (non-parallel profiles) between medaka exposed to the control and the greatest concentration of the mixture of TBPH/TBB but not the lesser concentration of TBPH/TBB (Figure C3.S1). Furthermore, a within-group repeated measures analysis indicated significant differences in daily production of eggs over time by medaka exposed to the greatest concentration of TBPH/TBB (Figure C3.S2A.), but not by medaka exposed to either the control diet or the diet containing the lesser concentration of TBPH/TBB. Across 21-repeated measures, a post hoc analysis with a Bonferroni adjustment was prohibitively conservative, so time points were grouped according to general trends in inflection points and statistically significant changes in daily production of eggs (Figure C3.S2B.). Days were grouped as follows: group 1 (days 1-5), group 2 (days 6-12), group 3 (days 13-16), and group 4 (days 17-21). Pooled group 1 was set at 100% fecundity, as this group represented the initial period of production of eggs during the exposure; statistically significant differences between initial production of eggs, group 1, and all subsequent groups (2, 3, and 4) were observed.

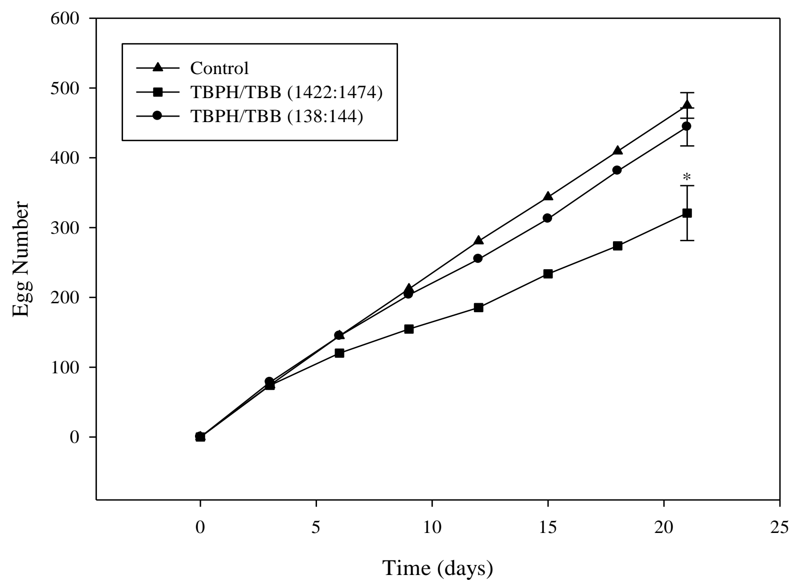

Figure 3.1. Cumulative production of eggs (fecundity) by medaka exposed to the high dose of the TBPH/TBB mixture (1422:1474 µg/ g food, w/w), the low dose of the TBPH/TBB mixture (138:144 µg/g food, w/w) and solvent control. The values represent the mean cumulative number of eggs per female over a 21-day period. The experiment included 4 replicate tanks, and each contained 8 female/male medaka. Asterisks (*) indicate a significant difference ($p < 0.05$) when compared to the control group.

3.4.3 Gene expression profiles of TBPH/TBB exposures

Abundances of transcripts of genes of the HPGL axis were quantified in male and female medaka exposed to the greatest concentration of TBPH/TBB because this concentration had the greatest effect on fecundity. In general, abundances of transcripts of target genes were lesser in males and females exposed to the greatest concentration of the mixture of TBPH/TBB compared to controls (Table 3.2), but profiles of gene expression and magnitude of effects were different between sexes. Although abundances of transcripts of several genes, such as sGnRH (-15.59-fold) and Activin BA (-11.70-fold), were much lesser in the brains and gonads from female medaka, neither effect was statistically significant because of the variability in the magnitude of effect (Table 3.2). There were incongruities in patterns of abundances of transcripts of ER and AR between male and female medaka. Abundances of transcripts of ERβ (-23.53-fold), ARα (-11.61-fold) and annexin max2 (-29.00-fold) were much lesser in livers from female medaka exposed to TBPH/TBB whereas abundances of these transcripts were not altered in brains or gonads. In contrast, abundances of transcripts of ERα, ERβ or ARα were not significantly different in livers from male medaka exposed to TBPH/TBB, whereas abundances of transcripts of ERα (-14.00-fold), ERβ (-9.37-fold), and ARα (-3.03-fold) were significantly lesser in gonads and ERβ (-3.11-fold) and ARα (-7.37-fold) were significantly lesser in brains. Abundances of transcripts of eight genes - ERα, ERβ, ARα, HDLR, HMGR, CYP 17, 3β-HSD, and activin BA - were significantly lesser in gonads from male medaka (Table 3.2 and Figure 3.2), but these effects were not observed in female medaka.

Table 3.2. Response profiles of genes of the hypothalamic-pituitary-gonadal-liver (HPGL) axis in Japanese medaka exposed to the greater dose of the TBPH/TBB mixture (1422:1474 μg/g food, w/w). Abundances of transcripts are expressed as fold change compared to corresponding solvent controls.

Tissue	Gene	Male	Female
Brain	*ERα*	-2.25	2.83
	ERβ	-3.11*	1.76
	ARα	-7.37*	-2.08
	Neuropep Y	-1.87	1.68
	cGnRH II	-1.24	-2.57
	mfGnRH	1.17	1.88
	sGnRH	-3.61	-15.59
	GnRH RI	-1.56	-4.71
	GnRH RII	-2.72	-4.34
	GnRH RIII	-2.25	-1.04
	GTHa	-4.59	-2.20
	LH- β	-13.54	-1.11
	CYP19B	-2.92	-4.45
Gonad	*ERα*	-14.00***	-1.92
	ERβ	-9.37***	-2.86
	ARα	-3.03**	1.11
	FSHR	-1.06	-5.55
	LHR	-1.04	-4.63
	HDLR	-5.22**	-7.33
	LDLR	-1.87	-1.09
	HMGR	-16.38*	1.17
	StAR	-1.79	-8.20
	CYP11A	-1.81	-1.44
	CYP17	-15.50***	-1.30
	CYP19A	-1.97	-1.67
	20β-HSD	-1.11	3.53
	3β-HSD	-2.85*	-4.08
	Inhibin A	-3.94	-1.84
	Activin BA	-2.32*	-11.70
	Activin BB	1.16	-2.32
Liver	*ERα*	-1.92	1.07
	ERβ	-1.01	-23.53*
	ARα	-1.78	-11.61*
	VTG I	1.31	1.22
	VTG II	1.91	8.91*
	CHG H	1.05	6.45*
	CHG HM	1.43	2.51
	CHG L	3.61	-2.55
	CYP3A	1.54	-2.03
	Annexin max2	-1.59	-29.00**

Animal replicate (n = 4-6). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

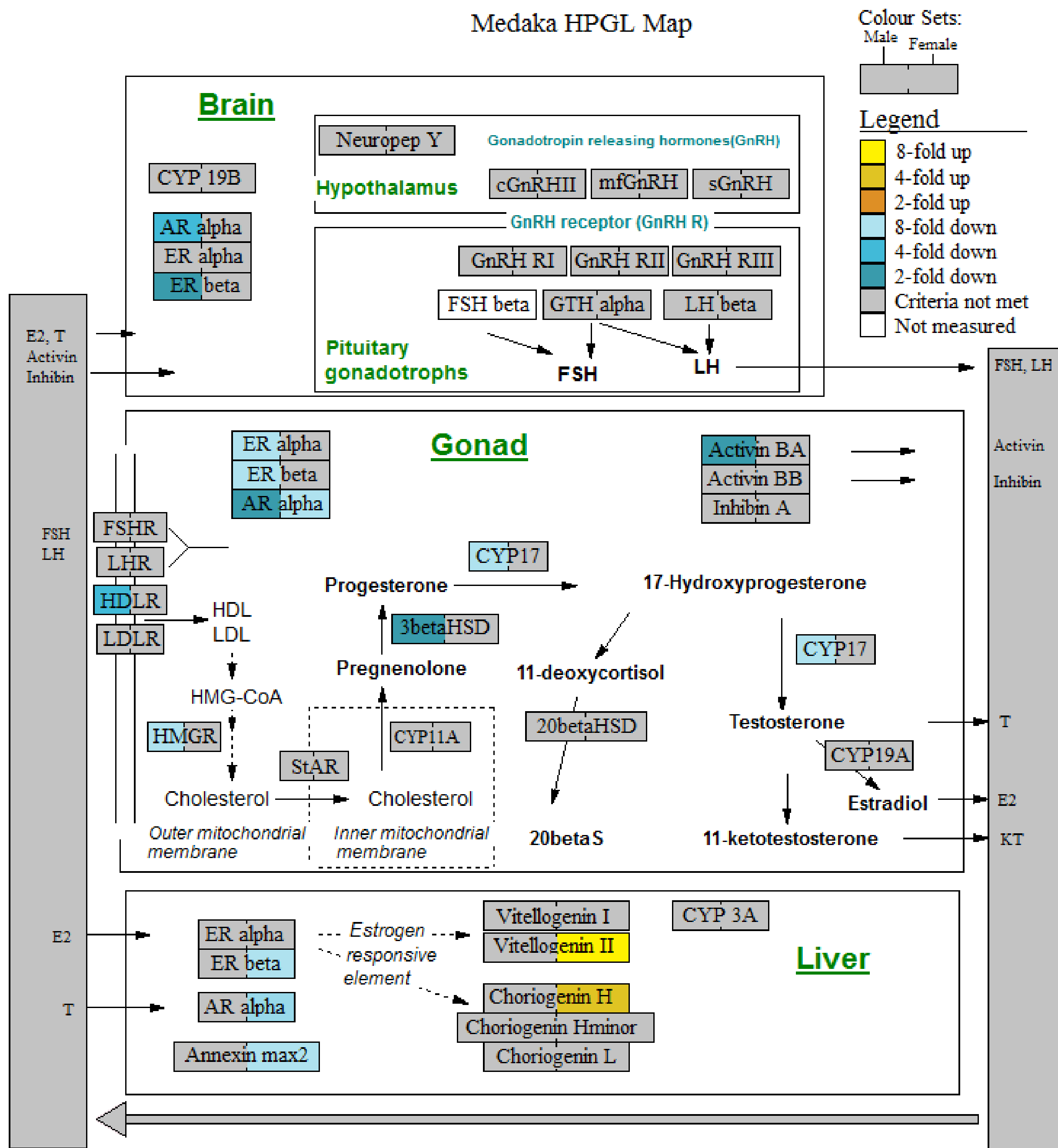

Figure 3.2. Graphical representation of the transcript response profile of the HPGL-axis in Japanese medaka exposed to the greater dose of the TBPH/TBB mixture (1422:1474 µg/ g food, w/w). Gene expression data are represented as striped colour sets with notches denoting sex of medaka. Eight colours were used to represent different fold-change thresholds. Criteria not met denotes a lack of statistical difference ($p < 0.05$) or lack of physiological relevance (< ±2-fold change). E2, 17β-estradiol; T, testosterone; KT, 11-ketotestosterone; FSH, follicle stimulating hormone; LH, luteinizing hormone; HDL, high-density lipoprotein; LDL, low density lipoprotein.

3.5 Discussion

There exist multiple lines of evidence from *in vitro* assays that TBPH and TBB might disrupt endocrine functions. Although *in vitro* assays are useful for screening of chemicals that might have endocrine disrupting effects, these preliminary tests cannot accurately represent the complexity of an *in vivo* system and it is necessary to perform definitive tests to determine whether chemicals affect the reproductive capacity of organisms. The current investigation is the first *in vivo* determination of endocrine disruption in fish exposed to the NBFRs TBPH/TBB.

3.5.1 Fecundity

Exposure to the mixture of TBPH/TBB impaired reproductive function of medaka. Cumulative fecundity of Japanese medaka exposed to the greatest concentration of TBPH/TBB was inhibited by 32%, but no significant effects of the lesser dose of the mixture were observed (Figure 3.1)

Changes in fecundity can be quantified as an integrative measurement endpoint for exposure to endocrine disrupting chemicals and provide a holistic measure of endocrine function. For example, the androgen 17β-trenbolone, two imidazole-type fungicides, prochloraz and ketoconazole, or the aromatase inhibitor fadrozole, inhibited cumulative fecundity of Japanese medaka[102-104]. Similar alterations in fecundity were observed in zebrafish (*Danio rerio*) exposed to DEHP, the structural analogue of TBPH[51]. Production of eggs by females exposed to the control diet was variable but similar to numbers reported in other studies[110]. Furthermore, a profile analysis which contrasts patterns of daily deposition of eggs among different treatment groups revealed significant differences between medaka exposed to the solvent control and medaka exposed to the greatest concentration of the mixture of TBPH/TBB (Figure C3.S1.). The profile analysis also illuminated the timeline of inhibition of deposition of eggs and differences in the overall pattern of fecundity. A within-group analysis of fecundity indicated that daily deposition of eggs by females exposed to the greatest concentration of the mixture changed across time (Figure C3.S2A.). Specifically, there appear to be two distinct phases of deposition of eggs, an initial toxic insult phase where deposition was significantly inhibited, and a compensatory phase where deposition recovered but remained lesser than initial numbers.

3.5.2 Abundances of transcripts

In teleost fishes, regulation of sexual reproduction is dependent on a complex signaling pathway mediated by the HPGL axis. The hypothalamus is a major site of initiating events and regulatory feedback of the HPGL signaling network as the organ integrates several endogenous and exogenous signals such as E2, T, photoperiod, and temperature. Gonadotropin-releasing hormones (GnRHs) synthesized in the hypothalamus in response to signaling events interact with GnRH receptors (GnRHRs) in the pituitary gland to regulate the synthesis and release of gonadotropin hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH consist of a non-covalently linked glycoprotein-hormone α-subunit (GTHα) and a specific β-subunit (FSHβ or LHβ)[111]. Male and female medaka exposed to the greater dose of TBPH/TBB demonstrated global down-regulation of mRNA of GnRHs (cGnRH II, sGnRH), GnRHRs (GnRH RI/II/III), and subsequent gonadotropin subunits, GTHα and LH-β (Table 3.2) which might have resulted in lesser synthesis and concentrations of FSH and LH in blood plasma. Although abundances of transcripts of many genes were not statistically different in medaka exposed to the mixture of TBPH/TBB compared to medaka exposed to the control diet, patterns of expression suggest that exposure to these compounds caused down-regulation of gene expression along the HPGL axis. Gonadotropins interact with gonadotropin receptors, FSHR and LHR, in gonads and initiate the release of second messenger molecules that regulate expression and activity of enzymes that catalyze the synthesis of sex hormones[112]. Interactions between gonadotropins and their receptors might have implications in the present study as expression of FSHR and LHR were significantly down-regulated in female and to a lesser extent in male medaka, which might provide another line of evidence of the potential decrease of gonadotropin hormones, FSH and LH. Alternatively, lesser abundances of transcripts of GnRHRs might have been caused by direct effects of the mixture on expression of these genes.

Exposure to the mixture of TBPH/TBB might have disrupted steroidogenesis. Significantly lesser abundances of transcripts of activin BA, CYP17 and 3β-HSD in male medaka might have inhibited synthesis of T. In female medaka, although there were no statistically significant effects on abundances of transcripts that encode enzymes of steroidogenesis, the trend of lesser abundances of several transcripts suggests that synthesis of E2 might have been impaired. If synthesis of E2 was impaired this effect might explain the

lesser fecundity by females exposed to the greatest concentration of the mixture of TBPH/TBB. Effects of the mixture on abundances of transcripts that encode enzymes of sex hormone steroidogenesis are similar to a previous study in which abundances of transcripts of CYP17 were lesser in a porcine primary testicular model exposed to 0.005 mg/L TBB, but not TBPH[100]. In contrast, the abundance of transcripts of CYP17 was greater in marine medaka and the Chinese rare minnow exposed to DEHP, a structural analogue of TBPH[49, 113]. The mechanism(s) of effects on steroidogenesis is not known, but exposure to the mixture of TBPH/TBB might have disrupted the activation of GnRHRs by GnRH and altered subsequent signal transduction cascades which affect expression of key steroidogenic enzymes including CYP11A, StAR, 3β-HSD, CYP19A and CYP17[111, 114]. Previous *in vitro* experiments have supported the observation that expression of CYP17, CYP21A and CYP11A, 3β-HSD, CYP19A are significantly altered following exposure to TBB and TBPH, respectively[100]. Alternatively, patterns of expression of genes involved in steroidogenesis in response to exposures to (anti)estrogenic and/or (anti)androgenic compounds might give insight to mechanisms of endocrine disrupting effects of TBPH/TBB. For example, expression of CYP17 and 3β-HSD[102], and HDLR, CYP17, activin BA, HMGR, and StAR was lesser in medaka exposed to the androgen, 17β-trenbolone[104] which is similar to the profile of expression in medaka exposed to the mixture of TBPH/TBB.

Any effects the mixture of TBPH/TBB might have had on steroidogenesis might have been caused, at least in part, by disruption of the metabolism of cholesterol. Among those proteins that play important roles in sex hormone steroidogenesis are those that function in the synthesis and transport of cholesterol. HMGR is the rate-limiting enzyme in the mevalonate pathway that is important for the synthesis of cholesterol; HDLR is a receptor protein which is essential to shuttle cholesterol to the cell from high density lipoproteins that transport cholesterol through blood; and StAR performs the rate limiting step in steroidogenesis of transporting cholesterol from the outer to inner mitochondrial membrane. Therefore, any alterations to the expression of genes encoding proteins involved in the synthesis and transport of cholesterol might increase or decrease the synthesis of E2 and T. In the current study, abundances of transcripts of HDLR and HMGR were lesser in male medaka exposed to the mixture of TBPH/TBB, while abundances of transcripts of HDLR and StAR were lesser in female medaka.

Effects of the greatest concentration of the mixture of TBPH/TBB on reproductive capacity of Japanese medaka might be related to effects on sex hormone receptor proteins. Estrogen and androgen receptors are ligand-activated transcription factors that interact with endogenous sex hormones to propagate endocrine signals. However, these receptor proteins can also interact with xenoestrogens and xenoandrogens, leading to disruption of endocrine functions. Sex steroid receptor proteins are important regulators of the HPGL axis, and *in vitro* investigations using the YES and YAS assays have demonstrated weak antagonistic effects of TBPH and TBB on human hERα and hARα, respectively[99]. However, because of differences in physiology of the ERs/ARs in humans and fishes, and differing complexities of the test systems, these results of the YES/YAS assays should be used only as an indicator of potential endocrine disrupting effects of TBPH/TBB. Teleost fishes have at least three estrogen receptors (ERα, ERβ1, and ERβ2) with differential tissue distributions[115], though only two have been included in the current rendition of the HPGL axis.

Exposure to the greatest concentration of the mixture of TBPH/TBB had a significant effect on the expression of ERβ and ARα. Significantly lesser abundances of transcripts of these receptors in brains from male medaka, but not brains from female medaka, suggest that effects were sex-dependent. Lesser abundances of transcripts of ERβ and ARα in brains from male medaka corresponded with the pattern of global down-regulation of gene expression in brains from male medaka (GnRHs, GnRHRs, GTHα, LH-β). ERs and ARs in the hypothalamus and pituitary interact with sex hormones as part of negative and positive feedback pathways and directly or indirectly regulate expression of gonadotropins and the subsequent production of sex hormones[107, 111, 116]. Furthermore, estrogen response elements (EREs) in the promoter region of ER genes auto-regulate their expression[117]. Therefore, lesser abundances of transcripts of the genes that encode these receptor proteins might be indicative of lesser concentrations of E2 in blood plasma or direct interaction of the mixture of TBPH/TBB with receptors, an effect which was observed *in vitro*[99].

Effects of the mixture of TBPH/TBB on abundances of transcripts of sex hormones receptors in livers were sex-specific. However, in contrast to brain, abundances of transcripts of ERβ and ARα were significantly lesser in livers from female medaka but were not different in livers from male medaka. In contrast to effects on ERβ, abundances of transcripts of ERα were

not significantly affected in female medaka exposed to the mixture of TBPH/TBB. There exists a complex interplay between ERα and ERβ and the current dogma suggests that ERα contributes almost exclusively to the induction of vitellogenesis through interactions with EREs in the promoter of the VTG gene[102, 118], whereas ERβ might function as a modulator of the expression of ERα [115]. Furthermore, several studies have demonstrated that increased expression of hepatic ERα is correlated to the induction of vitellogenesis[119, 120]. Though correlative relationships of induction exist between ERα and vitellogenesis, several investigations have also shown that expression of the VTG gene is not completely dependent on increases in the transcriptional activity of the ERα gene. In these studies, the increase in transcription of VTG required only basal or minimal concentrations of ERα, though prolonged induction of vitellogenesis was hypothesized to require increases in ERα transcripts[117, 121]. Patterns of gene expression in female medaka exposed to TBPH/TBB, which include the induction of VTG II, baseline transcript abundances of ERα, and lesser abundance of ERβ, a gene which likely does not affect vitellogenesis, corresponds to current knowledge regarding complex exchanges and endocrine disruption of ERs.

Greater expression of VTG is a sensitive biomarker of exposure to compounds that are agonists of ERs. Greater abundances of transcripts of VTG II and CHG H in female medaka indicate greater concentrations of E2 or activation of ER by the mixture of TBPH/TBB. However, the lesser abundances of transcripts of genes in the brain and gonads suggest that steroidogenesis might have been suppressed in these individuals, and therefore the greater abundances of transcripts of VTG II and CHG H might be due to activation of ER by the mixture[99]. Additionally, the lack of greater abundances of transcripts of VTG II and CHG H in male medaka suggests that effects of TBPH/TBB on ER signaling might be sex specific. Several other biomarkers of exposure to agonists of the ER including greater expression of CHG HM, CHG L, and VTG I were not induced in medaka exposed to the mixture of TBPH/TBB. However, VTG genes are differentially responsive to estrogens, and VTG II has been found to be more sensitive to estrogenic effects than VTG I[119].

3.5.3 Conclusions

The NBFRs TBPH and TBB are endocrine disrupting compounds. Lesser fecundity and altered expression of genes in medaka exposed to 1422:1474 μg/g food, w/w of a mixture of TBPH/TBB

are evidence of this effect. Effects of exposure to the mixture were sex-specific and altered expression profiles of key genes across signal initiation events in the brain, steroidogenesis in the gonad, and biomarkers of estrogenic effects in livers of female medaka. The global down-regulation of abundances of transcripts across all tissues of the HPGL axis is a unique signature of endocrine disruption resulting from exposures to TBPH/TBB which has manifested as inhibition of fecundity in female medaka. Fecundity is an integrated measure of endocrine disruption and has implications for population level effects as reductions in egg production could significantly alter population size and affect the survivability of the species.

4 CHAPTER 4: EFFECTS OF THE BROMINATED FLAME RETARDANT TBCO ON FECUNDITY AND PROFILES OF TRANSCRIPTS OF THE HPGL-AXIS IN JAPANESE MEDAKA

PREFACE

Chapter 2 demonstrated that TBCO did not activate the AhR, but elicited effects in EDC screening assays. TBCO produced antiandrogenic effects in the YAS assay and altered concentrations of the steroid hormone, E2, in the H295R assay system. Following the positive responses of TBCO in the *in vitro* screening level assessment, the goal of Chapter 4 was to characterize whole-organism endocrine-related adverse effects and mechanisms of action. In-depth characterization of adverse effects and mechanisms of action was critical to increase knowledge regarding the profile of toxicity of TBCO, to inform more accurate assessments of risk. This chapter was included in the first phase of this research program, the characterization of potential toxicities of TBPH, TBB, and TBCO.

The content of Chapter 4 was reprinted (adapted) from *Aquatic Toxicology*, (10.1016/j.aquatox.2015.01.018) D.M.V. Saunders, M. Podaima, Steve Wiseman, J.P. Giesy, "Effects of the brominated flame retardant TBCO on fecundity and profiles of transcripts of the HPGL-axis in Japanese medaka" 160, 180-187. Copyright 2015, with permission from Elsevier.

Author Contributions:

David M.V. Saunders (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Michelle Podaima (University of Saskatchewan) provided laboratory assistance with fish culture, maintenance and *in vivo* exposure.

Drs. Steve Wiseman and John P. Giesy (both at University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

4.1 Abstract

The novel brominated flame retardant, 1,2,5,6-tetrabromocyclooctane (TBCO) is an additive flame retardant which is marketed under the trade name Saytex® BCL-48. TBCO has recently been investigated as a potential alternative to the major use brominated flame retardant, hexabromocyclododecane (HBCD), which could have major implications for significant increases in amounts of TBCO used. Yet there is a lack of information regarding potential toxicities of TBCO. Recently, results of *in vitro* experiments have demonstrated the potential of TBCO to modulate endocrine function through interaction with estrogen and androgen receptors and via alterations to the synthesis of 17-β-estradiol and testosterone. Further research is required to determine potential endocrine disrupting effects of TBCO *in vivo*. In this experiment a 21-day fecundity assay with Japanese medaka (*Oryzias latipes*) was conducted to examine endocrine disrupting effects of TBCO *in vivo*. Medaka were fed a diet containing either 607 or 58 μg TBCO/g food, wet mass (wm). Fecundity, measured as cumulative deposition of eggs and fertilization of eggs, as well as abundances of transcripts of 34 genes along the hypothalamus–pituitary–gonadal–liver (HPGL) axis were measured as indicators of holistic endocrine disruption and to determine mechanisms of effects, respectively. Cumulative fecundity was 18% lesser by medaka exposed to 58 μg TBCO/g, wm food. However, fecundity of medaka exposed to 607 μg TBCO/g, wm food was not significantly different from that of controls. Organ-specific and dose-dependent alterations to abundances of transcripts were observed in male and female medaka. A pattern of down-regulation of expression of genes involved in steroidogenesis, metabolism of cholesterol, and regulatory feedback mechanisms was observed in gonads from male and female medaka which had been exposed to the greater concentration of TBCO. However, these effects on expression of genes were not manifested in effects on fertilization of eggs or fecundity. In livers from male and female medaka exposed to the lesser concentration of TBCO greater expression of genes that respond to exposure to estrogens, including vitellogenin II, choriogenin H, and ERα, were observed. The results reported here confirm the endocrine disrupting potential of TBCO and elucidate potential mechanisms of effects which include specific patterns of alterations to abundances of transcripts of genes in the gonad and liver of medaka.

4.2 Introduction

Brominated flame retardants (BFRs) are compounds that are added to consumer and industrial products to inhibit the propagation of fire. More than 175 brominated compounds are listed as flame retardants[2], including tetrabromobisphenol A (TBBPA), Deca-technical mixtures of polybrominated diphenyl ethers (PBDEs), and hexabromocyclododecane (HBCD). In 2001, these three compounds comprised approximately 59% (TBBPA), 26% (DecaBDE), and 8% (HBCD) for a combined 93% of the global market of BFRs[33]. Because of concerns about persistence, bioaccumulation, and toxicity (PBT) several governmental agencies and NGOs have reviewed the PBT profile and uses of HBCD[122-124]. HBCD is scheduled to be phased-out of European markets by 2015 and from North American markets in the near future[31]. However, to maintain compliance with consumer product flammability standards, replacement compounds such as novel brominated flame retardants (NBFRs) must be identified. Consequently, the uses and PBT profiles of potential replacement compounds have been reviewed. Assessments of alternatives to HBCD have included reports from the U.S. EPA Office of Pollution Prevention and Toxics, Design for the Environment (DfE), and Lowell Center for Sustainable Production[33, 125]. These reports have investigated several BFR alternatives that are promising substitutes for HBCD.

HBCD is used predominantly in building materials since the compound is mainly added to two insulating foams, extruded polystyrene (XPS) and expandable polystyrene (EPS). Increased demand for HBCD[33], which is fueled in part by growth of the construction sector, would also have implications for production volumes of any replacement compound. 1,2,5,6-Tetrabromocyclooctane (TBCO), which is marketed as Saytex® BCL-48, is an additive NBFR that has been investigated as an alternative to HBCD[33, 125]. Although the thermal stability of TBCO does not meet the operating temperature requirements for the manufacture of XPS foam, the compound might be incorporated into EPS foams and other materials to which HBCD is currently added[125]. Alternatives assessment reports have attempted to identify key health and environmental concerns for potential alternative products since the replacement compound should have lesser adverse effects on the health of humans and wildlife. Currently, there is little information regarding concentrations of TBCO in the environment or the compound's PBT profile. Thus, there is a lack of adequate information to include TBCO in an alternatives

assessment of HBCD. To date only three studies have attempted to detect TBCO in environmental matrices[126-128], and a single study investigated degradation of TBCO in the environment[129]. In an extensive assessment of potential effects on the environment that was conducted by the Environment Agency's Science Group of the United Kingdom[55], TBCO met EU criteria as a potential aquatic hazard, and met PBT criteria, specifically due to a large potential for persistence and bioaccumulation. Based on QSAR modeling, TBCO was classified as a potential acute toxicant to aquatic organisms with a predicted LC50 of <1 mg/L. That report also classified TBCO as having a low critical-tonnage, the amount of chemical which would have to be on the market to produce concern for an aquatic or terrestrial environment, and the compound has been identified as a priority for further substance-specific review.

There is insufficient toxicity data to properly assess the safety of TBCO as a replacement for HBCD. To date, there are only two studies of toxicity of TBCO, both of which investigated sub-lethal endpoints[99, 100]. TBCO was shown, by use of the yeast estrogen/androgen screening assays (YES/YAS), to be an antagonist of the human estrogen- and androgen-receptors (hERα/hARα). TBCO weakly antagonized the hERα, but antagonized the hARα in a dose dependent fashion with a maximal concentration of 300 mg/L resulting in a 74% inhibition of activity. In the same study, concentrations of 17β-estradiol (E2) were 3.3-fold greater in a H295R cellular assay system exposed to 15 mg/L of TBCO. In a second investigation, synthesis of testosterone (T) and E2, possibly because of greater expression of enzymes of steroidogenesis, was greater in porcine primary testicular cells exposed to 3.0 mg/L (2.1-fold) and 0.03 mg/L (5.9-fold), respectively[100].

Additional studies are required to augment existing aquatic toxicity data regarding TBCO for further alternatives assessments and to determine whether the compound has endocrine disrupting effects in an *in vivo* system. Therefore, in the present investigation an OECD 21-day short term fecundity assay[101] with medaka (*Oryzias latipes*) was used to quantify effects of TBCO on reproduction. Additionally, abundances of transcripts of 34 genes along the hypothalamic–pituitary–gonadal–liver (HPGL) axis were quantified to determine potential mechanisms of effects.

4.3 Materials and methods

4.3.1 Chemicals and reagents

1,2,5,6-Tetrabromocyclooctane (TBCO) and 6-fluoro-2,2′,4,4′-tetrabromodiphenyl ether (F-BDE-47) were obtained from Specs (Delft, SH, Netherlands) and AccuStandard (Connecticut, U.S.), respectively. All solvents including acetone, hexane, and dichloromethane (DCM) were of analytical grade and obtained from Fisher Scientific (Ontario, Canada).

4.3.2 Animal care

Embryos of Japanese medaka (*O. latipes*) were obtained from the aquatic culture unit at the US Environmental Protection Agency Mid-Continent Ecology Division (Minnesota, U.S.) and were shipped to the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan. Medaka were maintained in 30 L tanks under static-renewal conditions (27 °C 16:8 light/dark) and fed to satiation with flaked food and Artemia 3-times daily. All handling of fish and exposures were in accordance with protocols approved by the University of Saskatchewan Committee on Animal Care and Supply and Animal Research Ethics Board (UCACS-AREB; # 200090108).

4.3.3 Exposure protocol

Fish food was spiked with TBCO according to methods described previously[130]. Briefly, commercial flaked food (Nutrafin Basix Staple Food) was ground and spiked with a 150 mL solution of 2.34×10^{-3} M or 2.34×10^{-4} M TBCO in acetone, to make 1000 µg TBCO/g, wm food (greater dose), or 100 µg TBCO/g, wm food (lesser dose). Containers containing spiked food were shaken for 30 min to ensure thorough mixing of food and chemicals and subsequently air dried for 7 hr in a dark fume hood. A similar protocol was used to prepare the acetone-spiked control food. Concentrations of TBCO were based on previous *in vitro* studies of endocrine disruption[99, 100].

Exposure protocols were adapted from the fish short term reproductive assay, OECD test 229[101]. Medaka (14-wk-old) which ranged in mass from 0.3 to 0.6 g, live mass were randomly assigned to 10 L tanks under flow-through conditions. Eight males and eight females were placed into each tank and acclimated at 25 ± 2 °C with a 16:8 light/dark cycle and fed to satiation for 7-days prior to initiation of experiments. No mortalities were observed during the acclimation

period. Fish were exposed to either dose (lesser/greater) of TBCO or the solvent control (acetone prepared food) for 21-days. Fish were fed approximately 5% of body mass per day, and food was administered twice daily. At each 24 hr interval, total eggs from each tank were collected and counted and the number normalized to number of female medaka. Each treatment had four replicates. No mortalities were observed during the exposure period. Eggs that were collected at days 7, 14, and 21 were visualized by use of a dissecting microscope to assess success of fertilization. At termination of the 21-day experiment, fish were euthanized by cervical dislocation. Masses of whole body, livers and gonads were recorded to determine hepatic somatic index (HSI) and gonadal somatic index (GSI). Livers, brains (including pituitary), and gonads from each fish were frozen in liquid nitrogen and stored at −80 °C for quantification of abundances of transcripts.

4.3.4 Chemical analysis

Three replicates of each food type were homogenized with clean sodium sulphate and a mortar and pestle. Stainless steel extraction cells (33 mL) were packed with 1 g of fish food and an in-cell absorbent (activated alumina) to remove lipids (20:1, absorbent:lipid ratio)[106] then extracted by use of pressurized liquid extraction (ASE 200, Dionex, California, U.S.). Cells were extracted with a 1:1 solution of hexane and DCM at a temperature of 100 °C and 1500 psi for 10 min. The resulting extract was diluted 10x and 50 ng of F-BDE-47 was added as an internal standard. Three laboratory blanks and matrix spikes (spiked with 100 µg of TBCO) were extracted for quality assurance purposes.

Extracts were analyzed for TBCO by use of an Agilent (California, U.S.) 7890A gas chromatograph (GC) system coupled to an Agilent 5975C mass spectrometer (MS) operating in the electron impact ionization mode (EI). One microliter samples were injected at an injection port temperature of 280 °C in the splitless mode. Chromatographic separation was achieved with a 15 m x 250 µm i.d. Rtx-1614 fused silica capillary GC column, which had a 0.1 µm film thickness (Restek Corporation, Pennsylvania, U.S.). The carrier gas was helium at a constant flow of 1.5 mL/min. The following GC oven temperature program was used: 100 °C for 1 min, 5 °C/min to 190 °C for 2 min, 20 °C/min to 220 °C for 2 min, and 40 °C/min to 300 °C for 4 min. The GC/MS transfer line was maintained at 280 °C. Selected ion monitoring of *m/z* 267/187 and 343/234 was used for quantification/confirmation of TBCO and F-BDE-47. TBCO was

quantified by use of the internal standard method using F-BDE-47. TBCO was not detected in blank samples. The mean and standard error of the mean for TBCO recovery in the matrix spikes was 87 ± 0.12%.

4.3.5 Gene selection and graphical model

A total of 34 genes, which represent key signaling pathways in the brain, gonad, and liver, genes in steroidogenesis, and biomarkers of exposure to estrogens in the HPGL axis of Japanese medaka were selected for study based on previous results[102-104, 107, 130]. Primers were based on sequences available in the NCBI GeneBank database and were designed by use of NCBI Primer-Blast software. Sequences of nucleotide primers and efficiencies are given in the appendix (Table C4.S1.).

Graphical models depicting abundances of transcripts of 34 genes across the HPGL axis were produced by use of GenMapp 2.0 (Gladstone Institutes, California, U.S.) and were constructed and maintained by Dr. Xiaowei Zhang (Nanjing University, JS, China). Criteria for inclusion in the model were (a) ≥2-fold change in abundances of transcripts to represent physiological relevance, and (b) statistically significant changes in abundances of transcripts (Figure 2 and Figure 3).

4.3.6 Quantitative real-time PCR

Total RNA was extracted from livers, brains, and gonads by use of the RNeasy Plus Mini Kit (Qiagen, Ontario, Canada) according to the protocol provided by the manufacturer. Concentrations of RNA were determined by use of a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Delaware, U.S.) and stored at −80 °C. First strand cDNA was synthesized from 1 µg of RNA and by use of the QuantiTect Reverse Transcription Kit (Qiagen) according to the protocol provided by the manufacturer.

Real-time quantitative PCR (qPCR) was performed according to previously published methods[130]. Amplification of a single PCR product was confirmed by melt curve analysis and target gene transcript abundance was quantified by use of the ddCt method by normalizing to abundance of transcripts of the RPL-7 housekeeping gene.

4.3.7 Statistical analysis

Statistical analysis was conducted by use of SPSS statistics software (V.20). Normality of data was determined by use of the Kolmogorov–Smirnov test and homogeneity of variance was determined by use of Levene's test. Unless otherwise noted, data was analyzed by use of analysis of variance (ANOVA), followed by Tukey's test. Differences in daily production of eggs, within groups, was determined by use of a repeated-measures ANOVA. If assumptions of sphericity were violated a Greenhouse–Geisser correction was applied. Further, post hoc tests were corrected by use of a Bonferroni adjustment. Due to the conservative nature of the Bonferroni adjustment, data points were pooled according to data trends and statistical differences which were assessed by pairwise comparisons. All post hoc comparisons in repeated measures analysis were made to group 1 which represented the initial conditions of egg deposition in the experiment. Profile analyses were performed by use of a MANOVA test. A probability level of $p \leq 0.05$ was considered significant except in cases of Bonferroni adjustments. All data are shown as mean ± standard error of mean (S.E.M.).

4.4 Results

4.4.1 Concentrations of chemicals in food

Concentrations of TBCO differed from the reported nominal concentrations in two of three food types (Table 4.1). The measured concentration of TBCO was 58% and 61% of the desired nominal concentration in both types of spiked food. Concentrations of TBCO in food spiked with clean acetone were less than method limits of detection.

Table 4.1. Concentrations of TBCO in three diets used in the 21-day fecundity assay. Concentrations of TBCO are presented as mean ± standard error (μg/g, wm food). Three replicates were extracted and analyzed for each food type.

Feed [nominal]	TBCO
Control	ND
1000 μg/g, wm food	607±65.2
100 μg/g, wm food	57.7±4.95

ND: below limit of detection

4.4.2 Chemical-induced effects on fecundity of medaka

There were no significant differences in HSI or GSI of male or female medaka exposed to either concentration of TBCO compared to medaka exposed to the solvent control (Table C4.S2.). Fertility of male fish was not significantly affected since the proportion of fertilized eggs collected at days 7, 14, and 21 were not different between fish exposed to either concentration of TBCO or the solvent control.

Exposure to TBCO affected fecundity of medaka. There were significant differences in cumulative production of eggs between female medaka exposed to 58 μg TBCO/g, wm food and solvent controls, but there were no differences observed between medaka exposed to the greater concentration of TBCO and solvent controls (Figure 1). Numbers of eggs produced relative to solvent control were 95% ± 6.2 and 82% ± 4.0 by female fish exposed to greater and lesser concentrations of TBCO, respectively. Further statistical analyses were conducted to augment findings of the cumulative fecundity analysis. Profiles of daily production of eggs were significantly different between fish exposed to the control and the lesser concentration of TBCO but not the greater concentration of TBCO (Figure C4.S2.). A within-group repeated measures analysis also revealed significant differences in daily deposition of eggs over time by fish exposed to the lesser concentration of TBCO (Figure C4.S3A.), but not the greater concentration of TBCO or the solvent control. Across 21-repeated measures, a post hoc analysis with a Bonferroni adjustment was prohibitively conservative. To accommodate the conservative nature of the Bonferroni adjustment, time points were grouped according to statistical differences in daily deposition of eggs (Figure C4.S3B.). Pooled group 1 represented the initial period of deposition of eggs and was set at 100% fecundity. Statistically significant differences between initial depositions of eggs, group 1, and all subsequent groups (2–4) were observed.

4.4.3 Gene expression profiles

Abundances of transcripts of genes of the HPGL axis were quantified in male and female medaka exposed to the greater and lesser concentrations of TBCO. There were no statistically significant changes in abundances of transcripts in brains from male or female medaka exposed to either concentration of TBCO (Table 4.2).

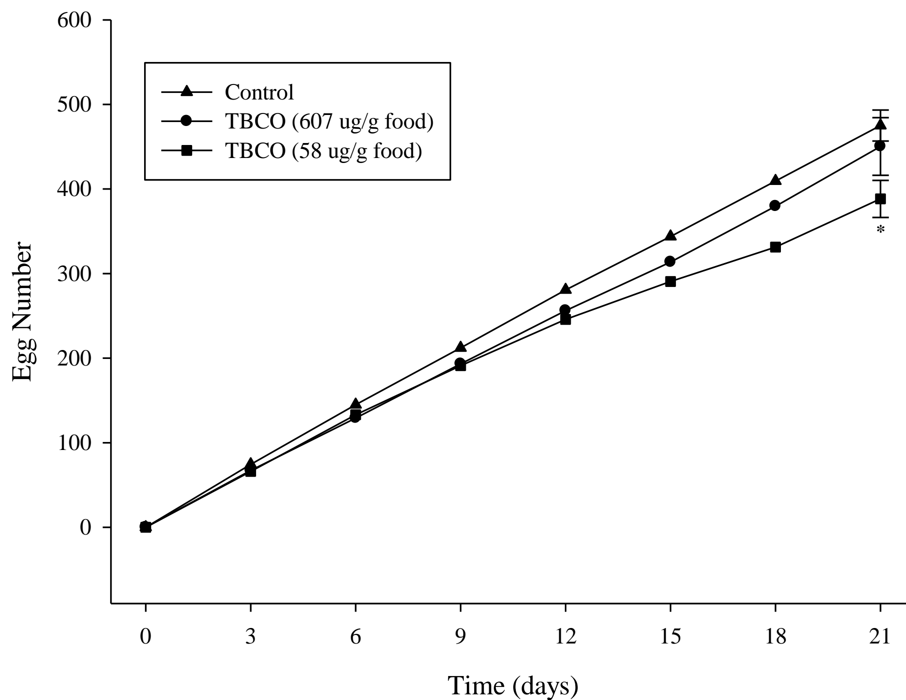

Figure 4.1. Cumulative production of eggs (fecundity) by medaka exposed to the greater concentration of the TBCO (607 µg/ g food, w/w), the lesser concentration of TBCO (58 µg/g food, w/w) and solvent control. The values represent the mean cumulative number of eggs per female over a 21-day period. The experiment included 4 replicate tanks, and each contained 8 female/male medaka. Asterisks indicate a significant difference ($p < 0.05$) when compared to the control group.

Table 4.2. Response profiles of genes of the hypothalamic-pituitary-gonadal-liver (HPGL) axis in Japanese medaka exposed to the greater (607 µg/g food) and lesser (58 µg/g food) concentrations of TBCO. Transcript responses are expressed as fold change compared to corresponding solvent controls

		Male		**Female**	
Tissue	**Gene**	**High Dose**	**Low Dose**	**High Dose**	**Low Dose**
Brain	*ERα*	-2.37	1.11	-3.62	-1.09
	ERβ	-2.12	1.13	-2.14	-2.07
	ARα	-1.99	1.70	-3.24	1.03
	Neuropep Y	-6.91	-1.28	1.13	1.37
	cGnRH II	1.22	2.71	2.13	1.45
	mfGnRH	3.33	1.49	5.39	2.72
	sGnRH	-4.92	1.40	-9.30	-1.51
	GnRH RI	11.51	1.10	-1.82	-3.73
	GnRH RII	-1.05	1.79	1.36	2.23
	GnRH RIII	3.46	1.42	4.67	1.69
	GTHa	-2.69	-8.55	-6.63	1.56
	LH- β	-1.39	-16.15	-3.48	1.49
	CYP19B	-5.45	1.61	1.13	1.70
Gonad	*ERα*	-5.19**	-4.35**	-6.72*	1.71
	ERβ	-14.77**	-1.44	-6.22*	1.12
	ARα	-2.45*	-2.36**	-8.94***	1.89
	FSHR	-2.49	-1.19	1.55	1.00
	LHR	-1.06	-3.60	-2.25	-2.16
	HDLR	-19.10**	-1.21	-21.20*	-1.45
	LDLR	-1.63	-1.59	-12.13*	2.92
	HMGR	-16.27**	-1.09	-7.37	1.47
	StAR	-16.37*	3.19	-16.26*	-4.79
	CYP11A	-1.53	1.04	1.60	1.01
	CYP17	-13.63**	-1.65	-2.71	-1.07
	CYP19A	1.33	-7.76	1.47	-1.53
	20β-HSD	-3.00	-3.66	-1.75	1.00
	Inhibin A	-6.04	-20.82*	-8.03*	2.01
	Activin BA	1.52	-18.70*	-12.94*	1.64
Liver	*ERα*	8.79	6.31	-10.09	11.83*
	ERβ	-5.23	-1.33	1.93	-16.86*
	ARα	-14.22	-7.63	-26.81	-12.06
	VTG I	1.60	3.82	2.32	5.04
	VTG II	1.75	12.99	-1.95	18.37*
	CHG H	-1.75	1.45	2.59	8.40*
	CHG HM	1.97	5.87*	3.44	2.46
	CHG L	1.70	13.70	-1.77	2.86
	CYP3A	2.54	28.29**	-5.46	-2.03
	Annexin max2	-2.52	-2.77	-1.01	-8.32*

Animal replicate (n = 4-6). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Exposure to TBCO affected expression of genes in gonads from male and female medaka. Some effects of TBCO on expression of genes in gonads were concentration-dependent. Abundances of transcripts of HDLR, StAR, and ERβ were significantly lesser in gonads from male and female fish exposed to the greater concentration of TBCO but not the lesser concentration of TBCO (Table 4.2 and Figures 4.2, 4.3). Some effects of TBCO on expression of genes were sex-specific. Abundances of transcripts of HMGR and CYP17 were lesser only in gonads from male medaka whereas the abundance of transcripts of LDLR was lesser only in gonads from female medaka (Table 4.2 and Figures 4.2, 4.3). Some effects of TBCO on gene expression were neither sex dependent nor concentration dependent. Abundances of transcripts of ERα and ARα were significantly lesser in males exposed to either concentration of TBCO and in females exposed to the greater concentration of TBCO (Table 4.2 and Figures 4.2, 4.3). Abundances of transcripts of Inhibin A and Activin BA were significantly less in males exposed to the lesser concentration of TBCO and in females exposed to the greater concentration of TBCO (Table 4.2 and Figures 4.2, 4.3).

TBCO affected abundances of transcripts of several genes in livers from male and female medaka exposed to the lesser concentration, but not the greater concentration, of TBCO (Table 4.2). Abundances of transcripts of ERα, VTG II, and CHG H were significantly greater, while ERβ and Annexin max2 were significantly lesser in female medaka exposed to the lesser concentration of TBCO (Figure 4.2). Abundances of transcripts of CHG HM and CYP3A were significantly greater in male medaka exposed to the lesser concentration of TBCO (Figure 4.2). The pattern of gene expression in livers from male and female medaka exposed to the lesser concentration of TBCO was very similar, though many alterations to abundances of transcripts were not statistically significant (Table 4.2).

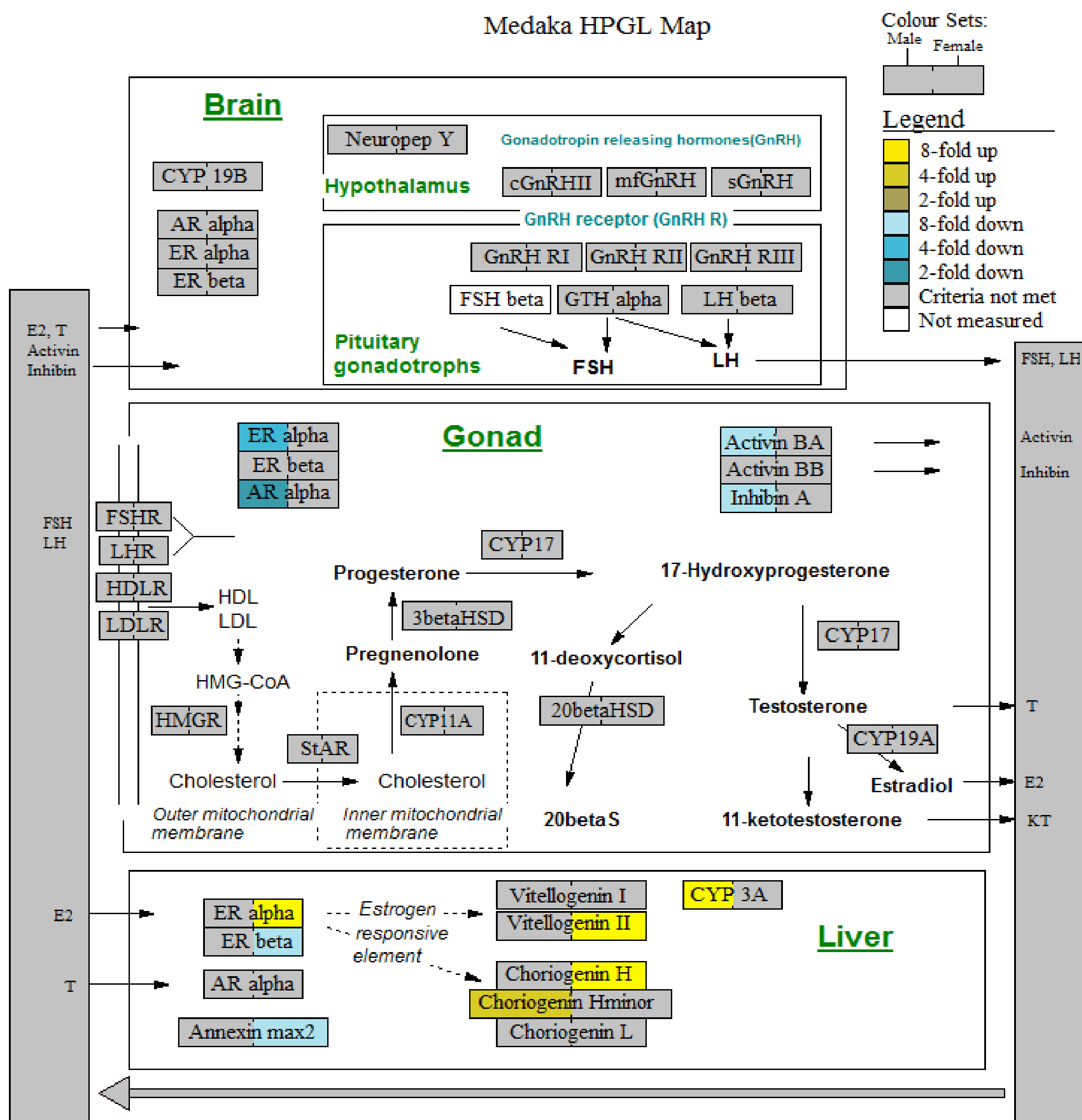

Figure 4.2. Graphical representation of the transcript response profile of the HPGL-axis in Japanese medaka exposed to the lesser concentration of TBCO (58 µg/ g food). Gene expression data are represented as striped colour sets with notches denoting sex of fish. Eight colours were used to represent different fold-change thresholds. Criteria not met denotes a lack of statistical difference ($p < 0.05$) or lack of physiological relevance (< ±2-fold change). E2, 17β-estradiol; T, testosterone; KT, 11-ketotestosterone; FSH, follicle stimulating hormone; LH, luteinizing hormone; HDL, high-density lipoprotein; LDL, low density lipoprotein.

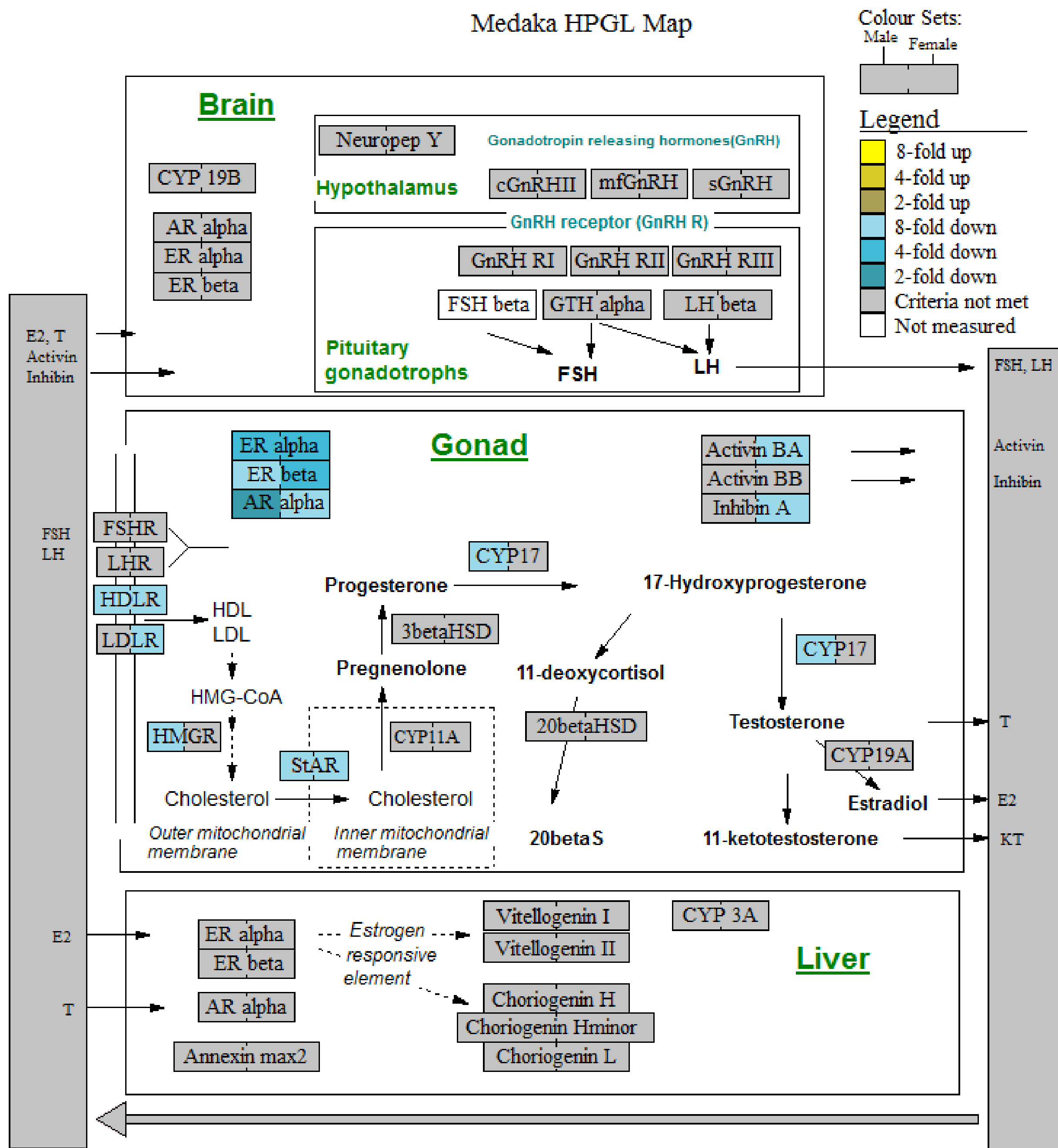

Figure 4.3. Graphical representation of the transcript response profile of the HPGL-axis in Japanese medaka exposed to the greater concentration of TBCO (607 µg/ g food). Gene expression data are represented as striped colour sets with notches denoting sex of fish. Eight colours were used to represent different fold-change thresholds. Criteria not met denotes a lack of statistical difference ($p < 0.05$) or lack of physiological relevance (< ±2-fold change). E2, 17β-estradiol; T, testosterone; KT, 11-ketotestosterone; FSH, follicle stimulating hormone; LH, luteinizing hormone; HDL, high-density lipoprotein; LDL, low density lipoprotein.

4.5 Discussion

TBCO is currently a low-production NBFR which has been assessed as a potential aquatic hazard with low-critical tonnage. Furthermore, TBCO is a potential replacement compound for HBCD, and due to the pending phase out of HBCD from global markets, the production volume of TBCO might drastically increase in the near future, which might increase risk to the environment including aquatic systems. It is of great importance to understand the persistence, bioaccumulation and toxic effects of TBCO prior to potential increases in production volumes. This investigation is an initial *in vivo* assessment of the endocrine disrupting potential of TBCO in a standard laboratory fish and is critical to generate meaningful data for risk and alternatives assessments.

4.5.1 Fecundity

Exposure to TBCO impaired reproductive performance of female medaka. Cumulative fecundity of medaka exposed to the lesser concentration of TBCO was inhibited by 18%, but no significant effects were observed in medaka exposed to the greatest concentration of TBCO (Figure 4.1). Similar disparities of effects on fecundity between fish exposed to the greater and lesser concentrations of TBCO were revealed by use of profile analyses and within-group repeated measures analyses. The profile analyses, which contrasts patterns of daily deposition of eggs among different treatment groups, revealed significant differences between medaka exposed to the lesser concentration of TBCO and controls, but not between medaka exposed to the greater concentration of TBCO and controls (Figure C4.S2.). The within-group repeated-measures analysis of fecundity showed that daily deposition of eggs by medaka exposed to the lesser concentration of TBCO changed over the duration of the study. This effect was not observed in medaka exposed to the greatest concentration of TBCO or controls (Figure C4.S3.). Furthermore, there were two distinct phases of deposition of eggs by medaka exposed to the lesser concentration of TBCO, an initial toxic insult phase where deposition was significantly inhibited, and a compensatory phase in which deposition slightly recovered but remained lesser than initial numbers (Figure C4.S3.).

4.5.2 Abundances of transcripts

Differences between effects of the greater and lesser concentrations of TBCO on fecundity might have been caused by differences in effects on expression of genes of the HPGL axis. Abundances of transcripts of several genes across the HPGL axis were altered in medaka exposed to either concentration of TBCO, but profiles of gene expression were unique between the two concentrations. With the exception of the brain, in which changes in gene expression were not significant, effects on gene expression were organ specific (Table 4.2). Based on the number of statistically significant changes in expression, gonad was the most sensitive tissue in males and in females exposed to the greater concentration of TBCO. But it is not known if changes in abundance of transcript in gonads from males exposed to TBCO affected fecundity of females. Livers from female medaka exposed to the lesser concentration of TBCO were more sensitive than gonads. These organ specific alterations might help to explain differences in inhibition of fecundity between medaka exposed to the lesser and greater concentrations of TBCO and have identified liver as the target tissue of effect.

Abundances of transcripts of several genes were significantly altered in gonads from male and female medaka exposed to the greater concentration of TBCO and in male medaka exposed to the lesser concentration of the compound. Abundances of transcripts of StAR, HDLR, HMGR, and LDLR, which are important for the synthesis and transport of cholesterol, were lesser in either male or female medaka exposed to the greater concentration of TBCO (Figure 4.3). Because cholesterol is the precursor of sex hormones, any significant alterations to abundances of transcripts encoding proteins involved in synthesis and transport of cholesterol might affect concentrations of T or E2. Several studies that utilized *in vitro* assays have shown that exposure to TBCO caused alterations to concentrations of T and E2. Concentrations of E2 increased in H295R cells exposed to 15 mg/L TBCO[99], while concentrations of T and E2 were significantly greater in primary porcine testicular cells exposed to 0.03 mg/L TBCO[100]. Additionally, genes involved in sex hormone steroidogenesis and regulatory networks in the HPGL axis, including CYP17, Inhibin A, Activin BA, ERα, ERβ, and ARα were down-regulated in male and/or female medaka exposed to the greatest concentration of TBCO (Figure 4.3). *In vitro* assessments of the endocrine disrupting effects of TBCO have shown alterations to abundances of transcripts of several genes involved in steroidogenesis, including CYP17 and

CYP21A[100], and antagonistic interaction of TBCO with the human ERα and ARα[99]. Significant alterations to the expression of genes involved in steroidogenesis and regulatory networks might disrupt reproductive performance in fish by affecting homeostasis of sex hormones and altering normal functions of the HPGL axis. Yet female medaka exposed to the greater concentration of TBCO did not demonstrate inhibition of fecundity or altered patterns of deposition of eggs, and there were no adverse effects on fertility of males. In this experiment, fecundity was assessed as an integrated measure of endocrine function, but concentrations of sex hormones were not measured. The pattern of lesser abundances of transcripts in gonads from medaka exposed to the greater concentration of TBCO would likely lead to reductions in concentrations of sex hormones. However, several compensatory networks present in the HPGL axis might have offset this effect thereby preventing effects on fecundity.

Abundances of transcripts of several genes were altered in livers from female medaka exposed to the lesser concentration of TBCO, which might have caused the lesser cumulative deposition of eggs. Furthermore, there were no significant alterations to abundances of transcripts in livers from male or female medaka exposed to the greater concentration of TBCO and no inhibition of cumulative deposition of eggs was observed. These results support the proposed link between altered expression of genes and inhibition of fecundity and provide a mechanistic explanation of effects on apical endpoints. Female medaka exposed to the lesser concentration of TBCO had significantly greater abundances of transcripts of ERα but lesser abundances of transcripts of ERβ (Figure 4.2). Current dogma suggests that activation of ERα by E2 stimulates vitellogenesis whereas ERβ might solely function as a modulator of expression of ERα. Several studies have demonstrated that increased expression of hepatic ERα is correlated to the induction of vitellogenesis[119, 120, 131] whereas expression of ERβ might be down-regulated by estrogenic compounds[115, 131]. A pattern of up-regulation of expression was observed in genes regulated by ERs and involved in vitellogenesis, which include VTGs and CHGs, though only VTG II and CHG H were significantly increased. However, VTG genes are differentially responsive to estrogens, and VTG II has been shown to be more sensitive to estrogenic effects than VTG I[119]. Because expression of VTGs and CHGs occurs in response to E2, greater abundances of these transcripts is likely a response to xenoestrogens or elevated concentrations of endogenous E2[132]. A similar pattern was observed in male medaka exposed to the lesser concentration of TBCO but the effects were not statistically significant (Table 4.2). It is

interesting to note that female medaka which presented inhibition of fecundity also presented increases in expression of VTG and CHG, two gene groups which are associated with production of eggs. Incongruities between gene expression and fecundity can likely be attributed to the complexity of HPGL signaling and regulatory networks, and timing of spawning patterns. The pattern of greater expression of ERα and lesser expression of ERβ is consistent with patterns of gene expression in response to xenoestrogens or endogenous E2 and, paired with increases in biomarkers of estrogenic exposure, provide further evidence for estrogenic effects of TBCO.

4.5.3 Conclusions

The NBFR, TBCO, is an endocrine disrupting compound and might alter estrogen signaling. Lesser fecundity observed in medaka exposed to the lesser concentration of TBCO, and patterns of gene expression that mimicked patterns of expression known to be caused by exposure to (xeno)-estrogens are evidence of this effect. Alterations to abundances of transcripts in medaka which experienced inhibition of fecundity occurred almost exclusively in the liver and were associated with vitellogenesis. In contrast, alterations to abundances of transcripts in medaka that experienced no inhibition of fecundity occurred almost exclusively in gonads and were associated with sex hormone steroidogenesis and metabolism of cholesterol. Differences in inhibition of fecundity experienced between dosing groups was likely attributable to different patterns of altered expression of genes.

Although there is little research regarding the toxicity of TBCO, the compound has been designated as a potential aquatic hazard, as having low critical-tonnage, and is an option to replace HBCD, a high production volume chemical[124]. Current research into the PBT characteristics of TBCO might represent a unique opportunity for researchers to accurately assess risk prior to incidences of environmental contamination or toxic insult.

5 CHAPTER 5: DETECTION, IDENTIFICATION, AND QUANTIFICATION OF HYDROXYLATED BIS(2-ETHYHEXYL)-TETRABROMOPHTHALATE ISOMERS IN HOUSE DUST

PREFACE

Following the determination of endocrine disrupting effects of TBPH, TBB, and TBCO in Chapters 2, 3, and 4, environmental concentrations of these NBFRs were required to define more accurate assessments of exposure and eventually inform assessments of risk. The objective of Chapter 5 was to develop a novel and improved analytical method by use of a newly acquired ultra-high resolution mass spectrometer (Q Exactive Orbitrap instrument) and determine concentrations of TBPH, TBB, and TBCO in dust collected from ECEs. Yet, upon analysis of analytical standards of TBPH and TBB, several unknown compounds were observed. Further investigation of the technical products Firemaster® 550 and BZ-54 confirmed the presence of these unknown contaminants. The amended objective of this chapter was to identify these novel compounds in analytical standards and technical products and investigate their presence and quantities in dust from ECEs. Though the hydroxylated contaminants of both TBPH and TBB were characterized in standards and environmental samples, the investigation of hydroxylated TBB was not included in this thesis. This chapter was included in the second phase of this research program, the characterization of exposure to TBPH and TBB in the indoor environment.

The content of Chapter 5 was reprinted (adapted) from *Environmental Science & Technology*, (10.1021/es505743d) H. Peng*, D.M.V. Saunders*, J. Sun, G. Codling, S. Wiseman, P.D. Jones, J. P. Giesy, “Detection, identification, and quantification of hydroxylated bis(2-ethylhexyl)-tetrabromophthalate isomers in house dust” 49, 2999-3006. Copyright 2015, with permission from the American Chemical Society.

Author Contributions:

* Indicates co-first authorship. Dr. Peng Hui and David Saunders were listed in alphabetical order.

David M.V. Saunders (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Dr. Hui Peng (University of Saskatchewan) designed the experiment, helped with data analysis and preparation of figures, and co-drafted the manuscript.

Dr. Jianxian Sun (University of Saskatchewan) laboratory aid in the preparation and processing of environmental samples.

Dr. Gary Codling (University of Saskatchewan) provided technical support for analytical instrumentation.

Drs. Steve Wiseman, Paul D. Jones, and John P. Giesy (all at University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

5.1 Abstract

Ultra-High Resolution LC/mass spectrometry (LC-UHRMS; Thermo Fisher Q-Exactive) was used to identify two novel isomers of hydroxylated bis(2-ethylhexyl)-tetrabromophthalate (OH-TBPH) which were unexpectedly observed in a commercial standard of TBPH. By combining ultra-high resolution (UHR) mass spectra (MS1), mass errors to theoretical $[TBPH-Br+O]^{-}$ were 2.1 and 1.0 ppm for the two isomers, UHR-MS2 spectra and NMR analysis; the structures of the two compounds were identified as hydroxylated TBPH with a hydroxyl group on the aromatic ring. Relatively great proportions of the two isomers of OH-TBPH were detected in two technical products, Firemaster® 550 (FM-550; 0.1% and 6.2%, respectively) and Firemaster® BZ 54 (BZ-54; 0.1% and 7.9%), compared to a commercial standard (0.4% and 0.9%). To simultaneously analyze OH-TBPH isomers and TBPH in samples of dust, a method based on LC-UHRMS was developed to quantify the two compounds, using negative and positive ion modes, respectively. The instrumental limit of detection for TBPH was 0.01 μg/L, which was 200–300 times better than traditional methods (2.5 μg/L) based on gas chromatography–mass spectrometry. The analytical method combined with a Florisil cleanup was successfully applied to analyze TBPH and OH-TBPH in 23 indoor dust samples from Saskatoon, Saskatchewan, Canada. Two OH-TBPH isomers, OH-TBPH1 and OH-TBPH2, were detected in 52% and 91% of dust samples, respectively. Concentrations of OH-TBPH2 (0.35 ± 1.0 ng/g) were 10-fold greater than those of OH-TBPH1 (0.04 ± 0.88 ng/g) in dust, which was similar to profiles in FM-550 and BZ-54. TBPH was also detected in 100% of dust samples with a mean concentration of 733 ± 0.87 ng/g. A significant ($p < 0.001$) log–linear relationship was observed between TBPH and OH-TBPH isomers, further supporting the hypothesis of a common source of emission. Relatively small proportions of OH-TBPH isomers were detected in dust (0.01% ± 0.67 OH-TBPH1 and 0.1% ± 0.60 OH-TBPH2), which were significantly less than those in technical products ($p < 0.001$). This result indicated different environmental behaviors of OH-TBPH and TBPH. Detection of isomers of OH-TBPH is important, since compounds with phenolic groups have often shown relatively greater toxicities than non-hydroxylated analogues. Further study is warranted to clarify the environmental behaviors and potential toxicities of OH-TBPH isomers.

5.2 Introduction

Brominated flame retardants (BFRs) have caused concern to regulatory agencies and the general public[133], particularly regarding polybrominated diphenyl ethers (PBDEs), which were the most widely used BFRs[134]. Previous studies have reported that PBDEs are ubiquitous in the environment[135-137], bioaccumulated into organisms[138-140], and can cause toxicity[141-143]. Since 2004, due to these concerns, production and sales of two major commercial PBDE products, Penta- and Octa-BDEs, have been voluntarily withdrawn or banned in some parts of the world[144]. Following the phase-out of PBDEs, the BFR industry has begun to use alternative brominated compounds to replace PBDEs. Investigations of the behaviors of these alternative BFRs and assessment of their potential health and ecological risk is thus of special interest.

Firemaster® 550 (FM-550) and Firemaster® BZ-54 (BZ-54) are two PBDE replacement mixtures, both of which contain 2-ethylhexyl-2,3,4,5-tetrabromo-benzoate (TBB) and bis(2-ethylhexyl)-tetrabromophthalate (TBPH). The percentage of TBPH in FM-550 and BZ-54 is 15% and 30%, respectively[30]. Since TBPH is a brominated analogue of bis(2-ethylhexyl)phthalate (DEHP), a well-studied compound that has exhibited a range of toxicities[145-147], concerns are emerging regarding the potential health risks of TBPH. For example, metabolic pathways for TBPH similar to those of DEHP have been observed. *In vitro* metabolism of TBPH has resulted in production of a monoester metabolite, mono-(2-ethyhexyl) tetrabromophthalate (TBMEHP)[78]. Furthermore, hepatotoxic effects and interaction with the peroxisome proliferator activated receptor (PPAR), known effects of DEHP, have also been elicited by the monoester metabolite of TBPH[54]. A recent study, using *in vitro* cellular assays, has also reported the antiestrogenic potency of TBPH[99]. In addition to potential toxicities, results of previous studies have demonstrated the widespread presence of TBPH in house dust[38], air[9], sediment[148], and tissues of wildlife[149]. Of particular importance, a recent study has reported the presence of TBPH in blood serum and milk of nursing women, which indicates its potential risk to the health of humans, particularly infants[150]. Since TBPH has been detected at relatively great concentrations in dust (geometric mean (GM) concentration was 234 ng/g, dry mass (dm)), which was comparable to concentrations of hexabromocyclododecane (HBCD; GM was 354 ng/g) in the same samples from the United States, indoor dust ingestion is expected to be one of

the primary routes of exposure[38]. Three other studies have also found TBPH in house dust from California and Norway at similar concentrations, ng/g, dm[56, 151, 152].

The study reported here developed a sensitive liquid chromatography ultrahigh resolution mass spectrometry (LC-UHRMS) method to analyze TBPH in dust samples collected from houses in Saskatoon, Saskatchewan (SK), Canada. Using UHRMS, two isomers of hydroxylated TBPH (Figure 5.1) in commercial standards and technical products were unexpectedly detected. Finally, the UHRMS method combined with a Florisil cartridge cleanup was used to simultaneously analyze TBPH and OH-TBPH isomers in 23 dust samples from eight homes in Saskatoon, SK, Canada.

5.3 Materials and methods

5.3.1 Chemicals and reagents

Native TBPH standard (purity, 98.1%) was purchased from AccuStandard (Connecticut, U.S.), and its surrogate, mass-labeled standard d_{34},$^{13}C_6$-TBPH (purity, >98%) was purchased from Wellington Laboratories Inc. (Ontario, Canada). Commercial standards of TBPH (purity, >95%) were purchased from Waterstone Technology (WST; Indiana, U.S.), and BZ-54 and FM-550 technical products were gifts from the Heather Stapleton Research Group at Duke University, Nicholas School of the Environment. OH-TBPH2 was purified by use of the HPLC fraction from BZ-54 technical products. Florisil (6 cm^3, 1 g, 30 μm) solid-phase extraction (SPE) cartridges were purchased from Waters (Massachusetts, U.S.). Ammonia solution (28–30%) was purchased from Alfa Aesar Chemical Industries (Ward Hill, Massachusetts, U.S.). Dichloromethane (DCM), methanol, and acetone were all “omni-Solv” grade and were purchased from EMD Chemicals (New Jersey, U.S.).

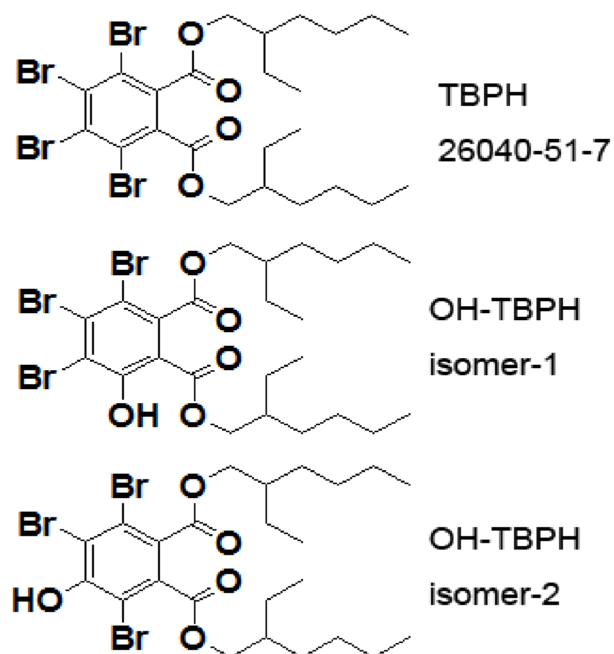

Figure 5.1. Chemical structures of TBPH and two identified OH-TBPH isomers.

5.3.2 Purification of OH-TBPH by HPLC fractionation

HPLC fractionation was used to isolate OH-TBPH from technical product BZ-54 which contained only TBB and TBPH compared to FM-550. Fractions were collected at 2-min interval from 0 min to 120 min, and then OH-TBPH2 in each fraction was quantified by use of UHPLC-Q Exactive after 10,000-fold dilution with a mixture of methanol and acetone (v/v, 1:1). Fractions which contained OH-TBPH2 were collected and combined, and then evaporated. Fractionation was conducted by use of a Betasil C18 column (5 µm; 22.1 mm x 150 mm; Thermo Fisher Scientific) which was maintained at 30 °C. The flow rate and the injection volume were 6 mL/min and 100 µL, respectively. Mixture of methanol and ultrapure water (v/v, 8:2) containing 0.1% NH4OH (v/v) was used as mobile phase. After purification, the OH-TBPH2 was characterized by ^{1}H NMR spectra (Figure C5.S4.). The purified OH-TBPH2 (0.1 mg/L) was also characterized using UHPLC-Q Exactive with full scan range from *m/z* 200-2000. The intensity of OH-TBPH2 was 100-folds higher than TBPH, indicated the relatively high purity of the OH-TBPH2 standard (Figure C5.S4.).

5.3.3 Collection of dust

Twenty-three samples of dust were collected from eight houses (2–3 dust samples per house) across Saskatoon, SK, Canada from May to August, 2013. Dust was collected by use of a Eureka Mighty-Mite vacuum cleaner (model 3670)[38, 153] into a cellulose extraction thimble (Whatman International, Pennsylvania, U.S.) which was inserted between the vacuum tube extender and suction tube and was secured by use of a metal hose clamp. Extraction thimbles were Soxhlet-extracted with DCM for 2 hr prior to use. The equivalent of the entire floor-surface area was sampled in each room. All sampling components upstream of the extraction thimble were cleaned after each sampling event.

5.3.4 Sample pretreatment and analysis

Approximately 0.1 g, dm of dust was transferred to a 15 mL centrifuge tube. Twenty microliters (20 µL) of 1 mg/L mass-labeled internal standard d_{34}, $^{13}C_6$-TBPH, and 5 mL of methanol were added to the house dust samples for extraction. Samples were vigorously shaken (Heidolph Multi Reax Vibrating Shaker, Brinkmann) for 30 min followed by sonication for an additional 30 min, and the methanol extract was separated by centrifugation at 1669*g* for 10 min and transferred to a

new tube. The extraction was repeated using 5 mL of DCM. The methanol and DCM extracts were combined and blown to dryness under a gentle stream of nitrogen. Extracts were dissolved in 500 μL of DCM and loaded onto Florisil cartridges, which had been sequentially conditioned by 6 mL of acetone and DCM. TBPH was eluted from the Florisil cartridges using 5 mL of DCM. Following a washing rinse of 4 mL of acetone, OH-TBPH isomers were eluted to a new tube using 5 mL of methanol/DCM mixture (v/v, 1:1). Final extracts were blown to dryness under a gentle stream of nitrogen and reconstituted with 200 μL of acetone for analysis.

5.3.5 Instrumental analysis

Aliquots of extracts were analyzed using a Q Exactive mass spectrometer (Thermo Fisher Scientific) interfaced to a Dionex UltiMate 3000 ultra high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific). Separation of TBPH and OH-TBPH was achieved with a Betasil C18 column (5 μm; 2.1 mm × 100 mm; Thermo Fisher Scientific) with an injection volume of 5 μL. Ultrapure water (A) and methanol containing 0.1% NH4OH (v/v) (B) were used as mobile phases. Initially, 20% B was increased to 80% in 3 min, then increased to 100% at 8 min and held static for 19.5 min, followed by a decrease to initial conditions of 20% B and held for 2 min to allow for column re-equilibration. The flow rate was 0.25 mL/min. The column and sample chamber temperatures were maintained at 30 and 10 °C, respectively. Data were acquired using full scan mode and selected ion monitoring (SIM). Briefly, MS scans (200–2000 *m/z*) were recorded at resolution $R = 70\,000$ (at *m/z* 200) with a maximum of 3×10^6 ions collected within 200 ms, based on the predictive automated gain control. SIM scans were recorded at a resolution $R = 70\,000$ (at *m/z* 200) with a maximum of 5×10^4 ions collected within 200 ms, based on the predictive automated gain control, with isolation width set at 2.0 *m/z*. For MS^2 identification, selected ions were fragmented in the collision cell utilizing higher-energy collisional dissociation (HCD). MS^2 scans with a target value of 1×10^5 ions were collected with a maximum fill time of 120 ms and $R = 35\,000$ (at *m/z* 200). The general mass spectrometry settings applied for negative ion mode were as follows: spray voltage, 2.7 kV; capillary temperature, 375 °C; sheath gas, 46 L/hr; auxiliary gas, 11 L/hr; probe heater temperature, 375 °C. The general mass spectrometry settings applied for positive ion mode were as follows: spray voltage, 3.0 kV; capillary temperature, 400 °C; sheath gas, 46 L/hr; auxiliary gas, 15 L/hr; probe heater temperature, 350 °C.

5.3.6 Quality assurance/quality control

Minor contamination of TBPH was detected during sample processing, so procedural blank experiments were performed along with each batch of samples. Standards were reinjected after four to six injections of samples, and acetone was injected after each standard injection to monitor background contamination. Due to minor background contamination, the method detection limit (MDL) for TBPH, defined as 3 times the procedural blanks, was 1.1 ng/g, dm. As for OH-TBPH without background contamination, its MDLs were calculated based on the peak-to-peak noise of the baseline near the analyte peak obtained by analyzing field samples on a minimum value of signal-to-noise of 3, and was 0.01 ng/g, dm. Compound-specific matrix spiking recoveries were calculated by spiking standards into samples of dust that contained the least concentrations of TBPH (n = 3). Standards were spiked at 500 ng TBPH/g and 10 ng OH-TBPH/g, dm. Recoveries from dust were 75 ± 12% and 86 ± 10% for TBPH and OH-TBPH, respectively. Quantification of TBPH was conducted by internal calibration curve by use of internal standard d_{34}, $^{13}C_6$-TBPH, for which recoveries from dust averaged 82 ± 27%. Concentrations of OH-TBPH were calculated without internal standard since recoveries were >80% and stable among recovery samples. Due to the lack of OH-TBPH1 standard, its concentrations were quantified using purified OH-TBPH2 standard. External calibration curves of target analytes were calculated for TBPH, 0, 49, 98, 195, 391, 781, 1563, 3125, 6250, 12 500, 25 000, 50 000, and 100 000 pg/mL and for OH-TBPH, 0, 63, 125, 250, 500, and 1000 pg/mL. Both calibration curves showed strong linearity (correlation coefficients >0.99).

5.3.7 Data analysis

Statistical analyses were carried out using SPSS 19.0. Values less than the MDLs were replaced by MDL/2. Normal distributions of chemical concentrations were assessed using the Shapiro-Wilk test, and a log-transformation was used to ensure the normality of the distribution of data. All 23 dust samples from eight homes were treated as independent data and were included in the log-regression analysis. Differences with $p < 0.05$ were considered significant.

5.4 Results and discussion

5.4.1 Observation and chemical structure identification of OH-TBPH in TBPH standards

The primary purpose of the study was to develop a LC-UHRMS method based on the LC-Q Exactive platform that could be used to quantify TBPH in environmental samples. Unexpectedly, three separate peaks were observed when ions were extracted at *m/z* 640.9946 (10 ppm window) from full scan mass spectra (200–2000 *m/z*) from the commercial TBPH standards in negative ion mode (Figure 5.2A). Based on full scan mass spectra, the *m/z* values for the three peaks were 640.9949, 640.9946, and 640.9948, with mass errors of 2.1, 1.0, and 1.9 ppm, respectively (Figure 5.2D) compared to the theoretical *m/z* value (640.9936; $[TBPH\text{-}Br+O]^{-}$) as reported previously[154]. Based on the results of the UHRMS data and the isotopic distribution pattern, the three peaks likely had the same formula, $C_{24}H_{35}Br_3O_5$, which could have been isomers or adducts of TBPH with the same protonated ion. The HPLC mobile phases were optimized, and the first two peaks (peak a and peak b) were eluted from the HPLC column earlier (5.85 and 6.92 min) with 0.1% NH_4OH as an additive in methanol compared to pure methanol (12.5 min, Figure C5.S1.), whereas the retention time of the third peak (c) remained unchanged (15.9 min). These results indicated that the first two peaks were likely acidic compounds, and their retention on C18 columns would likely be reduced with the use of basic mobile phases. To further identify the peak associated with TBPH, retention times of the identified peaks were compared to those of PBDEs under the same HPLC conditions; retention times of most PBDEs were 5–15 min (data not shown), which were earlier than the third peak (c) but later than peaks a and b. Considering that the log K_{OW} of TBPH (11.95) is greater than those of PBDEs (6.3–6.58 and 6.29 for Penta- and Octa BDE) and that the retention of compounds on C18 columns is correlated with K_{OW} values[155, 156], peak c was thought to most likely be TBPH. To further confirm this hypothesis, two strategies were used: (i) confirmation of the third peak with highly purified native and mass-labeled TBPH standards—the third peak (c) was specifically observed while peaks a and b were detected only at lesser intensities (Figure C5.S2.)—and (ii) analysis of the commercial standard in positive ion mode to detect TBPH adducts that were theoretically possible. The *m/z* of the amino adduct of TBPH ($[TBPH+NH_4]^{+}$, which was 719.9534 with 0.42 ppm mass accuracy) was only observed at a retention time similar to that of peak c (15.9 min) (Figures 5.3A, 5.3C).

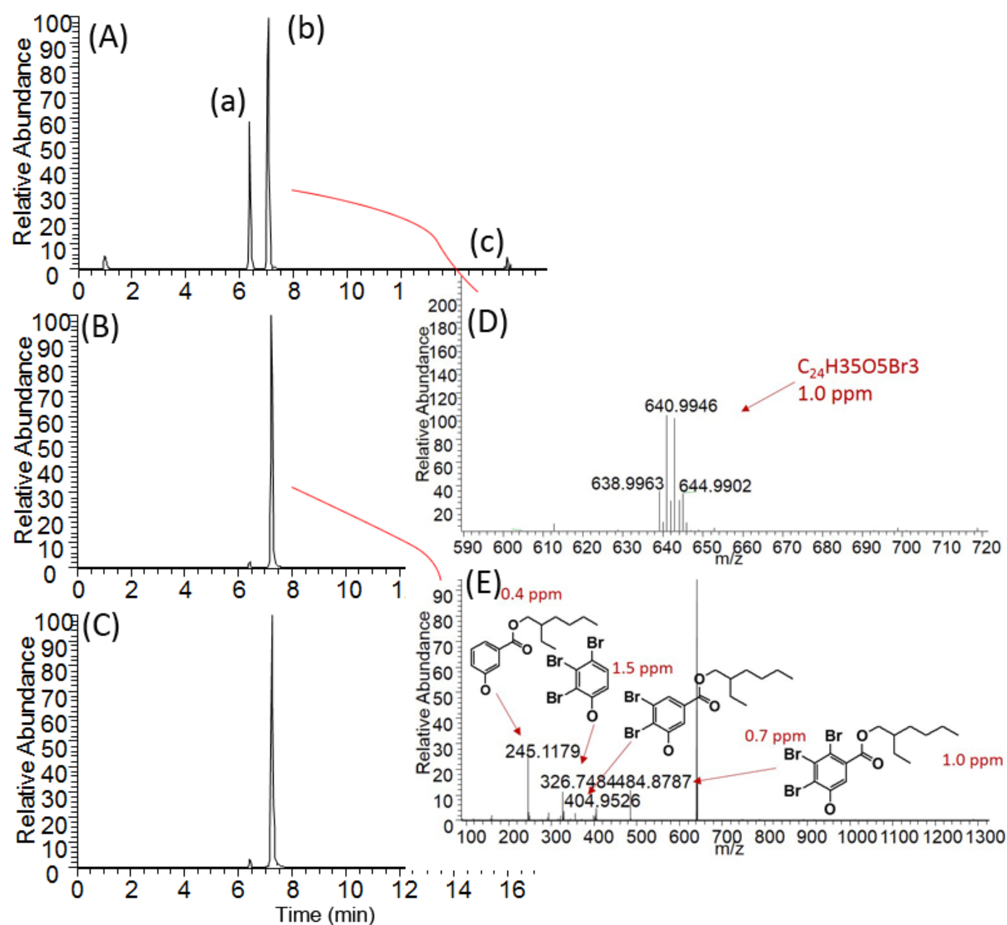

Figure 5.2. Chromatogram of extracted ions with *m/z* 640.9946 (10 ppm window) for (A) commercial standard (B) FM-550 technical product (C) BZ-54 technical product using Q Exactive in negative ion mode. (D) Mass spectra of OH-TBPH. (E) Product ion mass spectra of ion at *m/z* 640.9946. (a) OH-TBPH1, (b) OH-TBPH2, (c) TBPH.

Together, these four lines of evidence, alterations of retention times using basic mobile phases, retention times relative to PBDEs, relatively greater proportions in highly purified standards, and observation of ions of adducts in positive ion mode, were consistent with the third peak (c) at 15.9 min being TBPH. To further confirm the structure, high resolution MS^2 spectra (R = 35 000 at m/z 200) were analyzed in positive ion mode at 30–50 eV. As shown in Figure 5.3D, typical fragment ions of TBPH were observed with a mass error less than 10 ppm. The fragment ions clearly showed that TBPH precursor ions were fragmented at the alkyl side chains, while cleavage of the C–O bond yielded a predominant product ion at m/z 464.6611. Such routes of cleavage were similar to those of phthalates, which also produced a phthalic anhydride fragment when using collision induced dissociation (CID)[157], in triple quadruple mass spectrometry mode, despite the fact that HCD was used to fragment ions in the Q Exactive instrument.

To evaluate chemical structures of the two unknown peaks a and b, the Q Exactive was used in negative ion mode to obtain ultrahigh-resolution (R = 35 000 at m/z 200) product ion spectra. Several product ions with m/z at 245.1179, 326.7484, 404.9526, and 484.8787 were observed (Figure 5.2E). The structures of the product ions were evaluated based on elemental composition with a mass error of 0.4, 1.5, 0.7, and 1.0 ppm, respectively. Patterns of peaks associated with products of fragmentation were more complex than that of TBPH, and the addition of a hydroxyl substituent to the aromatic ring was observed for each of the four predominant fragments. To avoid the possibility that the addition of the hydroxyl moiety was due to a substitution reaction during negative ionization, as has previously been reported for brominated compounds[158], OH-TBPH was also analyzed in positive ion mode. When analyzed in positive ion mode, a sodium adduct of OH-TBPH ($[M + Na]^+$; m/z value of 666.9861 and mass error of 0.75 ppm) was observed (Figure C5.S3.). Based on all of this information, it was clear that the first two peaks were hydroxylated derivatives of TBPH with formulas $C_{24}H_{35}Br_3O_5$ and with hydroxylation at the aromatic ring which formed a phenolic group. To further confirm the chemical structures of the compounds, ^1H NMR was used to characterize the structures of OH-TBPH2 purified from the BZ-54 technical product using HPLC (Figure C5.S4.). A chemical shift characteristic of a phenolic group with carboxylic acid ester substituent was observed in the purified OH-TBPH2 standard at 8.6 ppm (Figure C5.S4.). Isomers of OH-TBPH were identified in purified standards of TBPH and technical products and had similar product ion spectra with TBPH; therefore, we proposed structures of these two novel compounds as shown in Figure 5.1.

To further investigate the widespread presence of OH-TBPH isomers as by-products of TBPH, we also determined OH-TBPH isomers in other standards. In addition to the commercial TBPH standard, OH-TBPH isomers were also observed in purified TBPH standards from AccuStandard and in technical materials. Percentages of OH-TBPH in the native standard from AccuStandard were <0.1% (Figure C5.S2.). Furthermore, small proportions, <0.1%, of mass-labeled OH-TBPH (d_{17}, $^{13}C_6$–OH-TBPH) were detected in the mass-labeled TBPH standard from Wellington Laboratories Inc. This result indicated potential widespread occurrence of isomers of OH-TBPH as byproducts in TBPH standards. These results indicated that OH-TBPH might also be present in technical products. To confirm this hypothesis, technical products, BZ-54 and FM-550, which, along with the flame retardant product, DP-45, are two of three potential major sources of TBPH in the environment[30], were analyzed for OH-TBPH. Isomers of OH-TBPH were detected in both BZ-54 and FM-550, but profiles of relative concentrations of the OH-TBPH isomers (Figures 5.2B, 5.2C) were different from those of the commercial TBPH standard. The relative contributions of OH-TBPH1, OH-TBPH2, and TBPH were 0.4%, 0.9%, and 98.7%, respectively, in the commercial standard, while their relative contributions were 0.1%, 6.2%, and 93.7% in FM-550 and 0.1%, 7.9%, and 92.0% in BZ-54 with relatively greater amounts of OH-TBPH2. The presence of isomers of OH-TBPH in FM-550 and BZ-54 technical products suggests potential emissions to the environment.

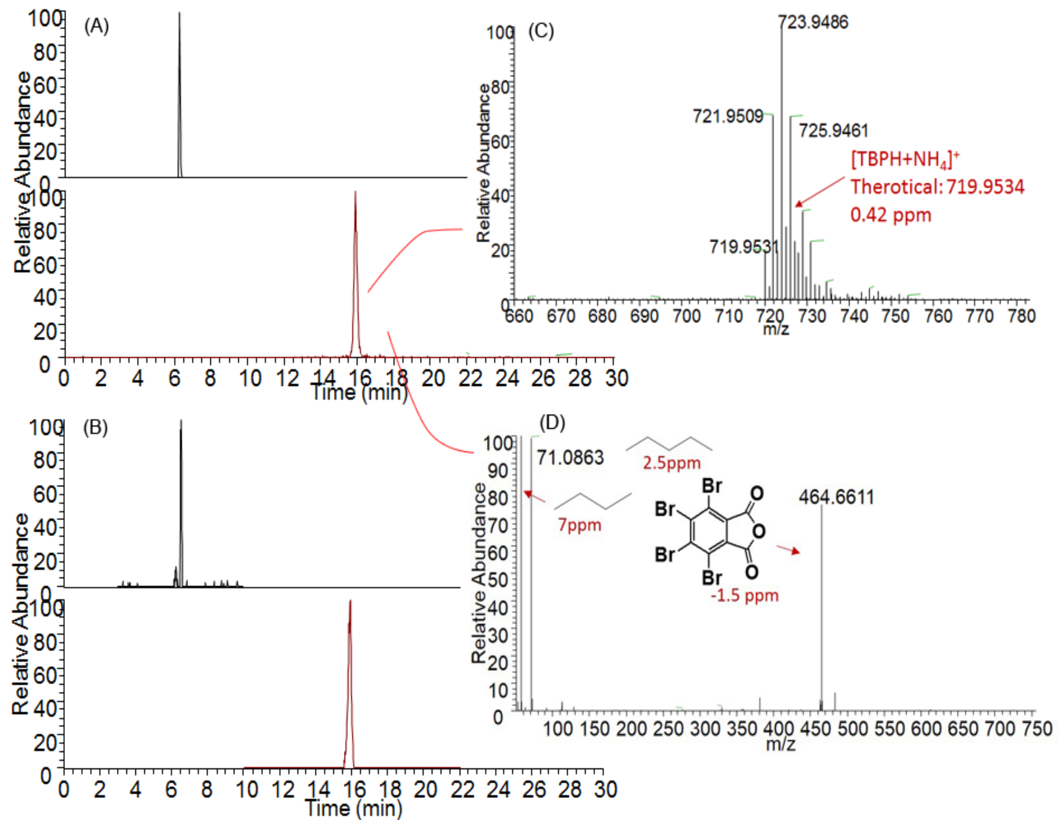

Figure 5.3. Chromatogram of extracted ions with *m/z* 640.9946 and *m/z* 723.9486 (10 ppm window) for (A) FM-550 technical product (B) house dust using Q Exactive (SIM) in both negative ion mode and positive ion mode. (C) Mass spectra of TBPH in positive ion mode. (D) Product ion mass spectra of ion at *m/z* 723.9486 in positive ion mode.

5.4.2 Development of analytical methods to measure TBPH and OH-TBPH in dust

To measure TBPH and OH-TBPH isomers in house dust, a LC-UHRMS method based on the Q Exactive Orbitrap was developed for simultaneous analysis of both compounds. A single study had previously reported a method for quantification of TBPH by use of LC-APCI (−)[154], while most studies have used GC-MS[148, 156]. In the current study, the Q Exactive operated in ESI (+) showed greater sensitivity for TBPH compared to analyses with ESI (−) or APCI (−) (>100 fold). An ammonium adduct of TBPH ($[TBPH+NH_4]^+$) was detected while $[TBPH+H]^+$, which was the expected ion in positive ion mode, was not observed even with 0.1% formic acid in methanol as the mobile phase. The IDL of the newly developed LC-UHRMS method was calculated to be 0.01 μg/L for TBPH, which was roughly 200- to 300-fold more sensitive than that of the LC-APCI (−) method (3.3 μg/L) and GC-MS method (2.5 μg/L; Table 5.1)[54, 154]. The $[M + Na]^+$ ion of OH-TBPH isomers was observed in positive ion mode, but the sensitivity was >50 fold less than ions produced in negative ion mode. Finally, a LC-HRMS method based on the Q-Exactive platform was established for simultaneous analysis of TBPH and isomers of OH-TBPH in both negative and positive ion modes. The greater sensitivity of the newly developed method for TBPH is important due to the low concentrations anticipated in human tissue (ND-164 ng/g lipid weight (lw) in human serum). The detection frequencies in humans are relatively low (<60%)[150], and information on concentrations of TBPH in these samples is limited.

Table 5.1. Instrumental detection limits (IDLs, μg/L), method detection limits (MDLs, ng/g, dm) and recoveries (n=3) of OH-TBPH isomers and TBPH of different methods

	IDLs (GC-MS)[a]	IDLs (LC-QE)	MDLs (GC-MS)[b]	MDLs (LC-QE)	Recoveries (n=3)
TBPH	2.5	0.01	4	1.1[d]	75±12%
OH-TBPH[c]	-	0.005	-	0.01	86±10%

[a] data from reference[78]

[b] data from reference[78]

[c] IDLs, MDLs and recoveries were just calculated for OH-TBPH2, due to the lack of standards for OH-TBPH1.

[d] MDL was relatively high compared to IDL since minor background contamination of TBPH was detected in procedural blanks.

To simultaneously analyze TBPH and OH-TBPH in house dust, a sequential liquid extraction method was developed by using methanol and DCM to extract polar OH-TBPH and nonpolar TBPH, respectively. To assess recoveries of the liquid extraction method, 500 μg/L of TBPH and 5 μg/L OH-TBPH were spiked into samples of house dust and left to equilibrate for 24 hr at room temperature. Extracts were diluted 20-fold with acetone and analyzed by LC-UHRMS directly without further sample pretreatment. Recoveries for TBPH and OH-TBPH, using the liquid extraction method, were 92 ± 5% and 95 ± 6%, respectively. Since matrix effects can be problematic in LC–MS/MS analysis, an SPE method was developed to clean extracts of samples. Several solvent mixtures for elution of OH-TBPH and TBPH from Florisil cartridges were tested. DCM was chosen to elute TBPH from the cartridge, while acetone, which could not elute OH-TBPH, was used to rinse potential interferences. Finally, OH-TBPH was eluted using a mixture of methanol/DCM (v/v, 1:1; Figure C5.S5.). The use of methanol as an elution solvent was different from cleanup methods for other phenolic compounds such as estrogens, which are generally eluted from Florisil using a mixture of acetone and DCM[159]. Thus, the use of acetone as a rinse prior to elution of OH-TBPH allowed removal of most of the yellow or blue interferences in extracts of house dust. Potential effects of matrices were also evaluated by spiking 1000 μg/L TBPH and 10 μg/L OH-TBPH into final extracts before analysis by Q Exactive LC-UHRMS. Suppression of signals of TBPH and OH-TBPH (n = 3) were minor at −5 ± 3% and −10 ± 6%, respectively. Finally, based on sample pretreatment methods, the recoveries for TBPH and OH-TBPH were 76 ± 12% and 86 ± 10%, respectively (Table 5.1).

5.4.3 Concentrations and profiles of TBPH and OH-TBPH in house dust

The newly developed methods were applied to quantify OH-TBPH and TBPH in 23 samples of house dust. Two OH-TBPH isomers were both detected in house dust (Figure 5.3B; Table 5.2), with detection frequencies of 52% and 91%, respectively. Use of SIM mode was necessary to detect OH-TBPH in house dust, since no peaks related to OH-TBPH were detected when using full scan mode (Figure C5.S6A.). This was likely due to the dynamic range of the Q Exactive and co-elution of numerous compounds that interfered with OH-TBPH. In the analysis of OH-TBPH, intensities of total ions were 1.5×10^{10} at a retention time similar to that for OH-TBPH and were much greater than those of OH-TBPH ($<10^5$ in SIM mode; Figure C5.S6.). Thus, OH-TBPH could not be detected in full scan mode because maximum injected ions, based on

predictive automated gain control, were limited to 3×10^6. During analysis by SIM, total injected ions were limited to a narrow isolation window (2.0 *m/z*), which greatly increased the number of injected ions for targeted chemicals. Therefore, SIM was used to analyze OH-TBPH and TBPH in house dust in subsequent experiments.

Profiles of relative concentrations of TBPH and OH-TBPH isomers in dust were compared to technical products to evaluate their potential emission source. As expected, the greater proportion of OH-TBPH2 compared to OH-TBPH1 in dust samples was similar to proportions in BZ-54 and FM-550 but different from the commercial TBPH standard in which proportions were roughly equal. Concentrations of OH-TBPH2 (GM ± GSD, 0.35 ± 1.0 ng/g, dm) were 10-fold greater than those of OH-TBPH1 (0.04 ± 0.88 ng/g, dm) in house dust (Table 5.2). TBPH was also detected in all samples of house dust with an average concentration, 734 ± 0.87 ng/g, dm. Concentrations of TBPH were comparable to dust samples from the UK (mean value was 381 ng/g, dm) but greater than dust samples from California (mean value 260 ng/g, dm)[56, 58]. It should be noted that the maximum detected concentration of TBPH in this study was 22251 ng/g, dm, several fold greater than previously reported maximum concentrations in dust from houses in the United Kingdom (6175 dm) and California (3800 ng/g dm)[56, 58]. In this study, dust samples were collected in 2013 and were more recent than those in previous studies. Since previous reports have shown trends of increasing concentrations of TBPH in house dust over time[151], greater concentrations of TBPH in more recent samples were expected in this study. In fact, in a more recent study by Stapleton et al., concentrations of TBPH (maximum concentration 20960 ng/g, dm) were similar to concentrations reported here[160]. In addition, as shown in Figure 5.4, relatively great concentrations of TBPH and OH-TBPH isomers were detected in three samples from the same home. Information provided in a brief survey showed that the house was constructed in 2004, and most electronics and furniture were recently purchased. Furthermore, several pieces of furniture with TB 117 labels were discovered in the home. The newly built house, recently purchased consumer items, and adherence to Californian furniture flammability standards might have contributed to the relatively great concentrations of TBPH in this home.

Table 5.2. Concentrations of OH-TBPH and TBPH (ng/g, dm) in samples of house dust from Saskatoon, Saskatchewan, Canada.

	GM±GSD	Min	Max	Detected%[b]	Contribution%[c]
TBPH	734±0.87	15	22251	100	99.9
OH-TBPH1	0.04±0.88	<0.01[a]	7.3	52%	0.01
OH-TBPH2	0.35±1.0	<0.01	27	91%	0.1

[a] the concentration was lower than MDL (0.01 ng/g, dm).
[b] detection frequencies of TBPH and OH-TBPH isomers in dust samples.
[c] relative contribution to the sum concentrations of TBPH and OH-TBPH isomers.

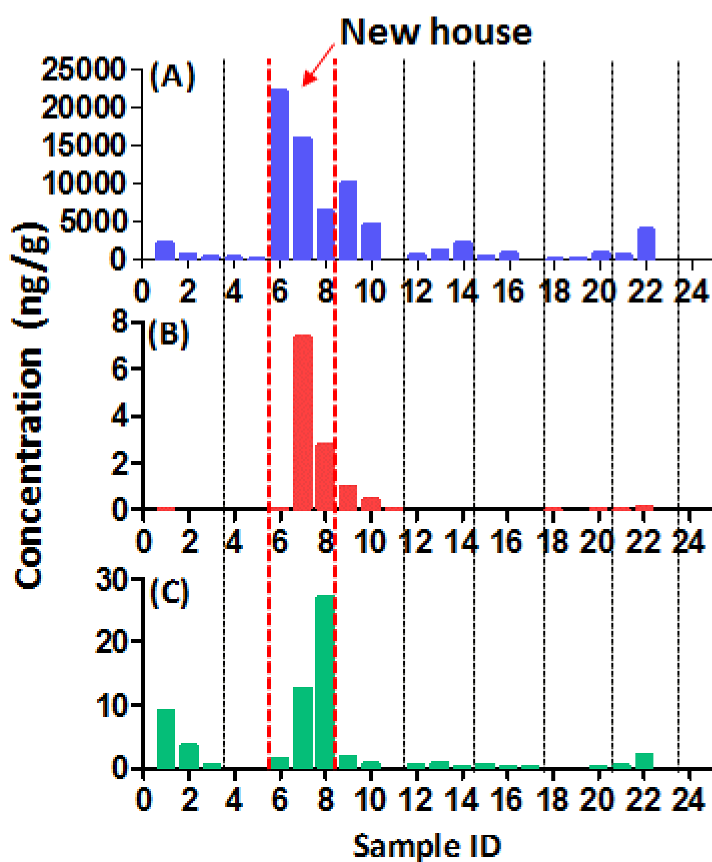

Figure 5.4. Concentrations of TBPH (A), OH-TBPH1 (B) and OH-TBPH2 (C) in 23 dust samples from 8 houses. Dotted lines were used to separate house dust samples among different houses. The samples between the two red dotted lines were from a house built in 2004, which had greater concentrations of TBPH and OH-TBPH isomers.

Isomers of OH-TBPH were not detected in two samples of house dust, and concentrations of TBPH in these samples were also small (74 ng/g and 158 ng/g). Furthermore, a log–linear regression analysis showed a significant relationship between concentrations of TBPH and OH-TBPH isomers ($r^2 = 0.67$ for OH-TBPH1 and $r^2 = 0.50$ for OH-TBPH2, $p < 0.001$ for both; Figure 5.5A), which, when paired with evidence of similar isomer profiles in dust and technical products, indicated common sources of OH-TBPH and TBPH. However, it should be noted that the percentage of isomers of OH-TBPH contributing to the sum of concentrations of OH-TBPH and TBPH in samples of dust were relatively small (0.01% ± 0.67 and 0.1% ± 0.60). Percentages of OH-TBPH in house dust were less than in BZ-54 (0.1% and 7.9%), FM-550 (0.1% and 6.2%), and the commercial standards (0.4% and 0.9% respectively; $p < 0.001$; Figure 5.5B), while relative contributions of TBPH in samples of house dust (99.9% ± 0.0) were significantly greater than in the technical product and commercial standard ($p < 0.001$). The relatively low contributions of OH-TBPH might be due to different physical–chemical properties and environmental fates during application, or mechanical or chemical emissions from products to the environment. Alternatively, the sample number for technical products was limited, and the proportions of OH-TBPH might vary among different manufacturers.

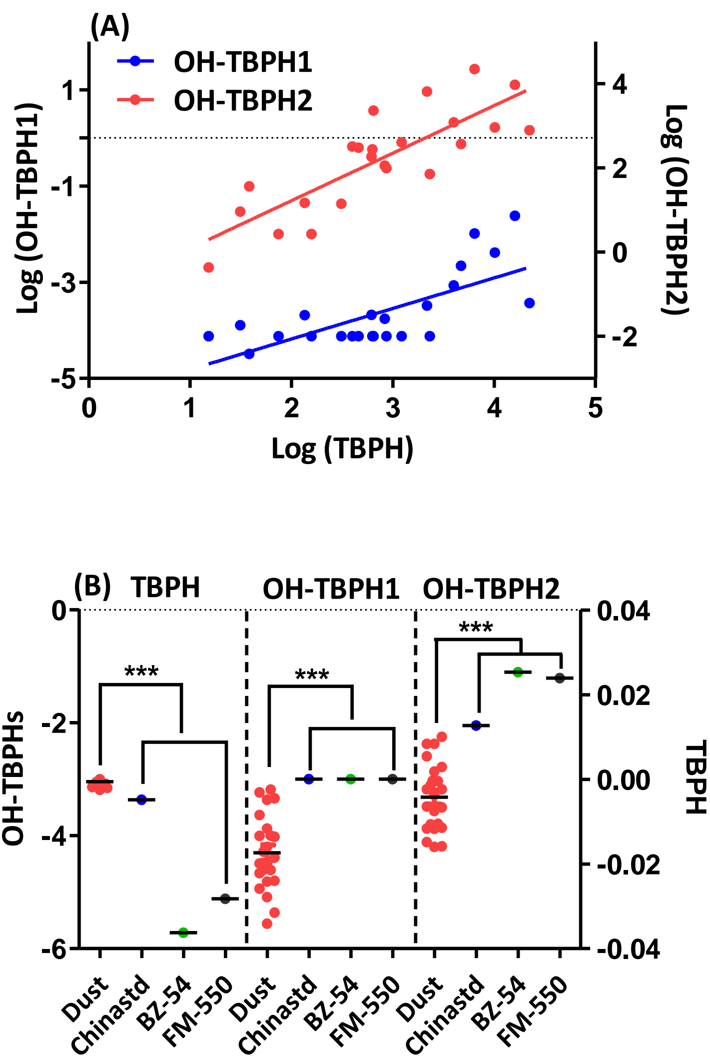

Figure 5.5. (A) Log−linear regression between concentrations of TBPH and OH-TBPH in 23 dust samples. (B) Comparison of relative contributions of TBPH and OH-TBPH isomers in dust samples, commercial standard, BZ-54 technical product, and FM-550 technical product. The *y* axis indicates the log-transformed percentages of TBPH and OH-TBPHs in different samples. Colors differentiate dust samples or standard. Chinastd indicates the commercial TBPH standard produced in China. A *t*-test was used to evaluate statistical difference. ***$p < 0.001$.

5.4.4 Implications

Detection of OH-TBPH contamination in analytical standards, technical products, and environmental dust samples is of great importance due to differences in the physical–chemical properties between TBPH and OH-TBPH and the implications for bioavailability and toxicity. For example, the toxic potency of OH-TBPH likely differs from that of TBPH as the addition of a phenolic group, as observed with OH-PBDEs, has been shown to increase the toxic potency of compounds[161]. Increased toxicities might be due to structural similarities to endogenous compounds including 17-β-estradiol, triiodothyronine, or thyroxine, and/or greater binding affinities with important receptors or transport proteins[162]. In addition, because of its extreme hydrophobicity (estimated log K_{OW}, 11.95), bioavailability of TBPH is likely relatively limited[29, 156]. The substitution of bromine for the hydroxyl group of OH-TBPH would lead to a lesser log K_{OW}, estimated at 9.56 (ChemDraw Ultra 8.0), and thus greater bioavailability. Therefore, the addition of a phenolic group, potential increased toxic potency, and increased bioavailability of OH-TBPH might lead to greater concerns about risks posed to the environment and human health.

6 CHAPTER 6: CONCENTRATION, SEASONALITY AND BIOACCESSIBILITY OF NOVEL BROMINATED FLAME RETARDANTS IN DUST FROM CHILDCARE FACILITIES IN SASKATOON, SK, CANADA

PREFACE

Following the detection and identification of novel hydroxylated isomers of TBPH (and TBB) in Chapter 5, further assessments of environmental concentrations and factors of exposure were necessary. The objective of Chapter 6 was to determine concentrations of TBPH, TBB, OH-TBPH1, OH-TBPH2, and ΣOH-TBBs in dust from ECEs to determine if children were exposed to greater amounts of these NBFRs in this environment. Other factors of exposure including seasonality of concentrations and microenvironment specific parameters which might lead to greater concentrations in dust, were assessed. Finally, bioaccessibility was calculated to estimate bioavailability of the compounds, a parameter which is important to accurate assessments of risk. This chapter was included in the second phase of this research program, the characterization of exposure to TBPH and TBB in the indoor environment.

The content of Chapter 6 is in preparation for submission for publications as D.M.V. Saunders, H. Peng, J. Sun, Wiseman, J. P. Giesy, "Concentration, seasonality, and bioaccessibility of novel brominated flame retardants in dust from childcare facilities in Saskatoon, Saskatchewan, Canada".

Author Contributions:

David M.V. Saunders (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Dr. Hui Peng (University of Saskatchewan) generated and analyzed data related to analytical chemistry.

Dr. Jianxian Sun (University of Saskatchewan) laboratory aid in the preparation and processing of environmental samples.

Drs. Steve Wiseman, and John P. Giesy (both at University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

6.1 Abstract

The novel brominated flame retardants (NBFRs) bis(2-ethylhexyl)-tetrabromophthalate (TBPH or BEH-TEBP) and 2-ethylhexyl-tetrabromobenzoate (TBB or EH-TBB) have been detected at some of the greatest concentrations in indoor dust from early childhood environments (ECEs) in Canada, the U.S., and Europe, though relatively few investigations have been conducted in Canada. Recently, hydroxylated isomers of these compounds, OH-TBPHs and OH-TBBs have been identified and quantified in indoor dust from Canada. Young children in the cold Canadian climate spend great proportions of time indoors, specifically in ECEs (i.e. day care centers) and are likely exposed to relatively great quantities of these compounds. Though increased exposure of children to these NBFRs is likely due to differences in behaviours (hand-to-mouth, hygiene), the greater concentrations of these contaminants in dust from ECEs has not been thoroughly assessed. In this study, concentrations of TBPH, TBB and their OH-isomers in specific microenvironments which contained permutations of greater/lesser amounts of children's products and foot-traffic were assessed in dust from several day care centers in Saskatoon, Saskatchewan, Canada. Further, seasonal differences in concentrations of NBFRs and bioaccessibilities were investigated. Day care centers in Saskatoon had some of the greatest concentrations of these NBFRs, globally; TBPH 734±0.87; TBB 992±0.82; OH-TBPHs 0.04±0.88 to 0.81±0.75; OH-TBBs 0.30±0.78 (GM±GSD, ng/g, dm). Though no significant seasonal differences were observed between summer and winter or TBPH or TBB, a non-statistically significant trend of increased concentrations of the OH-isomers was observed during the colder season (increases of 143-425%). Microenvironments in ECEs with greater numbers of toys and greater foot-traffic had greater concentrations of all NBFRs in winter, though no differences were observed in summer. Bioaccessibilities of TBPH, TBB, OH-TBPHs, and OH-TBBs in dust from day care centers were, 23, 53, 30, and 70%, respectively. The bioaccessibility of OH-TBBs was significantly greater than that of TBB which indicated that greater quantities of OH-TBBs would likely be absorbed. Results of the study presented here demonstrate that ECEs from Saskatoon, SK, Canada have some of the greatest concentrations of these NBFRs reported to date, which might be due, in part, to greater abundances of children's products. Furthermore, bioaccessibilities of these compounds are low to moderate, but OH-TBBs are likely more bioavailable.

6.2 Introduction

Bis(2-ethylhexyl)-tetrabromophthalate (TBPH or BEH-TEBP) and 2-ethylhexyl-tetrabromobenzoate (TBB or EH-TBB) are novel, additive brominated flame retardants (NBFRs) that are components of the flame retardant mixture Firemaster® 550 (FM-550) and BZ-54 which are replacements for the Penta-mixtures of polybrominated diphenyl ethers (PBDEs). Recently novel, hydroxylated isomers of TBPH and TBB have also been detected in FM-550 and BZ-54 technical products[163, 164]. As major replacements, FM-550 and BZ-54 are added to numerous consumer products, and specifically to flexible polyurethane foam. Polyurethane foams are widely used in children's products and furniture for juveniles and components of FM-550 have been detected at approximately 4.2% by weight in foam of some couches[41]. Additive flame retardants are known to migrate from consumer products to the surrounding environment through chemical (volatilization), direct contact with dust, or via mechanical (abrasion) processes. Several studies have documented relatively great concentrations of TBPH and TBB in dust[165, 166] and two recent studies have quantified their hydroxylated isomers (OH-TBPHs/TBBs) in the same matrix[163, 164]. Furthermore, due to the phase-out of Penta-BDE mixtures and the partial discontinuation of production of a prominent organophosphate flame retardant (TDCPP), concentrations of these compounds might increase in indoor environments. Changes in use of these major BFRs will likely increase use of FM-550 in consumer products. A recent investigation which approximately coincided temporally with the phase out of Penta-BDE mixtures, detected an approximate 2-fold increase in concentrations of TBPH and TBB in indoor dust in the U.S. collected from 2006-2011[56].

Indoor dust is considered a major route of exposure to brominated flame retardants (BFRs) and results of pharmacokinetic models have suggested that as much as 82% of total exposures might be via dust[57]. Because young children exhibit behaviours that increase the likelihood of exposure, they have greater potential for exposure to BFRs than do adults. Young children wash their hands less than adults and exhibit greater exploratory behaviours which include, greater hand-to-mouth activities and greater associations with floors/surfaces. Children generally have greater body burdens of BFRs than do adults[71-73]. For example, in a paired study of young U.S. children and their mothers, children had approximately 2.8-times greater concentrations of total PBDEs in blood[167]. This trend regarding greater body burdens of BFRs in

children has also been observed with NBFRs, including TBB. Recently, tetrabromobenzoic acid (TBBA) was identified as a metabolite of TBB detected in urine and validated as a marker of exposure to the compound [168]. In a subsequent study of paired children and adults, TBBA was detected in 27% of adults and 70% of children which suggested greater exposures to children[169].

In North America, children spend a great proportion of time away from home. Some young children spend as much as 50 h per week in childcare and preschool centres[75]. In Canada in 2011, 60% of all parents relied on some sort of childcare for children aged 2-4 yrs[170]. Due to the exploratory and clumsy nature of young children, products manufactured for use by children contain great quantities of polyurethane foam which might have been treated with FM-550. There is generally a direct relationship between the number of children in child care facilities and number of products for children. For example, a home with one child might have a single piece of furniture designed for juveniles, but a day care center might have several times more to facilitate concurrent use by children. This greater density of products designed for children might result in greater loadings of BFRs to the indoor environment and subsequently, greater exposure of children.

Exposure to TBPH and TBB might have implications for normal development of children. For example, a recent study has shown that prenatal exposure to several components of the Penta-BDE mixture, which are endocrine disrupting compounds (EDCs), were significantly associated with lower IQ and more hyperactivity of children[171]. TBPH, TBB and OH-TBPHs/TBBs likely are EDCs and might also adversely affect normal endocrine functions during key phases of early childhood development. In several studies, TBPH and TBB have been shown to interact antagonistically with the human estrogen receptor (ERα) and androgen receptor (ARα), alter sex hormone concentrations in *in vitro* cellular assays, and significantly alter fecundity and transcript abundances of genes associated with the highly conserved, hepatic-pituitary-gonadal-liver axis in Japanese Medaka[99, 130, 172]. Additionally, a study using rats showed that exposure to FM-550 resulted in obesogen-like effects and the mixture potentially contributed to an observed metabolic syndrome[52]. Also, the hydroxylated isomers, OH-TBBs, likely have endocrine disrupting effects. In a recent study, OH-TBB was detected as a strong agonist of the peroxisome proliferator-activated receptor gamma (PPARγ) and a weak agonist of the ER[164].

TBPH and TBB are hydrophobic compounds with theoretical Log K_{ows} of 11.95 and 8.75, respectively (estimated by use of KOWWIN, CLOGP, and Chemsketch programs), and might not be readily available for absorption or distribution, though the Log K_{ows} of the hydroxylated isomers are less (9.56 for OH-TBPH and 6.8 for OH-TBB) which might significantly alter bioavailability. One of the first exposure studies with FM-550, which used fathead minnows, calculated uptake to be 1% of daily dosage of TBPH or TBB, though uptake was not calculated for hydroxylated isomers[29]. A more recent study calculated bioaccessibility of TBPH and TBB from dust, by use of a colon extended physiologically-based extraction test (CE-PBET)[173]. The study concluded that approximately 25% and 50% of TBPH and TBB, respectively, were bioaccessible from dust, though there was no information regarding bioaccessibilities of OH-TBPHs/TBBs.

In this study, samples of dust from government licensed day care centers across Saskatoon, SK, Canada were collected in winter and summer and analyzed for TBPH, TBB and their hydroxylated isomers OH-TBPHs/TBBs. Dust was collected from three distinct environments in each day care center with permutations greater/lesser foot-traffic and greater/lesser numbers of children's products. Comparisons of concentrations of compounds in these environments could help to determine if increased amounts of children's products increase concentrations of NBFRs in dust. Concentrations of these compounds in samples collected during summer or winter months were also compared to deduce potential seasonal changes to concentrations of indoor contaminants. Any differences between seasons might have implications for exposures of humans, since Canadians spend approximately 97% of the day indoors during the winter[174]. Finally, bioaccessibilities of TBPH/TBB and OH-TBPHs/TBBs were assessed by use of a Tenax (TA) enhanced CE-PBET model. These tests generated data regarding uptake of the novel, potential EDCs, OH-TBPHs/TBBs. Young children are a susceptible population, and this study will help to more precisely evaluate risk of exposure of NBFRs to children via ingestion of dust.

6.3 Materials and methods

6.3.1 Chemicals and reagents

Native standards of TBPH and TBB were purchased from AccuStandard (Connecticut, U.S.), and their surrogates, mass labelled, *d*34, $^{13}C_6$-TBPH and *d*17, $^{13}C_6$-TBB were purchased from

Wellington Laboratories Inc. (Ontario, Canada). Procedures for the purification of OH-TBPHs/TBBs from BZ-54 technical mixture are included in our previous studies[163, 164]. Dichloromethane (DCM), acetone, and methanol were purchased from EMD Chemicals (New Jersey, U.S.) and an ammonia solution (28-30%) was purchased from Alfa Aesar Chemical Industries (Massachusetts, U.S.). Florisil, solid-phase extraction cartridges (6cc, 1 g, 30 μm) were purchased from Water (Massachusetts, U.S.).

6.3.2 Collection of dust samples

Forty-six (46) samples of dust were collected from 14 day care centers in Saskatoon, SK, Canada, from May 2013 to April 2014. This represented approximately 18% of licensed day care homes in the Saskatoon area. Ethical approval of all research and procedures has been awarded by the University of Saskatchewan Behavioural Research Ethics Board (Beh-REB). Dust was collected by use of a Eureka Might-Mite vacuum cleaner (model 3670) and stored at -20 ºC. The components upstream of the vacuum collection vessel were thoroughly cleaned between sampling events. The entire procedure for dust collection has been described in our previous publications[163, 164].

6.3.3 Pretreatment of dust

Approximately 0.1 g of dust was transferred to a 15 mL tube and 20 μL of each, 1 mg/L mass-labeled internal standards *d*34, $^{13}C_6$-TBPH and *d*17, $^{13}C_6$-TBB, and 10 mL methanol were added. Samples were shaken vigorously (Heidolph Multi Reax Vibrating Shaker, Brinkmann) for 30 min then sonicated for an additional 30 min. Dust was separated from the methanol by centrifugation at 1669 *g* for 10 min and the methanol was transferred to a new tube. The extraction procedure was repeated with 10 mL DCM. The methanol and DCM extracts were combined and blown to dryness under a stream of nitrogen. Extracts were dissolved in 500 μL of DCM and loaded onto Florisil cartridges, which had been conditioned by use of 6 mL acetone and DCM. TBPH, TBB, and the OH-isomers were eluted from Florisil cartridges by use of 5 mL DCM and a mixture of DCM/methanol (v/v, 1:1). Extracts were blown to dryness under a stream of nitrogen and reconstituted with 200 μL acetone for analysis.

6.3.4 Pretreatment of Tenax

The Tenax envelope, with approximately 0.3 g Tenax, was transferred to a 15 mL centrifuge tube and 20 µL of each, 1 mg/L mass- labeled internal standards *d*34, $^{13}C_6$-TBPH and *d*17, $^{13}C_6$-TBB, and 10 mL hexane were added. Samples were shaken vigorously (Heidolph Multi Reax Vibrating Shaker, Brinkmann) for 30 min, sonicated for an additional 30 min, then the hexane was transferred to a new tube. The extraction procedure was repeated with 10 mL acetone. The hexane and acetone extracts were combined and blown to approximately 1 mL under a stream of nitrogen. Extracts were loaded onto Florisil cartridges, which had been conditioned by use of 6 mL acetone and hexane. TBPH, TBB, and the OH-isomers were eluted from Florisil cartridges by use of 6 mL hexane and 6 mL acetone. Extracts were blown to dryness under a stream of nitrogen and reconstituted with 200 µL acetone for analysis.

6.3.5 Pretreatment of gastro-intestinal fluid

A sub-sample of total incubation fluid (30 mL), 20 µL of each, 1 mg/L mass- labeled internal standards *d*34, $^{13}C_6$-TBPH and *d*17, $^{13}C_6$-TBB, and 20 mL DCM were added to a 50 mL centrifuge tube. The mixture was shaken vigorously (Heidolph Multi Reax Vibrating Shaker, Brinkmann) for 30 min then sonicated for an additional 30 min. Following sonication, the mixture was stored at 4 ºC to allow for separation of DCM and GI-fluid. The GI-fluid and DCM were separated and DCM transferred to an evaporation tube and blown to complete dryness under a stream of nitrogen. The Extract was dissolved in 500 µL DCM and loaded onto Florisil cartridges, which had been conditioned by use of 6 mL DCM. TBPH, TBB, and the OH-isomers were eluted from Florisil cartridges by use of 5 mL DCM and a mixture of DCM/methanol (v/v, 1:1). Extracts were blown to dryness under a stream of nitrogen and reconstituted with 200 µL acetone for analysis.

6.3.6 Instrumental analysis

Instrumental analysis of the four target analytes has been described in our previous papers[163, 164]. Extracts were analyzed using a Q Exactive ultrahigh resolution mass spectrometer (Thermo Fisher Scientific) interfaced to a Dionex UltiMate 3000 ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific). Separation of the compounds was conducted by use of a Betasil C18 column (5 µm; 2.1 mm x 100 mm; Thermo Fisher Scientific)

with an injection volume of 5 µl. Ultrapure water (A) and methanol containing 0.1% NH4OH (v/v) (B) were used as mobile phases. Initially 20% of B was increased to 80% in 3 min, then increased to 100% at 8 min and held static for 19.5 min, followed by a decrease to initial conditions of 20% B and held for 2 min to allow for equilibration. The flow rate was 0.25 mL/min. Temperatures of the column and sample chamber were maintained at 30 °C and 10 °C, respectively. Data were acquired by use of selected ion monitoring (SIM) with an APCI or ESI ionization source. SIM mode was used to monitor the four target compounds to expand dynamic range. Briefly, MS scans (200-2000 *m/z*) were recorded at resolution R=70,000 (at 200 *m/z*) with a maximum of 3×10^6 ions collected within 200 ms (100 ms; APCI), based on the predictive automated gain control. SIM scans were recorded at a resolution of R=70,000 (at 200 *m/z*) with maximum of 5×10^4 ions collected within 200 ms (80 ms; APCI), based on the predictive automated gain control, with isolation width set at 2.0 *m/z*. For ESI, the general mass spectrometry settings applied for negative ion mode were as follows: spray voltage, 2.7 kV; capillary temperature, 375 °C; sheath gas, 46 L/h; auxiliary gas, 11 L/h; probe heater temperature, 375 °C. The general mass spectrometry settings applied for positive ion mode were as follows: spray voltage, 3.0 kV; capillary temperature, 400 °C; sheath gas, 46 L/h; auxiliary gas, 15 L/h; probe heater temperature, 350 °C. For APCI, the applied general mass spectrometric settings for APCI source were as follows: discharge current, 10 µA; capillary temperature, 225 °C; sheath gas, 20 L/h; auxiliary gas, 5 L/h; probe heater temperature, 350 °C.

6.3.7 Design of the Tenax bead incubation envelope

A previous study which added TA beads to the CE-PBET demonstrated the necessity of use of an incubation apparatus to isolate TA from dust samples[173]. In this experiment, Tenax beads (60-80 mesh, Supelco) were sieved through 100 mesh and cleaned by sonication by use of acetone:hexane (1:1, v/v). To limit loss of TA and the contamination of dust samples during incubation, an envelope was designed to isolate TA while allowing uninhibited flow of gastro-intestinal (GI) fluids, see appendix (Figure C6.S1). 100 mesh stainless steel was cut to approximately 9 x 6 cm (length x width) and rolled to create hollow cylinders. One end of the cylinder was folded and affixed with copper wire to form a TA pocket in the hollow cylinder. TA was weighed (0.3 g) and inserted into the hollow portion of the cylinder while the other end was folded and affixed with copper wire. To test their effectiveness to contain TA beads, several

envelopes were inserted and submerged in hexane:acetone, and sonicated/shaken for 30 min (n=4). Few TA beads were observed in the liquid or attached to the exterior of the envelope.

6.3.8 Tenax enhanced bioaccessible extraction

Methods used in this study were adapted from a recently developed *in vitro* CE-PBET technique and from an additional TA bead assisted method[173, 175]. Three surrogate GI fluids including stomach (pH 2.5), small intestine (pH 7.0), and colon (pH 6.5) were prepared, in the fed state, according to a previous study[175]. Concentrations of target compounds in samples of dust were determined prior to incubation to allow for mass balance analysis following *in vitro* digestion. OH-TBB isomers were not detected in the standard reference dust (SRM 2585; National Institute of Standards and Technology (NIST), Maryland, U.S.), so standards were spiked with 1 ng/g dust and homogenized to ensure complete mixing. Approximately 0.1 g of dust collected from day care centers or reference dust (SRM) and 0.3 g TA (in the prepared envelope) were added to 50 mL glass centrifuge tubes containing 45 mL of the pre-warmed, simulated stomach media and incubated at 37 ºC for 1 hr with constant agitation, to simulate peristaltic movement of the human gastro-intestinal tract. A sequential system, where dust was exposed to the three compartments in succession was employed. Following a 1 hr incubation period, bile salts (bovine and ovine, Sigma-Aldrich), pancreatin (porcine, Sigma-Aldrich), and sodium bicarbonate (to adjust to pH 7.0), were added to create the simulated intestinal media. Following incubation for 4 hr, the TA envelope was removed and dust was separated from the intestinal medium via centrifugation at 1000 *g* for 8 min. The TA envelope was re-added to the centrifuge tube containing dust, along with 45 mL of colon fluid, and incubated for 8 hr. Following incubation, the TA envelope was rinsed with deionized water to remove dust and both (dust & water) were added to colon fluid. Following this procedure, dust and fluid were separated as detailed above. Approximately 69% and 60% of dust was recovered following incubation in the CE-PBET and TA enhanced CE-PBET, respectively, and 95% of TA was recovered (Figure C6.S2).

6.3.9 Quality control

Minor contamination of TBPH was detected during processing of dust from day care centers, so procedural blank samples were included. Standards were typically re-injected after four to six injections of samples, and acetone was injected following each standard injection. Due to minor background contamination, the method detection limit (MDL) for TBPH in dust was 1.1 ng/g,

dm. No background contamination was detected for OH-TBPHs, TBB, or OH-TBBs. MDLs for these three compounds in dust were 0.01 ng OH-TBPH/g, dm, 0.12 ng TBB/g, dm and 0.005 ng OH-TBB/g, dm. Previous studies have investigated potential matrix effects by use of ESI or APCI[163, 164]. Recoveries of analytes were determined by spiking standards into samples of dust, TA, and the three GI media (n=12) prior to extraction. Concentrations of standards spiked into dust and TA, were 500 ng TBPH/g, dm, 500 ng TBB/g, dm, 5 ng OH-TBPHs/g, dm and 5 ng OH-TBBs/g, dm and 1 ng TBPH/TBB/OH-TBPHs/OH-TBBs/mL in GI fluid. Recoveries from dust, TA, and GI fluid ranged from 70-105% for TBPH, 65-94% for TBB, 83-101% for OH-TBPHs, and 85-115% for OH-TBBs. TBPH and TBB were quantified by use of internal standards, *d*34, $^{13}C_6$-TBPH and *d*17, $^{13}C_6$-TBB for which recoveries in dust, TA, and GI fluids were 82-95%., and 79-101%, respectively. Concentrations of OH-TBPHs and OH-TBBs were quantified without the use of internal standard due to the lack of commercial internal authentic standards and because recoveries were > 80% and stable across replicate recovery samples. Concentrations of both OH-TBPH1 and OH-TBPH2 were quantified by use of purified OH-TBPH2 standard, as described previously[163]. Concentrations of OH-TBBs were quantified by use of purified OH-TBBs standard, though, due to the lack of separation of the three OH-TBB isomers, total peak abundances were used for quantification. External calibration curves showed strong linearity for all four compounds ($r^2 > 0.99$), during the concentration series 0, 49, 98, 195, 391, 781, 1563, 3125, 6250, 12 500, 25 000, 50 000, and 100 000 pg/mL (TBPH/TBB) and 0, 63, 125, 250, 500, and 1000 pg/mL (OH-TBPHs/OH-TBBs).

6.3.10 Data analysis

Bioaccessibility was calculated by use of Equation 6.1[173].

Bioaccessibility = 1 – (NBFRs remaining in dust following incubation/Sum of NBFRs measured in dust, Tenax and digestive fluid)…………………………………………………………...(6.1)

Statistical analyses were completed by use of SPSS 19.0 software. Values less than MDLs were replaced by MDL/2. Contributions of individual compounds to sum of target analytes in dust were calculated as previously described[163, 164]. Concentrations were assessed using the Shapiro-Wilk test to determine if they followed a normal probability function. If the frequency distribution of a set of concentrations was not normally distributed, a log-transformation was used to ensure normality of distributions of values. When comparing bioaccessibility or

concentrations of compounds among samples/seasons, a paired t-test analysis was used. Differences with $p < 0.05$ were considered significant.

6.4 Results and discussion

6.4.1 Concentrations of NBFRs and their hydroxylated isomers in dust from day care centers

Concentrations of TBPH and TBB are consistently the greatest in indoor dust from the U.S. and Canada. Although there are numerous studies regarding concentrations of a range of legacy and novel flame retardants (FRs) in the U.S., Europe, and China, there have been fewer studies conducted in Canada. In this study, concentrations of TBPH, TBB and their hydroxylated isomers, OH-TBPH1, OH-TBPH2, and ΣOH-TBBs in dust from day care centers across Saskatoon, SK, Canada were among the greatest reported for these compounds. TBPH and TBB had geometric mean concentrations of 734 and 992 (ng/g, dm), respectively, in summer while OH-TBPH1, OH-TBPH2, and ΣOH-TBBs had greatest geometric mean concentrations of 0.17, 0.81, and 0.30 (ng/g, dm), respectively, in winter (Table 6.1). Concentrations of TBPH were similar, though generally greater, than those previously reported for dust from academic environments and homes in the U.S. and Canada[41, 160, 165, 176], while concentrations of TBB were similar, though generally greater, than those reported for dust in homes from the U.S. and Canada[41, 165]. Concentrations of the hydroxylated isomers have only been reported previously by our group[163, 164]. These isomers were initially detected in analytical standards and technical mixtures such as Firemaster® 550 and BZ-54, but were later confirmed in dust from day care centers. Though the relative contributions of OH-TBPH1, OH-TBPH2, and ΣOH-TBBs in FM-550 were approximately 0.1, 7.9, and 0.8%, their relative contributions in dust ranged from 0.01-0.13% OH-TBPH1/2, and 0.18-0.24% ΣOH-TBBs. Differences in relative contributions between technical mixtures and environmental samples might be due to differences in environmental partitioning to air or dust and in size of dust particles. OH-TBPH1, OH-TBPH2, and ΣOH-TBBs were detected in approximately 48, 84 and 88% of samples collected during winter, which were similar to frequencies of detection for TBPH (100%) and TBB (100%), though frequencies for OH-TBPH1 were significantly fewer. Reduced frequency of detection, lesser relative contributions, and lesser concentrations of OH-TBPH1 in dust indicate the compound is likely a very minor contaminant in technical mixtures of the flame retardant. Concentrations observed in

these ECEs are among the greatest reported, which indicates that products in these environments contain greater quantities of NBFRs or there are greater amounts of products which contribute to the concentrations detected in dust.

Table 6.1. Concentrations of TBPH, OH-TBPH, TBB, and ΣOH-TBB (ng/g, dm) in samples of dust collected from daycare centers in summer or winter of 2013 and 2014, respectively.

		TBPH	OH-TBPH1	OH-TBPH2	TBB	ΣOH-TBB
Summer[d,e]	GM ± GSD	734 ± 0.87	0.04 ± 0.88	0.35 ± 1.0	992 ± 0.82	0.21 ± 1.33
	Min.	15	<0.01[a]	<0.01[a]	25	<0.01[a]
	Max.	22251	7.3	27	37975	91
	Det. Freq. [b]	100%	52%	91%	100%	91%
	Contribution[c]	99.9%	0.01%	0.1%	99.8%	0.18
Winter	GM ± GSD	627 ± 0.66	0.17 ± 0.71	0.81 ± 0.75	841 ± 0.76	0.30 ± 0.78
	Min.	105	<0.01[a]	<0.01[a]	22	<0.01[a]
	Max.	19345	7.8	15.8	43035	87
	Det. Freq. [b]	100%	48%	84%	100%	88%
	Contributions[c]	99.8%	0.04%	0.13%	99.8%	0.23%

[a]The concentration was less than the MDL (0.01 ng/g).
[b]Detection frequencies in samples of dusts.
[c]Relative contribution to the sum of concentrations of TBPH or TBB and their OH-isomers.
[d,e]Concentrations of TBPH and TBB and their OH-isomers in samples collected from summer were reported in our previous studies[163, 164].
*Indicates a statistically significant difference between samples from summer and winter (p<0.05).

Children in the U.S. and Canada generally spend a great proportion of their time in early childhood environments (ECEs) and schools, while dust from these environments might contain relatively greater concentrations of BFRs than in homes. Though greater concentrations of BFRs in certain environments might be an important determinant of overall exposure of children to BFRs, relatively few assessments of concentrations of TBPH or TBB have been conducted on dusts from ECEs or primary schools. One such assessment in California, U.S., reported mean concentrations of TBPH and TBB of 431 and 1062 ng/g, dm, respectively, which is significantly less than reported here but greater than concentrations detected in homes in California[75]. Several other investigations have reported greater concentrations of BFRs including PBDEs (PentaBDE mixture) and hexabromocyclododecane (HBCD) in ECEs and schools relative to other environments including houses, cars, and apartments[70, 177-180]. These relatively great concentrations in dust might have originated from a greater number of children's products in these environments. For example, a screen of children's products collected from 2000 to 2010 detected components of the Firemaster® 550 mixture in 17% and tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) in 36% of products. Indeed, in a study of metabolites of organophosphate flame retardants (OPFRs) in urine, children who possessed > 16 children's products had 6.8 times greater concentrations and those infants which attended day care centers had 3.7 times greater concentrations of metabolites of some OPFRs[181]. A potential conclusion which can be inferred from these findings is that increased concentrations of FRs in dust might be partially due to the increased density of children's products in these environments and might be a relevant factor in the greater exposure of children to FRs.

6.4.2 Differences of concentrations of NBFRs and their hydroxylated isomers in dusts from specific microenvironments

Day care centers and other ECEs have relatively greater densities of children's products than homes and these products can contain significant amounts of legacy BFRs and NBFRs. Therefore, it was hypothesized that the greater number of children's products might lead to greater concentrations of these NBFRs in dust from ECEs, which would, in turn, result in greater exposures of children to BFRs. In this study, concentrations of compounds in dust from three microenvironments which represented permutations of greater/lesser numbers of children's products (toys/furniture) and more/less human activity and foot-traffic were investigated. The microenvironments were designated as higher traffic-higher toys (HT-HT), higher traffic-lower

toys (HT-LT), and lower traffic-lower toys (LT-LT). Microenvironments designated as higher/lower foot-traffic were major play/activity rooms or rooms with lesser foot-traffic (sleeping rooms), respectively, where microenvironments designated as higher/lower toys contained relatively greater amounts of children's products (storage or major activity rooms) and fewer children's products (sleep or craft rooms). By comparing concentrations of these NBFRs in dust from microenvironments which contain higher/lower number of children's products, information regarding potential sources of the compounds and increased risk of exposure might be inferred. Results of other studies have shown that high foot-traffic rooms can contain greater concentrations of BFRs in dust[153], which indicates that human activity, including air movement and transfer of dust, might alter concentrations of NBFRs in microenvironments.

In each day care center sampled during winter, dust from HT-HT microenvironments consistently had greatest concentrations of TBPH, TBB, OH-TBPH1, OH-TBPH2, and ΣOH-TBBs (Figures 6.1, 6.2). In assessments of TBPH, TBB and ΣOH-TBBs, concentrations in dust collected in winter from the HT-HT environments were greater than concentrations in the HT-LT and LT-LT environments while concentrations of OH-TBPH1 were greater in HT-HT than in HT-LT and concentrations of OH-TBPH2 were greater in the HT-HT than in LT-LT (Figures 6.1, 6.2). Greater concentrations of these FRs in the HT-HT environment could indicate that relatively more children's products might contribute to greater concentrations in dust, although it has also been previously described that high foot-traffic areas can contain greater concentrations of BFRs in dust. For example, in homes from Boston, concentrations of PentaBDEs and DecaBDEs were 72 and 97% greater in dust collected from the main living room than the bedroom[153]. Though higher foot-traffic in the area might contribute to concentrations of FRs in dust, there were no differences in concentrations of any compound in dust between the HT-LT and LT-LT microenvironments. These environments have similar numbers of children's products, but vary in intensities of human foot-traffic, which indicates that foot-traffic is not a major contributor to increased concentrations of NBFRs in dust. Differences in concentrations of OH-TBPH1 or OH-TBPH2 in microenvironments might also indicate that foot-traffic is not a major factor affecting concentrations of NBFRs. The common factor between microenvironments in which differences in concentrations of the hydroxylated isomers were detected was 'lower toys', no discernable pattern was observed for 'traffic' as a contributing factor. These microenvironments had similar numbers of electronics and furniture, though

considerable uncertainty exists in any analysis of contributing factors because NBFRs are added to numerous consumer products including flooring, electronics and insulating foams. The myriad of uses coupled with inconsistent masses applied and lack of information regarding types of FRs incorporated into the product can create difficulties in ascribing singular sources (children's products) as sole contributors to NBFRs in dust. These inconsistencies have been discussed in previous reports which encountered difficulties in relating concentrations of FRs in dust to numbers of furniture or electronics[182, 183]. Based on data derived from winter samples, microenvironments which contain more children's products generally have greater concentrations of NBFRs in dust and while the volume of foot-traffic might also contribute to these increased concentrations, a significant difference in concentrations of NBFRs were not detected between environments which varied only in the 'traffic' parameter (HT-LT vs. LT-LT).

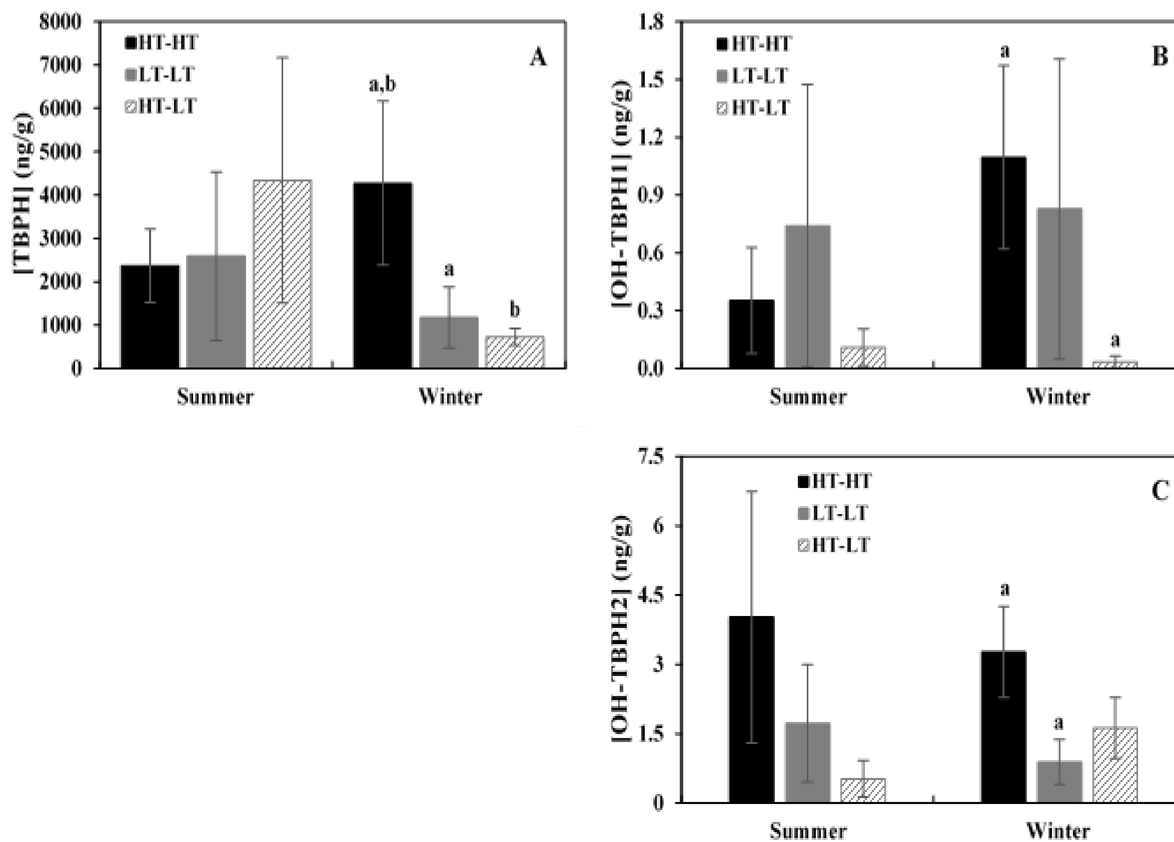

Figure 6.1. Mean concentrations of TBPH (A), OH-TBPH1 (B), and OH-TBPH2 (C) in dust from daycares across Saskatoon, SK, Canada (n=10). Dust was collected from higher traffic-higher toy environments (HT-HT), lower traffic-lower toy environments (LT-LT), and higher traffic-lower toy environments (HT-LT). Samples were collected in summer of 2013 and winter of 2014 (n=10, per room type/season). Error bars represent standard deviation, lower case letters represent statistically significant differences, $p < 0.05$.

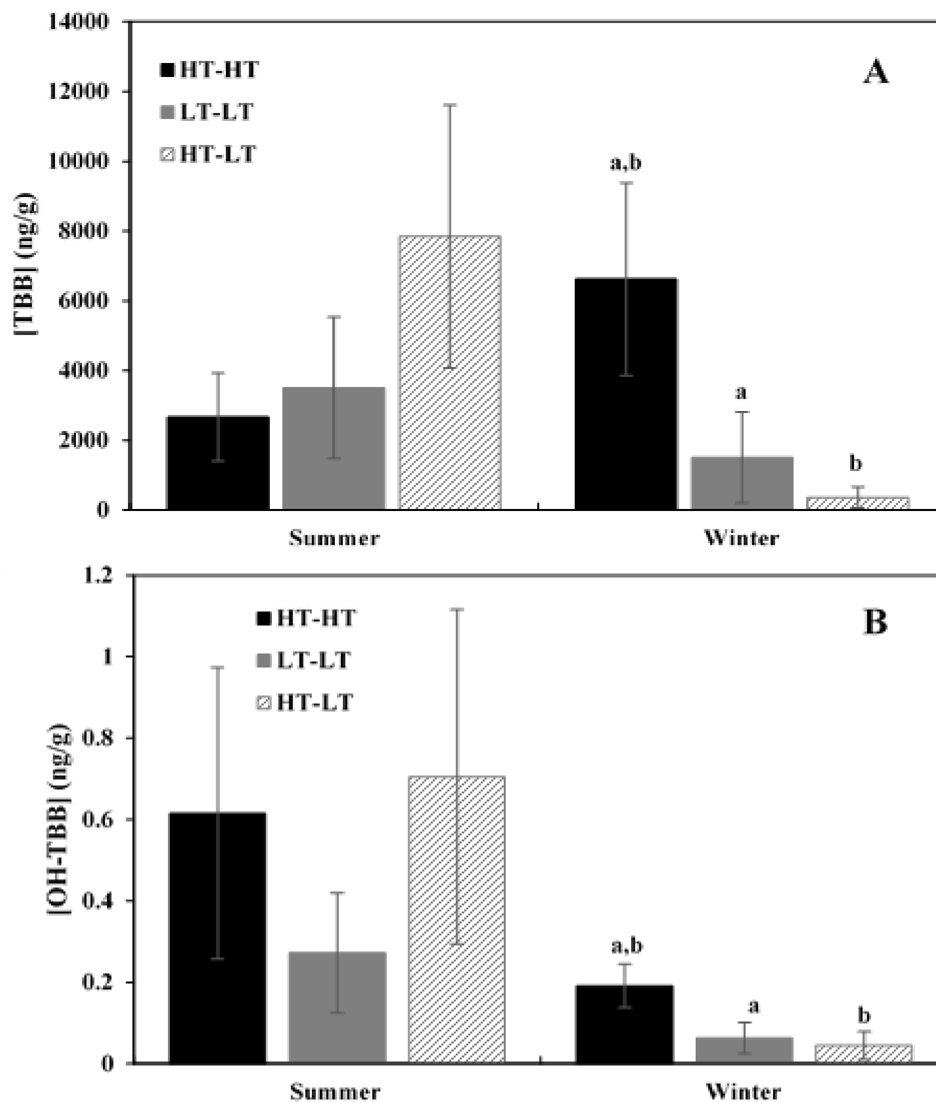

Figure 6.2. Mean concentrations of TBB (A) and ΣOH-TBBs (B) in dust from daycares across Saskatoon, SK, Canada (n=10). Dust was collected from higher traffic-higher toy environments (HT-HT), lower traffic-lower toy environments (LT-LT), and higher traffic-lower toy environments (HT-LT). Samples were collected in summer of 2013 and winter of 2014 (n=10, per room type/season). Error bars represent standard deviation, lower case letters represent statistically significant differences, $p < 0.05$.

6.4.3 Seasonal differences in concentrations of NBFRs and hydroxylated isomers in dust

Seasonal differences in concentrations of NBFRs in indoor dust are particularly important in colder climates, where people spend a great proportion of time indoors. Significant variability in concentrations of NBFRs among seasons could alter assessments of risk as exposure concentrations during cold seasons could be higher relative to warmer periods[177]. In particular, young children spend great proportions of time indoors, up to 97% in Canada[174], during the winter months, which might increase risk associated with these indoor contaminants.

Means of concentrations, frequencies of detection, and relative contributions of none of the compounds were statistically different between seasons, though a trend of increased concentrations of the hydroxylated isomers, OH-TBPH1, OH-TBPH2, and ΣOH-TBBs in winter was observed (Table 6.1). Concentrations of OH-TBPH1, OH-TBPH2, and ΣOH-TBBs were 425%, 231%, and 143% greater in winter than in summer. Furthermore, no seasonal differences were detected in comparison of microenvironments, which was expected as the amount of children's products and furniture were not significantly altered between the two sampling periods (Figures 6.1 and 6.2). Seasonality of concentrations of NBFRs in dust can be attributed to numerous phenomena including migration from consumer products[177, 184]. The processes are functions of specific physical-chemical properties including log K_{ow}, volatility, and molecular weight (MW)[182]. For example, BDE-209 (log K_{ow}, 12.11; vapour pressure, 6.22×10^{-10} Pa; MW, 959.17; EpiWeb 4.1) likely migrates from products via mechanical abrasion as it has a high log K_{ow} and low vapour pressure, where triphenyl phosphate (TPP) (log K_{ow}, 4.70; vapour pressure, 8.37×10^{-4} Pa; MW, 326.29; EpiWeb 4.1) another FR and major component of the Firemaster® 550 mixture, has a greater vapour pressure and lesser log K_{ow} and generally would migrate from products via volatilization-adsorption. Processes by which chemicals migrate from products partially dictates where a chemical will partition in the environment. For example, BDE-209 would likely remain adsorbed to abraded particles and incorporate into dust[185], while TPP would preferentially partition to the air-phase (vapour and particles). These mechanisms also dictate emissions of NBFRs, where TBPH, TBB, OH-TBPHs, and OH-TBBs have physical-chemical properties similar to BDE-209 and components of the PentaBDE mixture (BDE-47/99/153). As such, the NBFRs would likely remain adsorbed to abraded particles in dust and follow similar seasonal trends as BDEs-47/99/153/209. In previous studies of seasonality, concentrations of

PBDEs (47/99/153/209) in dust did not significantly change between cold and warm seasons[153, 186, 187]. For example, in one of the most in-depth and robust studies of seasonal variations of FRs, concentrations of PBDEs (47/99/153/209) and NBFRs (TBPH/TBB) remained stable between the summer and winter months, though a clear seasonal effect was observed in concentrations of OPFRs[184]. Increased concentrations of OPFRs in winter months are likely due to greater volatilities as compounds which partition to air would be affected by lower air flow and filtration in buildings, where this effect would be minor for non-volatile or lesser for semi-volatile compounds. Indeed, most investigations of seasonal differences of less volatile compounds indicate that introduction of new consumer products or changes in cleaning behaviours, instead of seasonal differences of heat and air flow, are important parameters for changes to concentrations in dust[188]. The lack of seasonal differences in concentrations of NBFRs and some PBDEs in dust is similar to results observed in this study, though increases in concentrations in winter months of the OH-isomers were not significant, the observed patterns could be due to greater volatility, lower MW and lower log Kow of the compounds relative to TBPH and TBB. Results of this study indicate that seasonality would not significantly alter assessments of risk for these NBFRs, though great variability in concentrations have been observed in repeated samples, so several sampling events would likely be required for accurate estimations of concentrations in dust.

6.4.4 Bioaccessibilities of NBFRs and their hydroxylated isomers in standard reference dust and dust collected from day care centers

In general, humans are exposed to NBFRs via three pathways, dermal absorption, inhalation, and ingestion. Due to the physical-chemical characteristics of TBPH and TBB, inhalation and ingestion are likely the major routes of exposure to these indoor contaminants. Though precise routes of exposure for TBPH and TBB are unclear, recent studies have shown that the dermis likely provides a significant barrier for hydrophobic compounds including TBPH and TBB, thus epidermal exposure is likely not a major route of concern[189, 190]. To properly assess exposure via ingestion the concentrations of target compounds in the exposure vector and their bioavailability must be accurately described. But, *in vivo* determinations of bioavailability are difficult and carry ethical and economic considerations associated with the use of animals. Thus, bioavailability data is seldom integrated into assessments of exposure when calculating risks. The surrogate measure, bioaccessibility via the *in vitro* CE-PBET method avoids these concerns and represents

an accurate estimation of *in vivo* bioavailability which can be integrated into these assessments[173].

Significant differences in bioaccessibilities were detected for all compounds when TA was/not added to the CE-PBET test system (Figure 6.3). For example, in the system enhanced with TA, bioaccessibilities of TBPH and TBB were 33 and 59%, respectively, while in the system lacking TA they were 3 and 12%, respectively. Results of previous studies have shown that predictions of *in vitro* bioaccessibility of PBDEs have been more similar to *in vivo* values with the use of a TA enhanced system[173]. This is likely because TA acts as a sorption sink to remove NBFRs mobilized into the GIT fluid from dust particles by mimicking the lipid membrane of intestinal cells[191]. As such, TA helps to maintain a desorption gradient between dust and the GIT fluid which would likely be observed in the gastro-intestinal system. Bioaccessibilities of TBPH, TBB, OH-TBPHs, and OH-TBBs from standard reference dust and dust collected from day care centers in winter and summer, were assessed with the system enhanced with TA. The NBFRs were detected in all samples, though OH-TBBs were not detected in the SRM and standards were subsequently spiked into the material. Bioaccessibilities of TBPH and OH-TBPHs in SRM and dust collected from day care centers in summer and winter ranged from 33-38% and 23-30%, respectively (Figures 6.3, 6.4; Table C6.S2). These values are similar to those from a previous study - 25% for TBPH - that used the TA assisted CE-PBET method. They were also similar to bioaccessibilities of PBDEs with similar physical-chemical characteristics as TBPH and OH-TBPHs; BDE-209 = 28% and 5-14% and BDE-153 = 28-34%[173, 192]. Bioaccessibilities of TBB and OH-TBBs in SRM and dust collected from day care centers in summer and winter were moderate, and ranged from 53-59% and 70-72%, respectively (Figures 6.3, 6.4; Table C6.S2). These values were similar, though greater than those from a previous study which used the TA assisted CE-PBET method. Bioaccessibility of TBB was 48% and was generally greater than bioaccessibilities of PBDEs with similar physical-chemical characteristics; BDE-153 = 55% and 28-34%, BDE-47 = 74% and 23-25%, and BDE-99 = 65% and 15-23%[173, 192]. No seasonal differences of bioaccessibility were observed for any compounds for dust collected in winter vs. summer (Figure 6.4). Differences of bioaccessibilities between studies could likely be explained by differences in composition of dust (organic content), the state of the *in vitro* model (fed vs. unfed) and use of TA as a sorptive sink. This study supports

the values of bioaccessibilities of TBPH and TBB reported in the only other assessment in the literature[173], and is the first to calculate bioaccessibilities of OH-TBPHs and OH-TBBs.

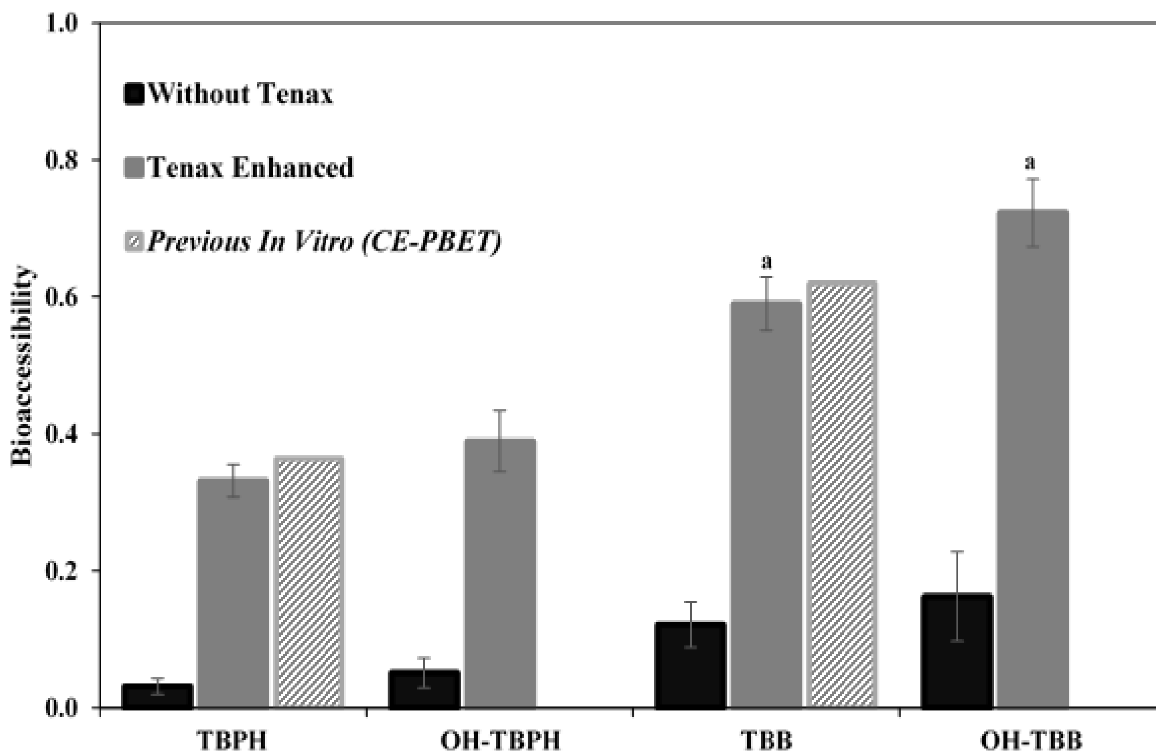

Figure 6.3. Bioaccessibilities of TBPH, TBB and their OH-isomers (ΣOH-TBPH1/2 and ΣOH-TBBs) in reference dust (n=4). Bioaccessibilities were tested with and without Tenax enhancement and compared to data from a previous *in vitro* study[173]. Error bars represent standard deviation, lower case letters represent statistically significant differences, $p < 0.05$.

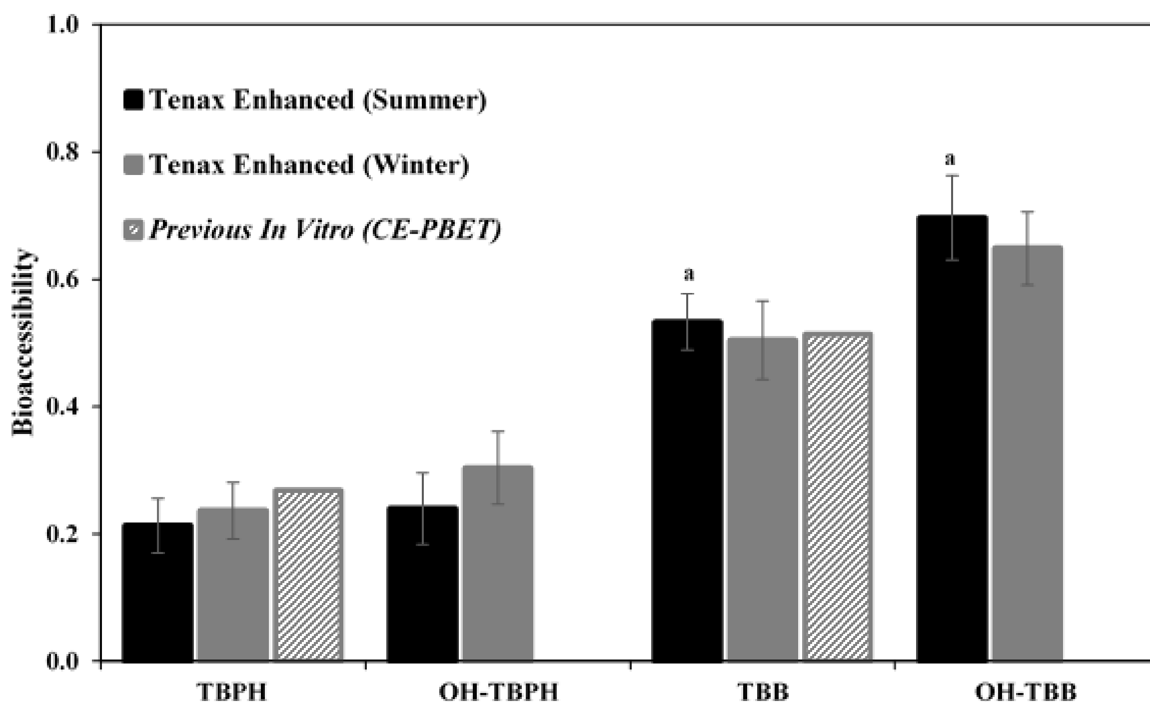

Figure 6.4. Bioaccessibilities of TBPH, TBB and their OH-isomers (ΣOH-TBPH1/2 and ΣOH-TBBs) in dust (n=14) collected in the summer of 2013 and winter of 2014 from daycares in Saskatoon, SK, Canada. Bioaccessibilities of TBPH and TBB were compared to data from a previous *in vitro* study[173]. Error bars represent standard deviation, lower case letters represent statistically significant differences, $p < 0.05$.

There were significant differences in bioaccessibilities of TBB and OH-TBBs from SRM and dust collected from day care centers, though no differences were observed between TBPH and OH-TBPHs (Figures 6.3, 6.4). Further, no differences in bioaccessibilities between TBB and OH-TBBs were observed in the CE-PBET system which did not integrate TA. This indicates that performance of the system was enhanced with the addition of TA. Molecular weight, solubility, and log K_{ow} are likely important factors which influence the bioaccessibility of compounds. Previous studies have shown a significant relationship between bioaccessibility and values of log K_{ow}, where bioaccessibility decreased as values of log K_{ow} increased, though bioaccessibilities of compounds with log $K_{ows} \geq 8$ were generally similar[173, 193]. This phenomena was also observed in this study, as bioaccessibilities of TBPH and OH-TBPH, which have similar log K_{ow} values of 11.95 and 9.56, respectively, were not significantly different. Bioaccessibilities of these compounds were similar to those of BDE-209 and BDE-153, which have log K_{ow} values of 12.11 and 8.55, respectively. Differences in bioaccessibilities of TBB and OH-TBBs is likely due to differences in log K_{ows}. Specifically, the log K_{ows} of TBB and OH-TBB are close to the asymptotic region of the relationship between bioaccessibility and log K_{ow}, where TBB > 8 > OH-TBB, which indicated that OH-TBB would likely be more bioaccessible than TBB. This increase in bioaccessibility of the OH-isomer is important to note because an increased amount of the compound will likely reach the systemic circulation, and the addition of a phenolic group generally increases the toxic potency of a chemical as observed with OH-PBDEs[161]. For example, in our recent study, OH-TBB demonstrated greater estrogenic response and induced greater PPARγ activity than native TBB in *in vitro* cellular assays[164]. The increased bioaccessibility of OH-TBB and increased toxic potency highlights the potential for increased risk to children.

6.4.5 Conclusion

This study was one of the first to quantify the NBFRs TBPH and TBB and their hydroxylated isomers, OH-TBPH1, OH-TBPH2, and ΣOH-TBBs in dust from early childhood environments. This study was also the first to investigate the seasonality of these compounds in dust from these environments and bioaccessibilities in an *in vitro* model. Results show that day care centers from Saskatoon, SK, Canada have some of the greatest concentrations of TBPH and TBB observed to date. This finding is concerning as these NBFRs have been shown to disrupt

endocrine functions which are important to normal development. As the majority of young children in Canada attend some version of an ECE, they might be exposed to increased concentrations of these NBFRs during a susceptible period of development.

Seasonal differences between concentrations of these NBFRs were minimal which might, in part, be due to their process of migration from consumer products. In our previous studies, which were the first to detect, identify, and quantify the OH-isomers of TBPH and TBB[163, 164], we proposed they might be more bioaccessible than the natural compounds due to their lower log K_{ows}. In this study we observed that OH-TBBs, though not OH-TBPHs, were indeed more bioaccessible than the native compounds. The increased bioaccessibility of these OH-isomers (OH-TBBs) is important as it has recently been shown that the compound has greater endocrine disrupting potency than the native TBB[164], and is more likely to enter systemic circulation and adversely affect normal endocrine functions in the developing child.

In an effort to obtain broad distribution of samples and appropriate sample sizes to determine seasonal and microenvironment specific differences in concentrations, approximately 60% of provincially licensed day care centers in Saskatoon, SK, Canada were canvassed for inclusion in this study. However, participation among day care centers was limited as there were perceived stigma related to scientific studies and implications for viability of small businesses. Participation by licensed day care centers was recorded at approximately 18%. Though a larger sample size would have helped to more accurately described trends in seasonal variation and differences in microenvironments, the study was successful with the acquired samples.

7 CHAPTER 7: GENERAL DISCUSSION

7.1 History and project rationale

7.1.1 Regulations, the use of BFRs, and research regarding Firemaster® 550

In 1972 the California Bureau of Electronic and Appliance Repair, Home Furnishing and Thermal Insulation responded to increases in devastating home fires by implementing a residential flammability standard known as Technical Bulletin 117 (TB 117). The terms of this standard required that fill materials in upholstered consumer products, including polyurethane foams (PUF), were able to withstand 12 s of open flame before ignition, in theory reducing the time to combustion of the material. Though this local standard was implemented in California, it has affected exposure to BFRs internationally. Due to the size and prominence of the Californian economy, manufacturers integrated this standard into most products destined for North American markets. At the initiation of this PhD program, most household furniture investigated in Saskatoon, SK, Canada contained the TB 117 label.

In recent years, there has been controversy regarding the TB 117 standard. Many scientists and engineers have noted that TB 117 was ineffective, specifically in the 12 s smoulder test which used naked polyurethane foam exposed to a candle sized flame. This standard did not represent a realistic scenario as fires generally started on the outer fabric which covered the PUF. Once contacted, the PUF would likely be exposed to flame significantly greater than those generated by a candle. In fire retardancy tests of PUF generated in the 1980s, researchers funded by manufacturers of BFRs claimed that the addition of FRs provided residents a 15-fold increase in time to escape residential fires. It was later exposed that concentrations of FRs used in these tests were significantly greater than those found in consumer products. Recently, scientists, regulators, media, and concerned citizens have questioned the use of chemical FRs, due to their associated toxicities, and have attempted to amend TB 117. In 2013, following a polarizing campaign which saw chemical manufacturers create fake citizen awareness groups and purchase false congressional testimony from a highly regarded specialist (who later relinquished their medical license), the California state government updated their fire safety standards. The new California standard, TB-117-2013, did not ban the use of BFRs, but it no longer required their use in upholstered furniture and suggested the use of smolder-resistant fabrics. This decision has the potential to significantly alter exposure to BFRs and NBFRs in North America as several

major retailers including Wal-Mart, Ashley Furniture, and Ikea have committed to eliminate BFRs and NBFRs from products.

Initially, compliance with TB 117 was partially achieved by application of flame retardant compounds such as PBDEs and OPFRs to consumer products. Studies which demonstrated their persistence in the environment, bioaccumulation potential, and toxicities lead to the phase-out of some of these compounds from U.S., European, and Canadian markets. Following the phase out of the Penta-BDE formulations, the FR mixture, FM-550, which contained TBPH and TBB was one of the primary replacements and their volumes of production have likely increased[31]. In 2011, when this program of study was initiated, there were six studies which characterized the presence and behaviour of TBPH and TBB in the environment[36, 38, 41, 43, 149, 194], and no studies which focused on toxicities of these compounds. In the two subsequent years, several investigations of toxicity, including the study presented in Chapter 2, were published[29, 52, 53, 78, 99]. These studies demonstrated potential endocrine disrupting effects, potential for DNA damage resulting from exposure to FM-550 or the individual components of the mixture, and characterized *in vitro* metabolism. Though these initial studies highlighted some of the potential toxicities of components of FM-550, further assessments of concentrations in the environment and toxic profiles of these compounds were greatly important.

7.1.2 History of research regarding TBCO

When this program was initiated in 2011 there were only three studies which had investigated the presence of TBCO in the environment[126, 127, 195], and though the compound had met EU criteria as a potential aquatic hazard, there were no assessments of its potential toxicities. TBCO was a low production volume chemical with great potential. The compound was structurally similar to HBCD, one of the major-use legacy BFRs, and had been identified by the U.S. EPA Design for the Environment program as a potential replacement. In 2001, use of HBCD comprised approximately 8% of the global market, but due to its toxicity, persistence in the environment, and bioaccumulation potential, the compound was added to the Stockholm Convention on POPs in 2014 and was phased out of use in European markets. Use of TBCO as a major or minor replacement of HBCD would greatly increase the compound's production volume and risk of exposure of humans and aquatic wildlife.

7.1.3 Project rationale

Information in the published literature regarding concentrations in the indoor-outdoor environment and potential toxicities of TBPH, TBB, and TBCO was sparse. Due to their inclusion as major and potential replacement compounds of legacy BFRs, production volumes of these chemicals were likely to increase. Thus, the rationale for this program of research was to increase knowledge of toxicities and concentrations of these compounds in the indoor environment. Such knowledge would improve assessments of risk and alternative assessments of these compounds. For this purpose, toxicity profiling similar to the U.S. EPA Endocrine Disruptor Screening Program was used. Screening level *in vitro* assessments of the compounds were conducted by use of a range of cellular assay systems (Chapter 2). Initial positive results indicated that further studies were required, thus, small fish models were used to characterize potential endocrine disrupting effects and elucidate mechanisms of action (Chapters 3 and 4). Following assessments of toxicities, concentrations of these compounds in dust from ECEs were determined and potential modulating factors of these concentrations and bioaccessibilities of the compounds were investigated (Chapters 5 and 6).

7.2 Toxicities of novel brominated flame retardants

7.2.1 Screening level *in vitro* assessments of endocrine disrupting effects of TBPH and TBB

The few initial assessments of toxicities of TBPH and TBB showed potential endocrine disrupting effects of these compounds (Chapter 2). Following these results, several *in vitro* studies were conducted to further verify and characterize toxicities of these compounds. Concurrent investigations into metabolism of TBPH and TBB were conducted as the mono-ester metabolite of DEHP, the non-brominated analogue of TBPH, was considered to be the toxicologically active form of the compound[79]. In tissue preparations of fish, rats, and humans, TBB was rapidly metabolized to form 2,3,4,5-tetrabromobenzoic acid (TBBA), while TBPH was metabolized to form mono(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (TBMEHP). Due to potential toxicities of these metabolites, some investigations have focused on these compounds[29, 52, 78]. The (anti)-estrogenic, (anti)-androgenic, and (anti)-thyroidal activities of TBPH, TBB and their metabolites TBBA and TBMEHP were assessed in numerous cellular assay systems at concentrations which ranged from 1 to 3000 μM. In corroboration of results from Chapter 2, studies showed that TBPH and its mono-ester metabolite, TBMEHP interacted with hormone

receptors to cause anti-androgenic and anti-thyroidal effects[196]; both compounds caused anti-estrogenic and anti-androgenic effects and inhibited deiodinase activity[54, 197]; and TBPH modulated levels of E2 and T in a porcine mixed cell model[100]; though TBPH did not cause (anti)-estrogenic or (anti)-androgenic effects at similar concentrations in the MCF-7 or YES/YAS cellular assay systems[53, 198]. TBPH and TBMEHP were potent agonists of the pregnane X receptor (PXR), while TBPH also elicited antagonistic effects[199].

Similar to results presented in Chapter 2, TBB and its metabolite TBBA interacted with hormone receptors to cause anti-androgenic and anti-thyroidal effects[196]; TBBA elicited anti-estrogenic and anti-androgenic effects in the YES/YAS assay system though no effects were observed for TBB[197]; TBB and TBBA were potent agonists of the pregnane X receptor (PXR) which caused significant increases in transcript abundances of CYP3A4[199]; though TBB did not modulate concentrations of E2 and T in a porcine mixed cell model[100]. Further, TBB, TBBA, and TBPH did not interact with the PPARα or PPARγ receptors *in vitro*[200-202], though TBMEPH caused moderate activity of both receptor sub-types and induced adipocyte differentiation while ΣOH-TBBs elicited strong activity of PPARγ[54, 164, 202, 203]. As observed in the screening level assessment of TBPH and TBB in Chapter 2, these compounds and their metabolites affected activities of several nuclear receptors which function within the endocrine system, and modulated concentrations of steroid hormones within these cellular assays. But, it is also important to note that there were several contradictory reports of these effects, which is common for compounds tested with different *in vitro* assay systems at varying concentrations. Cellular assay systems were created from diverse tissues and can vary greatly in their sensitivities, physiologies, and metabolism. For example, the MCF-7 cell line originated from mammary epithelium tissue which naturally express the hER and other cellular tools of the ER activation pathway, whereas YES/YAS were derived from yeast which contained a transfected receptor (hER/hAR), express none of the inherent enzymes associated with the ER activation pathway, and contain a cell wall which might alter absorption of chemicals relative to mammalian derived cellular systems[81].

Though *in vitro* assay systems are accepted tools for screening level assessments of toxicity, as exemplified in the U.S. EPA EDSP, there remains questions of confidence in results generated from these tests. Further, not all compounds which elicit responses in these *in vitro*

assays are EDCs and assessments beyond the limited complexity of *in vitro* binding assays are required. *In vitro* systems are inherently simple and cannot represent the complexity of an *in vivo* system which integrates dynamic networks of processes which are tightly regulated and controlled to maintain homeostasis. There are numerous examples of chemicals which interact with specific molecular endpoints in the endocrine system but have no adverse effects on tissue or the whole organism; vertebrate networks such as the endocrine system are incredibly adaptive to maintain function. Thus, further assessments of the endocrine disrupting toxicities of these NBFRs were conducted in *in vivo* model systems (Chapters 3,4).

7.2.2 *In vivo* assessments of endocrine disrupting effects of TBPH, TBB and TBCO

Fish models have been used extensively in screening programs of endocrine disruption, in part due to the significant conservation of basic aspects of the endocrine system between vertebrates. Thus, our assessment of these NBFRs used a small fish model to test fecundity and fertility to determine effects on the whole organism and identify mechanisms of toxic effect (Chapters 3,4). Exposure to a mixture of TBPH/TBB or TBCO resulted in a decrease in cumulative fecundity of 32% for the TBPH/TBB mixture, and 18% for TBCO, though neither exposure resulted in modulation of fertility[130, 172]. Analysis of 34 genes across the HPGL-axis did not provide a specific mechanism of effect for the TBPH/TBB mixture, though a pattern of global down-regulation of transcripts of upstream signals including gonadotropin releasing hormones, gonadotropin releasing hormone receptors, and brain ER/AR was observed. No compelling mechanism of toxic effect was observed from the analysis of transcript abundances of the HPGL-axis of fish exposed to TBCO, though sex and organ specific differences were noted. In female fish increased expression of genes that responded to exposure to estrogens, which included vitellogenin II, choriogenin H, and ERα, were observed. This indicated that TBCO might have caused increased production of E2. This hypothesis was supported by the 3.3-fold increase in production of E2 observed in the H295R assay following exposure to TBCO[99] (Chapter 2). The studies conducted in Chapters 3 and 4 supported the hypotheses that TBPH, TBB, and TBCO were endocrine disrupting compounds, and though the mechanisms of toxicity were not fully elucidated, significant alterations to expression of genes across the HPGL-axis were observed.

Several investigations have attempted to characterize toxicities of FM-550 and its components in *in vivo* systems. Components of the FM-550 mixture including TBPH, TBB,

triphenyl phosphate (TPP), and a complex mixture of ortho-, meta, and para- substituted isomers of mono-, di-, tri-, and tetra isopropylated triaryl phosphates (ITPs) were tested for developmental toxicity in a zebrafish embryo model[204]. In this screening level assessment, exposure of TBPH or TBB up to 10 µM resulted in no significant effects on embryonic survival or development, whereas exposure to the phosphate components resulted in effects on cardiac function during embryogenesis. In a separate study which investigated the neurobehavioural effects of the FM-550 mixture, chronic exposures of 0.01 to 1.0 mg/L FM-550 in developing zebrafish or acute exposures of 1.0 to 3.0 mg/L FM-550 in adolescent zebrafish caused hypoactivity and a significant reduction in social behaviour, though within 1-week of acute exposure, effects were completely attenuated[205]. This was one of the first studies which investigated potential neurobehavioural effects of FM-550 and reported that the mixture might cause persistent alterations to social behaviour. This study also found that exposures during susceptible periods of development were likely more harmful than acute exposures during adolescence.

Due to the similarity of the mono-ester metabolite of TBPH to the bioactive metabolite of DEHP (MEHP), the toxicities of TBMEHP were assessed in pregnant rats[54]. Rats were treated by gavage with 200 or 500 mg/kg of TBMEHP on gestational days 18 and 19, and dams and fetuses were evaluated for toxicity. The 48 hr exposure of TBMEHP produced hypothyroidism and decreased concentrations of serum T3, produced an increase in maternal hepatotoxicity, and an increase in multinucleated germ cells (MNGs) in fetal testes. These effects were similar to those observed following exposure to MEHP[88]. The increase in MNGs was likely an indication of effects on the seminiferous cords which can cause anti-androgenic effects and developmental toxicity[206]. The lack of inhibition of production of T was dissimilar to previous studies where TBPH was shown to antagonize the AR *in vitro* and modulate concentrations of T in the H295R assay[99]. In our study regarding toxicities of the mixture of TBPH/TBB in fish, we did not observe hepatotoxic effects. There were no significant differences in the HSI of exposed and control fish, and though that simple measurement is generally indicative of hepatic health, we did not conduct confirmatory histological studies. Further, hepatotoxicity of DEHP is likely caused by activation of the PPARα[207], an activity which has been confirmed for TBMEHP[54]. Differences in hepatotoxicity between our assessment of fish and assessment in rats could be due to direct exposure of TBMEHP vs. exposure to TBPH (and subsequent metabolism to TBMEHP)

or due to the known PPAR agonistic effects of the OPFR components of FM-550. In a separate study, the FM-550 mixture was fed to pregnant rats at 100 or 1000 µg/day until the 8th gestational day[52]. Exposure to FM-550 caused increased concentrations of serum thyroxine in dams and induced metabolic syndrome, advanced female puberty, weight gain, cardiac hypertrophy, and altered exploratory behaviours in offspring. These effects indicated that perinatal exposure might affect normal development and alter adipogenic pathways. Effects on development might be mediated through disruption of endocrine function including modulation of concentrations of hormones or antagonism of hormone receptors, which has been characterized for TBPH, TBB, and their metabolites. Weight gain and metabolic syndrome could be attributed to activation of PPARs, as they are known to affect adipocyte differentiation and deposition and might contribute to phenotypes of metabolic syndrome[208]. TBMEHP, ΣOH-TBBs, and the phosphate ester components of the FM-550 mixture have been shown to activate PPARs in *in vitro* experiments[54, 164, 201]. Though many of these *in vivo* assessments highlighted the endocrine disrupting effects of the FM-550 mixture, it was impossible to determine if effects were attributable to TBPH or TBB. Indeed, effects including cardiotoxicity, hepatoxicity and activation of the PPARs observed in these assessments have previously been attributed to several components of the FM-550 mixture[52, 54, 204]. As such, it was difficult to confirm results from our previous *in vivo* assessments.

There are limited studies which have investigated toxicities of TBCO, and though it is a low production volume chemical, its potential as a replacement for HBCD justified prescriptive assessments of potential toxicity. The initial *in vitro* assessment (Chapter 2) indicated the compound might have endocrine disrupting effects, which was confirmed in a subsequent *in vivo* study (Chapter 4). Further characterization of this potential aquatic hazard has focused on early life stage exposures to fish. Early stages of development are likely the most sensitive to toxic effects and represent an important period for which to assess toxicity of chemicals. Embryos of Japanese medaka were exposed with 10 to 1,000 µg/L TBCO from 2 hr post fertilization until 1-day post-hatch, and both time to- and success of- hatch were impaired[209]. Modulation of the transcriptome and proteome of medaka exposed to 100 µg/L TBCO was investigated to determine potential causes of toxic effects. Medaka exposed to TBCO produced lesser abundances of proteins involved in pathways associated with embryo development and hatching which could explain effects on time to hatch, with lower success. Further analysis of the

transcriptome and proteome revealed potential impairment of visual performance and contraction of cardiac muscle which was confirmed in separate exposures by use of targeted bioassays. Targeted analysis of several genes from the HPGL-axis showed significant up-regulation of VTG II, CHG-H, and the AR, a similar pattern of gene expression observed in our previous study[172]. This study represented the only other investigation of toxicity of TBCO in an *in vivo* system. The results confirmed the modulation of transcripts or proteins across the HPGL-axis and identified disruption of normal development and hatching, which were regulated by the endocrine system. The study confirmed TBCO as a potential endocrine disrupting compound and demonstrated significant impairment in the development of cardiac muscle and vision. Further research is required to characterize toxic effect and mechanisms of action of TBCO.

7.2.3 Epidemiological studies of legacy BFRs and potential for 'read-across'

There is significant structural similarity between the NBFRs, TBPH, TBB and TBCO, and the legacy compounds, PBDEs, DEHP, and HBCDs to appropriately use a qualitative 'read-across' approach to inform potential toxicities of these chemicals. This is a particularly useful method because it can address gaps in data for these compounds as there are currently few investigations regarding their toxicities. The 'read-across' approach has been utilized in the high production volume chemical program under the U.S. EPA, and has been adopted by REACH as a method to screen chemicals. Legacy BFRs including PBDEs and HBCD as well as DEHP are known endocrine disrupting compounds with effects similar to those predicted for TBPH, TBB, and TBCO. For example, rats exposed to Octa-BDEs experienced significant modulation of the thyroid system[210] whereas both TBPH and TBB interacted antagonistically with the thyroid receptor[196]. DEHP is a reproductive toxicant, as it causes lesions in the testis, decreases rates of pregnancy, and increases rates of miscarriage[211]. DEHP's active metabolite, MEHP is also an endocrine disrupting compound, as it has decreased serum concentrations of E2, decreased activity of aromatase and inhibited cellular signaling of FSH[46, 212]. Though these specific effects have not been thoroughly tested for TBPH, TBB, or TBCO, studies have shown that they might have similar toxicities as they interact with the thyroid hormone receptor, modulate concentrations of E2, affect transcript abundances of FSH, and impair fecundity of fish[99, 130, 172, 196]. Epidemiological investigations have highlighted the population level effects of PBDEs and DEHP, but these studies require strong evidence of molecular toxic effects. There have been

relatively few targeted studies of TBPH, TBB, or TBCO to facilitate these large scale assessments. Thus, it might be useful to review epidemiological studies of compounds with similar structures or molecular effects. For example, an epidemiological study of MEHP, a compound with a similar structure to TBB and TBMEHP, showed a negative association between concentrations of MEHP and concentrations of serum T3 in the U.S. population[213]. Modulation of the thyroid system via the TR receptor has been described for TBB and TBMEHP[196]. Numerous studies have linked behavioural alterations in children to elevated concentrations of BFRs in serum, an effect which has been observed in rats and fish exposed to the FM-550 mixture[52, 205]. For example, prenatal exposure to several components of the Penta-BDE mixture were significantly associated with lower IQ and greater hyperactivity of children[171]. The Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) completed a longitudinal birth cohort study of families in California and found that both prenatal and childhood exposure to PBDEs were associated with impaired attention and cognition. Children at age 7 were found to have deficits in verbal IQ and issues with attention and perceptual reasoning[214]. Neurodevelopmental, behavioural, and reproductive effects have been linked to endocrine disruption during early stages of development[215]. Unfortunately, this stage of development corresponds with elevated body burdens of (N)BFRs including PBDEs and TBB relative to adolescents or adults[169, 216, 217]. TBPH, TBB, and TBCO are likely endocrine disrupting compounds but researchers have inchoate knowledge of their effects. From the investigations outlined above, these compounds have similar potential for population level effects as BFRs and DEHP. Yet, physiological or molecular effects of a compound alone do not define its toxicity. The exposure and toxicokinetics of compounds are important to determine potential risk to human and ecological health.

7.3 Exposure to novel brominated flame retardants

7.3.1 Routes of exposure

Humans are exposed to NBFRs via three pathways, dermal absorption via direct contact with products or dust which contain NBFRs, inhalation of NBFRs in the gaseous phase or associated with small particles, and ingestion of food or dust which contain NBFRs. The dermal absorption of TBPH, TBB and HBCD, a surrogate for TBCO, has recently been investigated. TBPH, TBB, and HBCD were applied to a human skin *ex vivo* model and though significant amounts were

recovered in the upper skin layers, relatively little of the compounds penetrated the skin[190]. A significant relationship between penetration of compound and log K_{ow} was observed, where a greater log K_{ow} resulted in less penetration into the skin. This might be important for exposure to OH-TBBs and OH-TBPHs which have lower log K_{ow} than TBPH and TBB. The dermal absorption of TBPH and TBB were also tested in human and rat skin *ex vivo* systems[189]. In this study, human skin absorbed approximately 24% of TBB, while only 0.2% penetrated the tissue, where rat skin was slightly more permeable as 2% of TBB penetrated the tissue. Further, TBB was metabolized to TBBA in these *ex vivo* systems. Approximately 12% of TBPH was absorbed into human skin, while rat skin was significantly more permeable and absorbed 41% of TBPH, though < 0.01% of the compound penetrated the tissue in the rat or human systems. Overall, < 1% of TBPH or TBB was estimated to reach the systemic circulation via dermal absorption, which indicated that the dermis provided a significant barrier for these highly lipophilic compounds[189, 190]. Dermal absorption might account for some exposure to these NBFRs, though it likely does not represent a major pathway of exposure for humans. It is important to note that exposure via dermal absorption is likely attenuated via hand-washing, as such, total exposure of children is likely more affected by this route than adolescents or adults.

Exposure to NBFRs via inhalation is possible via two sources, the gaseous phase and small particles suspended in air. The low vapour pressure and relatively high log K_{ow} of TBPH and TBB indicated that these compounds would likely remain in dust, as they would preferentially migrate from consumer products via mechanical abrasion processes and become incorporated into the dust matrix. Exposure to these NBFRs via inhalation would likely be via small particles as the compounds would preferentially partition to this phase. For example, compounds with similar physical-chemical properties such as Penta-Hepta BDEs, and BDE-209 have been predicted to remain almost exclusively in the particle phase; 60-90% for Penta-Hepta BDEs, and 100% for BDE-209[218]. TBPH and TBB have been detected in respirable (< 4 μm) and inhalable (> 4 μm) fractions of air[166]. These NBFRs were major contributors to ΣFRs (approx. 60%) detected in respirable fractions. Further analysis of the inhalable particulate fraction of air showed a 10-fold increase in concentrations of these NBFRs, which indicated that these compounds were generally associated with larger particles. This association would result in exposure via absorption of NBFRs in the mucus membranes of the respiratory and digestive tracts as these large particles would not likely penetrate deep into the lung[166]. TBCO, OH-TBBs,

and OH-TBPHs have lower log K_{ows} and greater vapour pressures than TBPH and TBB and might have greater fractions which partition to the respirable particle phase. Inhalation of particles contaminated with TBPH, TBB, or TBCO is likely a relevant vector of exposure, though due to the large particle size, a fraction of this exposure would occur in the digestive tract.

Ingestion is a major route of exposure to BFRs, and most studies to date have focused on ingestion via diet or indoor dust[210]. Ingestion via diet was predicted to be a main route of exposure for some BFRs, such as PBDEs[219]. Yet, there is little information regarding concentrations of NBFRs in food and exposure to humans. TBPH and TBB have been detected in food from an e-waste site in Eastern China[220]. The study examined meat from pork and several avian and aquatic species and detected the greatest concentration of TBB in fish (62.2 ng/g, lw), though the main source of exposure to TBPH and TBB was via pork (34% and 54% for adults and children, respectively). Of the NBFRs tested, TBB followed by TBPH had the greatest estimated exposure from diet for both children and adults (18.9 ng/kg/day and 8.03 ng/kg/day, respectively). This was the first account of these NBFRs in food though it might not represent a representative scenario as animals were cultured near highly contaminated sites of e-waste recycling. The authors also noted that no regulatory health based limit values existed for consumption of TBPH or TBB, perhaps reflecting the limited information regarding toxicity and estimates of exposure via food. An investigation of serum concentrations in Swedish mother-toddler pairs, and exposure via diet and dust did not detect TBPH or TBB in any dietary items from Sweden, though the compounds were detected at relatively moderate concentrations in indoor dust[221]. Thus, indoor dust was considered to be the primary route of exposure for TBPH and TBB. In 2012, the European Food Safety Authority conducted a meta-analysis regarding concentrations of legacy and novel BFRs in European diets, but could not find information regarding dietary exposure to TBPH, TBB, or TBCO[222]. Though few studies have attempted to detect TBPH, TBB or TBCO in the diet, due to their detection in wildlife the compounds are likely present in food. Ingestion of dust is likely a major route of exposure of TBPH and TBB and might be more important than dietary routes in children compared to adults.

7.3.2 Toxicokinetics and human exposure

Ingestion of indoor dust contaminated with NBFRs is likely a major route of exposure for humans. As such, it was important to characterize potential metabolism, bioaccessibility and bioaccumulation of these compounds, as the toxicokinetics of ingested chemicals would affect risk to human health. *In vitro* and *in vivo* assessments of metabolism of TBPH and TBB have identified two major metabolites, TBBA and TBMEHP[52, 54, 78]. Two recent studies have investigated the disposition and metabolism of TBPH and TBB in rats and mice[223, 224]. Within 24 hr of oral exposure of radio-labelled TBPH, rats eliminated approximately 75% of the unchanged compound in feces, though bioaccumulation was observed in liver and adrenals following a prolonged (10-day) exposure. Further, a significant increase in dose (100-fold) of orally administered TBPH did not alter metabolism or uptake, though when administered intravenously TBPH was eliminated as a mixture of parent and metabolite compounds. These results indicated that TBPH was poorly absorbed in the GIT and supported results presented in Chapter 6 which tested bioaccessibility of TBPH *in vitro*. These studies supported the findings that the CE-PBET system was an accurate measure of bioavailability of TBPH in rats and mice. Bioaccessibility was predicted at 23% while bioavailability from the GIT was observed at approximately 25%. Following a single oral exposure of radio-labelled TBB, rats eliminated greater than 90% of the compound as metabolites (TBBA, TBBA-sulfate, TBBA-glycine) in urine and feces, while less than 1% of the total dose remained in tissues at 72 hr post exposure[223]. At greater doses (100 to 1000-fold), the main route of elimination shifted from urine to feces which indicated lesser absorption from the gut. At lower exposure doses, TBB was rapidly absorbed from the gut with a half-life of approximately 4 hr and an absorption of approximately 85%. Further, limited bioaccumulation of TBB was observed at all doses, which indicated a low likelihood of bioaccumulation upon chronic exposure[223]. These results indicated that TBB was readily bioavailable in the GIT, though the assessment presented in Chapter 6 indicated a moderate bioaccessibility of 53%. Differences between *in vivo* and *in vitro* results were likely due to methods of delivery of TBB. The purpose of the *in vivo* assessment was to determine toxicokinetic characteristics of TBB, thus rats were exposed by gavage with a liquid formulation, where the *in vitro* system was seeded with TBB adsorbed to dust to determine bioaccessibility from this matrix. To date, no studies have examined the toxicokinetics of OH-TBPHs or OH-TBBs. Though these compounds were minor contaminants in FR technical formulations and

indoor dust, our recent assessments indicated that OH-TBBs would likely be more bioaccessible than TBB (Chapter 6). Further, it would be interesting to identify metabolites of these compounds as current studies indicate that esterase enzymes hydrolyze TBPH and TBB, where the substitution of a Br atom with a hydroxyl substituent might present a favourable site for conjugation.

Few studies have detected TBPH, TBB, or TBCO in humans. TBPH and TBB were detected in 94 and 98% of hair samples, in 86 and 96% of fingernail samples, and 16 and 92% of serum samples, respectively, from participants from Indiana, U.S.[225]. TBPH was also detected in 50% of samples of feces from children in Sweden, though TBB was not detected[226]. Analysis of feces might be a useful tool to monitor exposure to TBPH as a large proportion of TBPH was excreted, unchanged via this matrix[224]. TBPH and TBB were detected in 17 and 57% of serum samples from nursing women in Quebec, Canada[150]. In this study, concentrations of TBPH and TBB in serum were of similar magnitude to components of the Penta-BDE mixture, BDE-153. Further, the fraction of TBB of total FM-550 components in serum was significantly less than in dust, which might be due to the relatively rapid metabolism of TBB compared to TBPH[78]. TBPH was detected in a single sample of serum of residents of Laizhou Bay, China which was within 10 km of a production site of halogenated flame retardants[227]. The concentration of TBPH was approximately double the maximum concentration detected from residents of Quebec, Canada. TBPH and TBB were not detected in serum from paired mothers and toddlers from Uppsala, Sweden, and babies from North Carolina, U.S.[228, 229]. Non-detection of these compounds might have been due to patterns of NBFR use in Europe or the period in which samples were collected (U.S. samples, 2008 to 2010). We were not aware of any study which has attempted to quantify TBCO in samples from humans. TBPH and TBB have been detected in serum from humans and though investigations of their toxicokinetics have shown they are generally not accumulated at great quantities, due to their known endocrine disrupting effects and continual exposure, they might represent risk to human health. Thus further studies to monitor potential increases in serum concentrations, and assess differences across geographic populations and between age-groups, would be beneficial.

Numerous studies have suggested that the ingestion of dust is the most important route of exposure for children, as higher serum concentrations of BFRs have been reported in children

compared to adults[216, 217, 221, 228]. Concentrations of BFRs measured on hand-wipes and indoor dust were the strongest predictors of concentrations of PBDEs in blood in North America[73]. This trend regarding greater body burdens of BFRs in children has also been observed with NBFRs, including TBB. Recently, a metabolite of TBB (TBBA), was quantified in urine and was strongly associated with concentrations of TBB measured in hand-wipes[160]. A subsequent study of paired children and adults showed that TBBA was detected in 70% of children, though only 27% of adults[169]. Further, applications and environmental concentrations of TBPH and TBB are likely increasing which might lead to greater exposures for humans[230]. These are important findings as studies in this thesis (Chapters 5 and 6) have detected some of the greatest concentrations of TBPH and TBB in indoor dust globally and have characterized the novel flame retardants OH-TBPHs and OH-TBBs. Studies from Chapter 6 also demonstrated the potential for increased concentrations of these NBFRs in microenvironments with greater quantities of children's products. Due to the importance of dust as a route of exposure, these relatively high concentrations of NBFRs might indicate that children in ECEs from Saskatoon, Canada are at greater risk of exposure.

7.4 Assessment of risk of TBPH and TBB

This thesis attempted to characterize the endocrine disrupting effects of TBPH, TBB, and TBCO and investigated concentrations of these compounds in early childhood environments. Though the compounds elicited endocrine disrupting effects in several assay systems and were detected at great quantities in indoor dust from ECEs, there has been little analysis of potential risk to children. As such, an abbreviated assessment of risk which focused on children was conducted. This assessment integrated data generated in the thesis with important information from the literature.

The assessment of exposure focused exclusively on contaminated indoor dust from ECEs. Mean concentrations of TBPH, TBB, OH-TBBs, and OH-TBPHs in dust from ECEs reported in Chapters 5 and 6, were used. Exposure of children to NBFRs was assessed by use of equations 7.1 and 7.2

$$ADD_{pot} = (C \times IR \times AF \times EF)/BM \quad (7.1)$$

Where, ADDpot = potential average daily dose (mg/kg-day), C = average contaminant concentration in dust (mg/g), IR = intake rate of dust (mg/day), AF = bioaccessibility factor, EF = exposure factor, and BM = body mass (kg). The exposure factor represented the intermittent basis on which children were exposed to dust from early childhood environments as it accounted for time spent in these environments. The EF was calculated by use of equation 7.2,

$EF = (F \times ED)/AT$..(7.2)

Where, F = frequency of exposure (hrs/day), ED = exposure duration (1-day), and AT = averaging time (24 hrs). Rate of intake from dust was derived from U.S. EPA guidelines of the upper percentile of the general population, aged 1-6 yrs[69], and mass of children was derived from the European Food Safety Authority[231]. Bioaccessibility factors were those derived for TBPH, TBB, OH-TBBs, and OH-TBPHs in dust from ECEs, presented in Chapter 6. Frequency of exposure represented time spent in childcare during a typical work day in Saskatchewan (9 hrs), while exposure duration represented a single day.

The exposure assessment used maximum concentrations in dust from ECEs and maximum values for variables of exposure (ingestion and bioaccessibility) to constitute a highly conservative scenario. Daily exposures to TBPH, TBB, OH-TBBs, and OH-TBPHs were calculated as $5.28\text{x}10^{-4}$, $1.64\text{x}10^{-3}$, $6.56\text{x}10^{-7}$, and $3.75\text{x}10^{-8}$ mg/kg bm, per day, respectively, while exposures assuming 100% bioaccessibility were 2.2910^{-3}, $3.10\text{x}10^{-3}$, $9.38\text{x}10^{-7}$, and $1.30\text{x}10^{-7}$ mg/kg bm, per day. The assessment of exposure to compounds with limited bioaccessibility including TBPH and OH-TBPHs were significantly increased (order of magnitude) when 100% bioaccessibility was assumed. These results demonstrated the significance of integration of accurate estimates of bioaccessibility in assessments of risk. A similar assessment could not be completed for TBCO as there have been no studies which have detected the compound in indoor dust.

Assessment of risk is the process of estimating the magnitude and probability of adverse impacts based on assessments of exposure and effects[232]. Risk characterization is generally an iterative process which used a weight-of-evidence approach to aggregate multiple lines of evidence of toxicity and exposure including concentration of the contaminant, duration of exposure, and severity of response, though an abbreviated process was used in this thesis. To complete the assessment of risk, further information regarding hazards of NBFRs was required.

Most *in vivo* assessments of toxicity of TBPH and TBB exposed test organisms to the technical mixtures FM-550 or BZ-54. As these mixtures contained flame retardants in addition to TBPH and TBB, information from these studies could not be used in this exercise. Further, no reference doses were readily available for TBPH or TBB. Though it represented a pharmacological study of a mixture of TBPH and TBB, toxicity data from Chapter 3 was used in this assessment as it was the only available information derived from an *in vivo* study of these compounds. The lowest observed adverse effect level (LOAEL) which altered fecundity and profiles of transcripts of the HPGL-axis were 1422 mg/kg and 1474 mg/kg for TBPH and TBB, respectively. There have been several recent commentaries regarding the use of NOAELs and LOAELs in risk assessment, and this scenario was an excellent example for such critiques. Due to the abbreviated form of this assessment, a simple hazard quotient (HQ) was used to estimate risk of TBPH and TBB to children. A hazard quotient is a mathematical function which integrates an environmental concentration (EC), in this instance, the calculated concentrations of exposure of NBFRs to children and a toxicological benchmark concentration such as a LOAEL. If the HQ was ≥ 1, then an effect was expected to occur. Hazard quotients were calculated by use of equation 7.3,

$$HQ = EC/LOAEL \qquad (7.3)$$

The HQs of TBPH and TBB were $3.71x10^{-7}$ and $1.12x10^{-6}$, respectively, while HQs where 100% bioaccessibility was assumed were $1.61x10^{--6}$ and $2.10x10^{-6}$. From this simple assessment of risk it was calculated that exposure to NBFRs at concentrations detected in dust from ECEs in Saskatoon, Canada would not elicit endocrine disrupting effects in children. This assessment represented the worst-case-scenario for exposure of children to these NBFRs, but due to limited information regarding toxicities, was likely not representative of actual risk. Thus, the calculated HQ could be referenced as guidance, but could not inform decisions regarding use of these compounds.

7.5 Future work

The research presented in this thesis generated important information regarding the NBFRs, TBPH, TBB, and TBCO. However, this research also provided a foundation for several areas of further study:

1) Endocrine disrupting effects of TBPH and TBB manifested in the integrative measurement of fecundity, and though a global pattern of alterations to transcript abundances was discovered, the mechanisms of effect were not apparent[130, 172]. Targeted research which integrated time point information from gene analysis, quantification of hormones, and gonad/brain histology would generate information required to ascertain mechanisms of action. Further, these mechanisms could be analyzed through the adverse outcome pathway organizational framework to determine organismal or population level effects of these compounds.

2) Novel hydroxylated isomers of TBPH and TBB were characterized in Chapter 5 and their concentrations in ECEs and bioaccessibilities were assessed in Chapter 6. It was noted that the ratio of OH-TBPHs or OH-TBBs to TBPH or TBB in indoor dust was significantly less than in the technical mixtures[163, 164]. This was likely due to differences in physical-chemical characteristics which affected environmental fates and emissions of the compounds. Emissions of OH-isomers could be investigated by use of mass migration test chambers which analyze concentrations of compounds in the gaseous and particle phases[185] and by experiments of mechanical abrasion which use X-ray fluorescence imaging coupled to mass spectrometry[233]. These studies would determine if differences in ratios were due to differences in emission pathways.

3) The OH-isomers have the potential for greater potency of toxic effects, as observed with hydroxylated PBDEs[161]. An initial assessment of OH-TBB indicated that the compound was a strong agonist of the PPARγ[164] and showed that the OH-isomers elicited toxicities not observed from exposures to TBPH or TBB. Differences in potencies of effects might have been due to structural similarities to endogenous compounds including E2, T3, or thyroxine and/or greater binding affinities with receptors or transport proteins[162]. Characterization of toxic effects and potencies of these potential endocrine disrupting compounds is required. OH-TBPHs and OH-TBBs should be initially assessed by a range of *in vitro* assays which test for receptor moderated (ER/AR/TR) and hormone modulating effects. Following positive results, the compounds should be further assessed by use of *in vivo* assay systems.

4) Further research into the toxicity, toxicokinetics and environmental behaviour of TBCO is required. TBCO is structurally similar to HBCD, which has several diastereoisomers that might be differentially metabolized in biota and transformed in the environment. For example,

technical formulations of HBCD consist primarily of the γ-isomer, though α-HBCD is the predominant isomer detected in biota and might have a greater potency of toxic effect[234, 235]. Diastereoisomers of TBCO might behave similarly in the environment and biota, thus it is important to characterize bioaccumulation and metabolism, environmental fate, and potencies of toxic effects across isomers.

5) Following the *in vivo* study presented in Chapter 4, significant maternal transfer of TBCO to eggs of exposed fish was detected. TBCO is an endocrine disruptor and has previously altered expression of genes associated with oocyte meiosis[209]. Thus, exposure during early life stages resulting from maternal transfer might cause deleterious effects on normal development and reproductive function. Due to the short time to sexual maturity of small fish, the assessment of developmental toxicities from maternal exposures to TBCO could be investigated. Analysis of endocrine and reproductive function during early life stages and sexual maturity of fish exposed via maternal deposition of TBCO could expose potential developmental toxicities and persistent effects. Additionally, continuous breeding of subsequent generations with/out exposure to TBCO could function as a method to investigate potential multigenerational or transgenerational effects of TBCO.

7.6 Final thoughts

Since the initiation of this PhD program, TBPH was listed as a high production volume chemical by the U.S. EPA, and both TBPH and TBB have been detected almost ubiquitously in the environment. In fact, recent studies from the IADN have shown that atmospheric concentrations of TBPH and TBB have continued to increase since previous assessments[230]. TBCO has been detected in the environment. TBCO was detected in marine animals in the San Francisco Bay area, and in sediments and fish in the North Sea, and is the focus of investigation of research groups in Illinois, U.S.[236]. Additionally, TBPH, TBB and their metabolites have been detected in serum and other biological matrices of humans. It has also been highlighted that brominated flame retardants, at the concentrations applied to consumer products and scenarios in which PUF would ignite, might not be effective at reducing flammability. Further, incidences of fires in the U.S. have decreased by 22% from 2004 and incidence of death has decreased by 21%[237]. These insights into the effectiveness of BFRs and incidences of fires demand the reassessment of the use of these compounds – is the use of brominated flame retardants beneficial or harmful to

humans and the environment? The recent amendments to TB 117 have highlighted these questions as changes to this standard have the potential to significantly alter use of these compounds and subsequent exposure to BFRs and NBFRs in North America.

Work presented in this thesis was some of the first to investigate potential toxicities of these novel brominated flame retardants. These investigations have helped to confirm their endocrine disrupting effects, identified novel compounds which had not been previously characterized, and created information regarding factors which affected concentrations in dust from ECEs and bioavailability. This work will help to inform more accurate assessments of risk and regulations regarding these compounds.

REFERENCES

1. Alaee, M.; Barcelo, D.; Birnbaum, L.; de Boer, J.; Covaci, A.; Dirtu, A. C.; Dominguez, A. A.; Eljarrat, E.; Feo, M. L.; Guerra, P.; Hearn, L.; Herzke, D.; Kierkegaard, A.; Law, R. J.; Lepom, P.; Mueller, J. F.; Ricklund, N.; Roosens, L.; Sellström, U.; Sjodin, A.; Toms, L.-M. L.; Voorspoels, S.; Wikoff, D. S.; De Wit, C. A., *Brominated flame retardants*. Springer-Verlag: **2011**.

2. Alaee, M., An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. *Environment International* **2003,** *29*, (6), 683-689.

3. Andersson, P. L.; Oberg, K.; Orn, U., Chemical characterization of brominated flame retardants and identification of structurally representative compounds. *Environmental Toxicology and Chemistry* **2006,** *25*, (5), 1275 - 1282.

4. de Wit, C., An overview of brominated flame retardants in the environment. *Chemosphere* **2002**, (46), 583-624.

5. Covaci, A.; Gerecke, A. C.; Law, R. J.; Voorspoels, S.; Kohler, M.; Heeb, N. V.; Leslie, H.; Allchin, C. R.; de Boer, J., Hexabromocyclododecanes (HBCDs) in the Environment and Humans: A Review. *Environmental Science & Technology* **2006,** *40*, (12), 3679-3688.

6. Marvin, C. H.; Tomy, G. T.; Armitage, J. M.; Arnot, J. A.; McCarty, L.; Covaci, A.; Palace, V., Hexabromocyclododecane: Current understanding of chemistry, environmental fate and toxicology and implications for global management. *Environmental Science & Technology* **2011,** *45*, (20), 8613-8623.

7. Wang, Y.; Jiang, G.; Lam, P. K. S.; Li, A., Polybrominated diphenyl ether in the East Asian environment: A critical review. *Environment International* **2007,** *33*, (7), 963-973.

8. Hites, R. A., Polybrominated diphenyl ethers in the environment and in people: A meta-analysis of concentrations. *Environmental Science & Technology* **2004,** *38*, (4), 945-956.

9. Möller, A.; Xie, Z. Y.; Cai, M. H.; Zhong, G. C.; Huang, P.; Cai, M. G.; Sturm, R.; He, J. F.; Ebinghaus, R., Polybrominated diphenyl ethers vs alternate brominated flame retardants and dechloranes from East Asia to the Arctic. *Environmental Science & Technology* **2011,** *45*, (16), 6793-6799.

10. Hale, R., Polybrominated diphenyl ether flame retardants in the North American environment. *Environment International* **2003,** *29*, (6), 771-779.

11. Hyotylainen, T.; Hartonen, K., Determination of brominated flame retardants in environmental samples. *Trends in Analytical Chemistry* **2002,** *21*, (1), 13-29.

12. Salamova, A.; Hites, R. A., Discontinued and alternative brominated rlame retardants in the atmosphere and precipitation from the Great Lakes Basin. *Environmental Science & Technology* **2011,** *45*, (20), 8698-8706.

13. Gouin, T.; Thomas, G.; Chaemfa, C.; Harner, T.; Mackay, D.; Jones, K., Concentrations of decabromodiphenyl ether in air from Southern Ontario: Implications for particle-bound transport. *Chemosphere* **2006,** *64*, (2), 256-261.

14. Hoh, E.; Hites, R. A., Brominated flame retardants in the atmosphere of the East-Central United States. *Environmental Science & Technology* **2005,** *39*, 7794-7802.

15. Harrad, S.; Abdallah, M. A. E.; Rose, N. L.; Turner, S. D.; Davidson, T. A., Current-use brominated flame retardants in water, sediment, and fish from English Lakes. *Environmental Science & Technology* **2009,** *43*, (24), 9077-9083.

16. Remberger, M.; Sternbeck, J.; Palm, A.; Kaj, L.; Stromberg, K.; Brorstrom-Lunden, E., The environmental occurrence of hexabromocyclododecane in Sweden. *Chemosphere* **2004,** *54*, 9-21.

17. Sellström, U.; Kierkegaard, A.; De Wit, C. A.; Jansson, B., Polybrominated diphenyl ethers and hexabromocyclododecane in sediment and fish from a Swedish river. *Environmental Science & Technology* **1998,** *17*, 1065-1072.

18. Sellström, U.; Kierkegaard, A.; Alsberg, T.; Jonsson, P.; Wahlberg, C.; De Wit, C. A., Brominated flame retardants in sediments from European estuaries, the Baltic Sea and in sewage sludge. *Organohalogen Compd.* **1999,** *40*, (383-386).

19. Law, R.; Allchin, C.; Deboer, J.; Covaci, A.; Herzke, D.; Lepom, P.; Morris, S.; Tronczynski, J.; Dewit, C., Levels and trends of brominated flame retardants in the European environment. *Chemosphere* **2006,** *64*, (2), 187-208.

20. Vorkamp, K.; Bester, K.; Riget, F. F., Species-specific time trends and enantiomer fractions of hexabromocyclododecane (HBCD) in biota from East Greenland. *Environmental Science & Technology* **2012,** *46*, (19), 10549-10555.

21. de Wit, C.; Alaee, M.; Muir, D., Levels and trends of brominated flame retardants in the Arctic. *Chemosphere* **2006,** *64*, (2), 209-233.

22. Johnson-Restrepo, B.; Adams, D. H.; Kanna, K., Tetrabromobisphenol A (TBBPA) and hexabromocyclododecanes (HBCDs) in tissues of humans, dolphins, and sharks from the United States. *Chemosphere* **2008,** *70*, (11), 1935-1944.

23. Thomsen, C.; Froshaug, M.; Leknes, H.; Becher, G., Brominated flame retardants in breast milk from Norway. *Organohalogen Compd.* **2003,** *64*, 33-36.

24. Weiss, J.; Meijer, L.; Sauer, P.; Linderholm, L.; Athanasiadis, I.; Bergman, Å., PBDE and HBCD levels in blood from Dutch mothers and infants: Analysis of a Dutch Groningen infant cohort. *Organohalogen Compd.* **2004,** *66*, 2677-2682.

25. McDonald, T. A., A perspective on the potential health risks of PBDEs. *Chemosphere* **2002,** *46*, 745-755.

26. La Guardia, M. J.; Hale, R. C.; Harvey, E.; Mainor, T. M.; Ciparis, S., *In situ* acccumulation of HBCD, PBDEs, and several alternative flame-retardants in the bivalve (*Corbicula fluminea*) and gastropod (*Elimia proxima*). *Environmental Science & Technology* **2012,** *46*, (11), 5798-5805.

27. Covaci, A.; Harrad, S.; Abdallah, M. A. E.; Ali, N.; Law, R. J.; Herzke, D.; de Wit, C. A., Novel brominated flame retardants: A review of their analysis, environmental fate and behaviour. *Environment International* **2011,** *37*, (2), 532-556.

28. Wu, J.-P.; Guan, Y.-T.; Zhang, Y.; Luo, X.-J.; Zhi, H.; Chen, S.-J.; Mai, B.-X., Several current-use, non-PBDE brominated flame retardants are highly bioaccumulative: Evidence from field determined bioaccumulation factors. *Environment International* **2011,** *37*, (1), 210-215.

29. Bearr, J. S.; Stapleton, H. M.; Mitchelmore, C. L., Accumulation and DNA damage in fathead minnows (*Pimephales promelas*) exposed to 2 brominated flame-retardant mixtures, Firemaster® 550 and Firemaster® BZ-54. *Environmental Toxicology and Chemistry* **2010,** *29*, (3), 722-729.

30. Ma, Y. N.; Venier, M.; Hites, R. A., 2-ethylhexyl tetrabromobenzoate and bis(2-ethylhexyl) tetrabromophthalate flame retardants in the Great Lakes atmosphere. *Environmental Science & Technology* **2012,** *46*, (1), 204-208.

31. de Wit, C. A.; Kierkegaard, A.; Ricklund, N.; Sellström, U., Emerging brominated flame retardants in the environment. *Environmental Chemistry* **2011,** *16*, (241-286).

32. Riddell, N.; Arsenault, G.; Klein, J.; Lough, A.; Marvin, C.; McAlees, A.; McCrindle, R.; Macinnis, G.; Sverko, E.; Tittlemier, S.; Tomy, G. T., Structural characterization and thermal stabilities of the isomers of the brominated flame retardant 1,2,5,6-tetrabromocyclooctane (TBCO). *Chemosphere* **2009,** *74*, (11), 1538-1543.

33. Morose, G., An overview of alternatives to tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD). In Production, L. C. F. S., Ed. University of Massachusetts Lowell, **2006**.

34. Möller, A.; Xie, Z.; Sturm, R.; Ebinghaus, R., Polybrominated diphenyl ethers (PBDEs) and alternative brominated flame retardants in air and seawater of the European Arctic. *Environmental Pollution* **2011,** *159*, (6), 1577-1583.

35. Gentes, M.-L.; Letcher, R. J.; Caron-Beaudoin, É.; Verreault, J., Novel flame retardants in urban-feeding Ring-Billed Gulls from the St. Lawrence River, Canada. *Environmental Science & Technology* **2012,** *46*, (17), 9735-9744.

36. Harju, M.; Heimstad, E. S.; Herzke, D.; Sandanger, T.; Posner, S.; Wania, F., Emerging "new" brominated flame retardants in flame retarded products and the environment. In Authority, N. P. C., Ed. Oslo, Norway, **2009**.

37. Lee, S.; Sverko, E.; Harner, T.; Schachtschneider, J. A.; Zaruk, D.; DeJong, M.; Barresi, E. In *"New" flame retardants in the global atmosphere under the GAPS network*, BFR2010 Workshop, Kyoto, Japan, 7-9 April, 2010, 2010; Kyoto, Japan, **2010**.

38. Stapleton, H. M.; Allen, J.; Kelly, S.; Konstantinov, A.; Klosterhaus, S.; Watkins, D. J.; McClean, M. D.; Webster, T. F., Alternate and new brominated flame retardants detected in U.S. house dust. *Environmental Science & Technology* **2008,** *42*, 6910-6916.

39. Ali, N.; Dirtu, A. C.; Eede, N. V. d.; Goosey, E.; Harrad, S.; Neels, H.; ’t Mannetje, A.; Coakley, J.; Douwes, J.; Covaci, A., Occurrence of alternative flame retardants in indoor dust from New Zealand: Indoor sources and human exposure assessment. *Chemosphere* **2012,** *88*, (11), 1276-1282.

40. Stapleton, H. M.; Klosterhaus, S.; Keller, A.; Ferguson, P. L.; van Bergen, S.; Cooper, E.; Webster, T. F.; Blum, A., Identification of flame retardants in polyurethane foam collected from baby products. *Environmental Science & Technology* **2011,** *45*, (12), 5323-5331.

41. Stapleton, H. M.; Klosterhaus, S.; Eagle, S.; Fuh, J.; Meeker, J.; Blum, A.; Webster, T. F., Detection of organophosphate flame retardants in furniture foam and U.S. house dust. *Environmental Science & Technology* **2009,** *43*, 7490-7495.

42. Lam, J. C. W.; Lau, R.; Murphy, M.; Lam, P. K. S., Hexabromocyclododecanes (HBCDs) and polybrominated diphenyl ethers (PBDEs) and detection of two novel flame retardants in marine mammals from Hong Kong, South China. *Environmental Science & Technology* **2009,** *43*, 6944-6949.

43. Davis, E.; Stapleton, H. M., Photodegradation pathways of nonabrominated diphenyl ethers, 2-ethylhexyltetrabromobenzoate and di(2-ethylhexyl)tetrabromophthalate: Identifying potential markers of photodegradation. *Environmental Science & Technology* **2009,** *43*, (15), 5739-5746.

44. Mankidy, R.; Wiseman, S.; Hong, M.; Giesy, P. J., Biological impact of phthalates. *Toxicology Letters* **2013,** *217*, (1), 50-58.

45. Rozati, R.; Reddy, P. P.; Reddanna, P.; Mujtaba, R., Role of environmental estrogens in the deterioration of male factor fertility. *Fertility and Sterility* **2002,** *78*, (6), 1187-1194.

46. Davis, B. J.; Maronpot, R. R.; Heindel, J. J., Di-(2-ethylhexyl) phthalate suppresses estradiol and ovulation in cycling rats. *Toxicology and Applied Pharmacology* **1994,** *128*, 216-223.

47. Kruger, T.; Long, M.; Bonefeld-Jorgensen, E., Plastic componenets affect the activation of the aryl hydrocarbon and the androgen receptor. *Toxicology* **2008,** *246*, 112-123.

48. Maradonna, F.; Evangelisti, M.; Gioacchini, G.; Migliarini, B.; Olivotto, I.; Carnevali, O., Assay of VTG, ERs and PPARs as endpoints for the rapid *in vitro* screening of the harmful effect of di-(2-ethyhexyl)-phthalate (DEHP) and phthalic acid (PA) in zebrafish primary hepatocyte cultures. *Toxicology in Vitro* **2013,** *27*, 84-91.

49. Wang, X.; Yang, Y.; Zhang, L.; Ma, Y.; Han, J.; Yang, L.; Zhou, B., Endocrine disruption by di-(2-ethylhexyl)-phthalate (DEHP) in Chinese rare minnow (*Gobiocypris rarus*). *Environmental Toxicology and Chemistry* **2013**.

50. Kim, E.-J.; Kim, J.-W.; Lee, S.-K., Inhibition of oocyte development in Japanese medaka (*Oryzias latipes*) exposed to di-2-ethylhexyl phthalate. *Environment International* **2002,** *28*, 359-365.

51. Carnevali, O.; Tosti, L.; Speciale, C.; Peng, C.; Zhu, Y.; Maradonna, F., DEHP impairs zebrafish reproduction by affecting critical factors in oogenesis. *PLoS ONE* **2010,** *5*, (4), 1-7.

52. Patisaul, H. B.; Roberts, S. C.; Mabrey, N.; McCaffrey, K. A.; Gear, R. B.; Braun, J.; Belcher, S. M.; Stapleton, H. M., Accumulation and endocrine disrupting effects of the flame retardant mixture Firemaster®550 in rats: An exploratory assessment. *Journal of Biochemical and Molecular Toxicology* **2013,** *27*, (2), 124-136.

53. Ezechiáš, M.; Svobodová, K.; Cajthaml, T., Hormonal activities of new brominated flame retardants. *Chemosphere* **2012,** *87*, (7), 820-824.

54. Springer, C.; Dere, E.; Hall, S. J.; McDonnell, E. V.; Roberts, S. C.; Butt, C.; Stapleton, H. M.; Watkins, D. J.; McClean, M. D.; Webster, T. F.; Schlezinger, J. J.; Boekelheide, K., Rodent thyroid, liver, and fetal testis toxicity of the monoester metabolite of bis-(2-ethylhexyl) tetrabromophthalate (TBPH), a novel brominated flame retardant present in indoor dust. *Environmental Health Perspectives* **2012**.

55. Fisk, P. R.; Girling, A. E.; Wildley, R. J., Prioritisation of flame retardants for environmental risk assessment In Agency, E., Ed. Wallingford, UK, **2008**.

56. Dodson, R. E.; Perovich, L. J.; Covaci, A.; Van den Eede, N.; Ionas, A. C.; Dirtu, A. C.; Brody, J. G.; Rudel, R. A., After the PBDE phase-out: A broad suite of flame retardants in repeat house dust samples from California. *Environmental Science & Technology* **2012,** *46*, (24), 13056-13066.

57. Lorber, M., Exposure of Americans to Polybrominated Diphenyl Ethers. *Journal of Exposure Science and Environmental Epidemiology* **2008,** *18*, 2-19.

58. Ali, N.; Harrad, S.; Goosey, E.; Neels, H.; Covaci, A., ''Novel'' brominated flame retardants in Belgian and UK indoor dust: Implications for human exposure. *Chemosphere* **2011,** *83*, 1360-1365.

59. Ali, N.; van den Eede, N.; Dirtu, A. C.; Neels, H.; Covaci, A., Assessment of human exposure to indoor organic contaminants via dust ingestion in Pakistan. *Indoor Air* **2011,** *22*, (3), 200-211.

60. Harrad, S.; De Wit, C. A.; Abdallah, M. A. E.; Bergh, C.; Bjorklund, J.; Covaci, A.; Darnerud, P. O.; De Boer, J.; Diamond, M.; Huber, S.; Leonards, P.; Mandalakis, M.; Ostman, C.; Huag, L. S.; Thomsen, C.; Webster, T. F., Indoor contamination with hexabromocyclododecanes, polybrominated diphenyl ethers, and perfluoroalkyl compounds: An important exposure pathway for people? *Environmental Science & Technology* **2010,** *44*, (9), 3221-3231.

61. Jones-Otazo, H.; Clarke, J. P.; Diamond, M.; Archbold, J.; Ferguson, G.; Harner, T.; Richardson, G. M.; Ryan, J. J.; Wilford, B., Is house dust the missing exposure pathway for PBDEs? An analysis of the urban fate and human exposure to PBDEs. *Environmental Science & Technology* **2005,** *39*, (14), 5121-5130.

62. Frederiksen, M.; Vorkamp, K.; Thomsen, M.; Knudsen, L., Human internal and external exposure to PBDEs – A review of levels and sources. *International Journal of Hygiene and Environmental Health* **2009,** *212*, (2), 109-134.

63. Zota, A. R.; Rudel, R. A.; Morello-Frosch, R. A.; Brody, J. G., Elevated house dust and serum concentrations of PBDEs in California: Unintended consequences of furniture flammability standards? *Environmental Science & Technology* **2008,** *42*, (21), 8158-8164.

64. Karlsson, M.; Julander, A.; van Bravel, B.; Hardell, L., Levels of brominated flame retardants in blood in relation to levels in household air and dust. *Environment International* **2007,** *33*, (1), 62-69.

65. Roosens, L.; Abdallah, M. A. E.; Harrad, S.; Neels, H.; Covaci, A., Exposure to hexabromocyclododecanes via dust ingestion, but not diet, correlates with concentrations in human serum. *Environmental Health Perspectives* **2009,** *117*, 1707-1712.

66. Frederiksen, M.; Thomsen, C.; Froshaug, M.; Vorkamp, K.; Thomsen, M.; Becher, G.; Knudsen, L., Polybrominated diphenyl ethers in paired samples of maternal and umbilical cord blood plasma and associations with house dust in a Danish cohort. *International Journal of Hygiene and Environmental Health* **2010,** *213*, (4), 233-242.

67. Wu, N.; Herrmann, T.; Paepke, O.; Tickner, J.; Hale, R. C.; Harvey, E.; La Guardia, M. J.; McClean, M. D.; Webster, T. F., Human exposure to PBDEs: Associations of PBDE body

burdens with food consumption and house dust concentrations. *Environmental Science & Technology* **2007,** *41*, (5), 1584-1589.

68. Frederiksen, M.; vorkamp, K.; Thomsen, M.; Knudsen, L., Correlations of PBDEs in house dust at different sampling time and association of BDE-47 in house dust and human placental tissue. *Organohalogen Compd.* **2009,** *71*, 2172-2176.

69. U.S.EPA, Exposure factors handbook 2011 edition (Final). In EPA, U. S., Ed. Washington, DC, **2011**.

70. Harrad, S.; Goosey, E.; Desborough, J.; Abdallah, M. A. E.; Roosens, L.; Covaci, A., Dust from U.K. primary school classrooms and daycare centers: The significance of dust as a pathway of exposure of young U.K. children to brominated flame retardants and polychlorinated biphenyls. *Environmental Science & Technology* **2010,** *44*, (11), 4198-4202.

71. Rose, M.; Bennett, D.; Bergman, Å.; Fangstrom, B.; Hertz-Picciotto, I., PBDEs in 2–5 year-old children from California and associations with diet and indoor environment. *Environmental Science & Technology* **2010,** *44*, (7), 2648-2653.

72. Roze, E.; Meijer, L.; Bakker, A.; Van Braekel, K.; Sauer, P.; Bos, A., Prenatal exposure to organohalogens, including brominated flame retardants, influences motor, cognitive, and behavioral performance at school age. *Environmental Health Perspectives* **2009,** *117*, (12), 1953-1958.

73. Stapleton, H. M.; Eagle, S.; Sjodin, A.; Webster, T. F., Serum PBDEs in a North Carolina toddler cohort: Associations with handwipes, house dust, and socioeconomic variables. *Environmental Health Perspectives* **2012,** *120*, (7), 1049.

74. Toms, L.-M. L.; Harden, F.; Paepke, O.; Hobson, P.; Ryan, J. J.; Mueller, J. F., Higher accumulation of polybrominated diphenyl ethers in infants than in adults. *Environmental Science & Technology* **2008,** *42*, (19), 7510-7515.

75. Bradman, A.; Castorina, R.; Gaspar, F.; Nishioka, M.; Colon, M.; Weathers, W.; Egeghy, P.; Maddalena, R.; Williams, J.; Jenkins, P.; McKone, T., Flame retardant exposure in California early childhood education environments. *Chemosphere* **2014,** *116*, 61-66.

76. Tulve, N.; Jones, P.; Nishioka, M., Pesticide measurements from the first National Environmental Health Survey of Child Care Centers using a multi-residue GC/MS analysis method. *Environmental Science & Technology* **2006,** *40*, 6269-6274.

77. Guo, W.; Park, J.; Wang, Y.; Gardner, S.; Baek, C.; Petreas, M.; Hooper, K., High polybrominated diphenyl ether levels in California house cats: House dust a primary source? *Environmental Toxicology and Chemistry* **2012,** *31*, (2), 301-306.

78. Roberts, S. C.; Macaulay, L. J.; Stapleton, H. M., *In vitro* metabolism of the brominated flame retardants 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB) and bis(2-ethylhexyl) 2,3,4,5-

tetrabromophthalate (TBPH) in human and rat tissues. *Chemical Research in Toxicology* **2012,** *25*, (7), 1435-1441.

79. Inada, H.; Chihara, K.; Yamashita, A.; Miyawaki, I.; Fukuda, C.; Tateishi, Y.; Kunimatsu, T.; Kimura, J.; Funabashi, H.; Miyano, T., Evaluation of ovarian toxicity of mono-(2-ethylhexyl) phthalate (MEHP) using cultured rat ovarian follicles. *The Journal of Toxicological Sciences* **2012,** *37*, (3), 483-490.

80. Kessler, W.; Numtip, W.; Volkel, W.; Seckin, E.; Csanady, G. G.; Putz, C.; Klein, D.; Fromme, H.; Filser, J. G., Kinetics of di(2-ethylhexyl) phthalate (DEHP) and mono(2-ethylhexyl) phthalate in blood and of DEHP metabolites in urine of male volunteers after single ingestion of ring-deuterated DEHP. *Toxicology and Applied Pharmacology* **2012,** *264*, (2), 284-291.

81. Routledge, E.; Sumpter, J., Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental Toxicology and Chemistry* **1996,** *15*, (3), 241-248.

82. El-Fouly, R.; Giesy, P. J.; Denison, M., Production of a novel recombinant cell line for use as a bioassay system for detection of 2,3,7,8-tetrachlorodibenzi-p-dioxin-like chemicals. *Environmental Toxicology and Chemistry* **1994,** *13*, 1581-1588.

83. Garrison, P. M.; Tullis, K.; Aarts, J. M. M. J. G.; Brouwer, A.; Giesy, P. J.; Denison, M., Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals. *Fundamental and Applied Toxicology* **1996,** *30*, 194-203.

84. Hilscherova, K.; Machala, M.; Kanna, K.; Blankenship, A. L.; Giesy, P. J., Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. *Environ. Sci. Pollution. Res* **2000**, (7), 159-171.

85. Horii, Y.; Khim, J. S.; Higley, E.; Giesy, P. J.; Ohura, T.; Kannan, K., Relative potencies of individual chlorinated and brominated polycyclic aromatic hydrocarbons for induction of aryl hydrocarbon receptor-mediated responses. *Environmental Science & Technology* **2009,** *43*, (6), 2159-2165.

86. OECD, *Test No. 456: H295R steroidogenesis assay*. OECD Publishing: **2011**.

87. Hecker, M.; Giesy, P. J., Novel trends in endocrine disruptor testing: The H95R Steroidogenesis assay for identification of inducers and inhibitors of hormone production. *Anal bioanal Chem* **2008**, (390), 287-291.

88. Foster, P. M. D.; Mylchreest, E.; Gaido, K. W.; Sar, M., Effects of phthalate esters on the developing reproductive tract of male rats. *Human Reproduction Update* **2001,** *7*, (3), 231-235.

89. Lyche, J. L.; Gutleb, A. C.; Bergman, Å.; Eriksen, G. S.; Murk, A. J.; Ropstad, E.; Saunders, M.; Skaare, J. U., Reproductive and developmental toxicity of phthalates. *Journal of Toxicology and Environmental Health, Part B* **2009,** *12*, (4), 225-249.

90. Piché, C. D.; Sauvageau, D.; Vanlian, M.; Erythropel, H. C.; Robaire, B.; Leask, R. L., Effects of di-(2-ethylhexyl) phthalate and four of its metabolites on steroidogenesis in MA-10 cells. *Ecotoxicology and Environmental Safety* **2012,** *79*, 108-115.

91. David, R., Proposed mode of action for *in utero* effects of some phthalate esters on the developing male reproductive tract. *Toxicologic Pathology* **2006,** *34*, 209-219.

92. Parks, L.; Ostby, J.; Lambright, C.; Abbott, B.; Klinefelter, G.; Barlow, N.; Gray, J. L. E., The plasticizer diethyhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicological Sciences* **2000,** *58*, 339-349.

93. Corton, C.; Lapinskas, P., Peroxisome proliferator-activated receptors: Mediators of phthalate ester-induced effects in the male reproductive tract. *Toxicological Sciences* **2005,** *83*, 4-17.

94. Gazouli, M.; Yao, Z.-X.; Boujrad, N.; Corton, C.; Culty, M.; Papadopoulos, V., Effects of the peroxisome proliferators on leydig cell peripheral-type benzodiazepine receptor gene expression, hormone-stimulated cholesterol transport, and steroidogenesis: Role of the peroxisome proliferator-activated receptor α. *Endocrinology* **2002,** *143*, 2571-2583.

95. Rusyn, I.; Peters, J.; Cunningham, M., Modes of action and species-specific effects of di-(2-ethylhexyl)phthalate in the liver. *Critical Reviews in Toxicology* **2006,** *36*, 459-479.

96. Boelsterli, U., *Mechanistic toxicology: The molecular basis of how chemicals disrupt biological targets, second ed.* Informa Healthcare: **2007**.

97. Hokanson, R.; Hanneman, W.; Hennessey, M.; Donnelly, K.; McDonald, T.; Chowdhary, R.; Busbee, D., DEHP, bis(2)-ethyhexyl phthalate, alters gene expression in human cells: Possible correlation with initiation of fetal developmental abnormalities *Human & Experimental Toxicology* **2006,** *25*, 687-695.

98. Voss, C.; Zerban, H.; Bannasch, P.; Berger, M. R., Lifelong exposure to di-(2-ethylhexyl)-phthalate induces tumors in liver and testes of Sprague-Dawley rats. *Toxicology* **2005,** *206*, 359-371.

99. Saunders, D. M. V.; Higley, E. B.; Hecker, M.; Mankidy, R.; Giesy, J. P., *In vitro* endocrine disruption and TCDD-like effects of three novel brominated flame retardants: TBPH, TBB, & TBCO. *Toxicology Letters* **2013,** *223*, (2), 252-259.

100. Mankidy, R.; Ranjan, B.; Honaramooz, A.; Giesy, J. P., Effects of novel brominated flame retardants on steroidogenesis in primary porcine testicular cells. *Toxicology Letters* **2014,** *224*, (1), 141-146.

101. OECD, *Test No. 229: Fish short term reproductive assay*. OECD Publishing: **2012**.

102. Zhang, X.; Hecker, M.; Park, J.-W.; Tompsett, A. R.; Newsted, J.; Nakayama, K.; Jones, P. D.; Au, D.; Kong, R.; Wu, R. S. S.; Giesy, P. J., Real-time PCR array to study effects of chemicals on the hypothalamic-pituitary-gonadal axis of the Japanese medaka. *aquatic Toxicology* **2008,** *88*, 173-182.

103. Zhang, X.; Hecker, M.; Jones, P.; Newsted, J.; Au, D.; Kong, R.; Wu, R. S. S.; Giesy, P. J., Response of the medaka HPG axis PCR array and reproduction to prochloraz and ketoconazole. *Environmental Science & Technology* **2008,** *42*, 6762-6769.

104. Zhang, X.; Hecker, M.; Park, J.-W.; Tompsett, A. R.; Jones, P. D.; Newsted, J.; Au, D.; Kong, R.; Wu, R. S. S.; Giesy, P. J., Time-dependent transcriptional profiles of genes of the hypothalamic-pituitary-gonadal axis in medaka (*Oryzias latipes*) exposed to fadrozole and 17β-trenbolone. *Environmental Toxicology and Chemistry* **2008,** *27*, (12), 2504-2511.

105. Wan, Y.; Liu, F.; Wiseman, S.; Zhang, X.; Chang, H.; Hecker, M.; Jones, P.; Lam, M. H. W.; Giesy, P. J., Interconversion of hydroxylated and methoxylated polybrominated diphenyl ethers in Japanese medaka. *Environmental Science & Technology* **2012,** *44*, (22), 8729-8735.

106. Kettle, A., Use of accelerated solvent extraction with in-cell cleanup to eliminate sample cleanup during sample preparation. In Thermo Fisher Scientific: **2013**.

107. Villeneuve, D. L.; Larkin, P.; Knoebl, I.; Miracle, A. L.; Kahl, M. D.; Jensen, K. M.; Makynen, E. A.; Durhan, E. J.; Carter, B. J.; Denslow, N. D.; Ankley, G. T., A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. *Environmental Science & Technology* **2007,** *41*, 321-330.

108. Doering, J.; Wiseman, S.; Beitel, S.; Giesy, P. J.; Hecker, M., Identification and expression of aryl hydrocarbon receptors (AhR1 and AhR2) provide insight in an evolutionary context regarding sensitivity of white sturgeon (Acipenser transmontanus) to dioxin-like compounds. *Aquatic Toxicology* **2014,** *150*, 27-35.

109. Zhang, Z.; Hu, J., Development and validation of endogenous reference genes for expression profiling of medaka (*Oryzias latipes*) exposed to endocrine eisrupting chemicals by quantitative real-time RT-PCR. *Toxicological Sciences* **2007,** *95*, (2), 356-368.

110. Papoulias, D. M.; Tillitt, D. E.; Talykina, M. G.; Whyte, J. J.; Richter, C. A., Atrazine reduces reproduction in Japanese medaka (*Oryzias latipes*). *Aquatic Toxicology* **2014,** *154*, 230-239.

111. He, Y.; Wiseman, S. B.; Wang, N.; Perez-Estrada, L. A.; El-Din, M. G.; Martin, J. W.; Giesy, J. P., Transcriptional responses of the brain–gonad–liver axis of fathead minnows exposed to untreated and ozone-treated oil sands process-affected water. *Environmental Science & Technology* **2012,** *46*, (17), 9701-9708.

112. Kusakabe, M., Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. *Journal of Endocrinology* **2006,** *189*, (3), 541-554.

113. Ye, T.; Kang, M.; Huang, Q.; Fang, C.; Chen, Y.; Shen, H.; Dong, S., Exposure to DEHP and MEHP from hatching to adulthood causes reproductive dysfunction and endocrine disruption in marine medaka (*Oryzias melastigma*). *Aquatic Toxicology* **2014,** *146*, 115-126.

114. DiMuccio, T.; Mukai, S.; Clelland, E.; Kohli, G.; Cuartero, M.; Wu, T.; Peng, C., Cloning of a second form of activin-betaA cDNA and regulation of activin-betaA subunits and activin type II receptor mRNA expression by gonadotropin in the zebrafish ovary. *General and Comparative Endocrinology* **2005,** *143*, (3), 287-299.

115. Yost, E. E.; Lee Pow, C.; Hawkins, M. B.; Kullman, S. W., Bridging the gap from screening assays to estrogenic effects in fish: Potential roles of multiple estrogen receptor subtypes. *Environmental Science & Technology* **2014,** *48*, (9), 5211-5219.

116. Liu, X.; Ji, K.; Jo, A.; Moon, H.-B.; Choi, K., Effects of TDCPP or TPP on gene transcriptions and hormones of HPG axis, and their consequences on reproduction in adult zebrafish (*Danio rerio*). *Aquatic Toxicology* **2013,** *134-135*, 104-111.

117. Lei, B.; Wen, Y.; Wang, X.; Zha, J.; Li, W.; Wang, Z.; Sun, Y.; Kang, J.; Wang, Y., Effects of estrone on the early life stages and expression of vitellogenin and estrogen receptor genes of Japanese medaka (*Oryzias latipes*). *Chemosphere* **2013,** *93*, (6), 1104-1110.

118. Sabo-Attwood, T.; Kroll, K.; Denslow, N. D., Differential expression of largemouth bass (*Micropterus salmoides*) estrogen receptor isotypes alpha, beta, and gamma by estradiol. *Molecular and Cellular Endocrinology* **2004,** *218*, (1-2), 107-118.

119. Yamaguchi, A.; Ishibashi, H.; Kohra, S.; Arizono, K.; Tominaga, N., Short-term effects of endocrine-disrupting chemicals on the expression of estrogen-responsive genes in male medaka (*Oryzias latipes*). *Aquatic Toxicology* **2005,** *72*, (3), 239-249.

120. Marlatt, V. L.; Martyniuk, C. J.; Zhang, D.; Xiong, H.; Watt, J.; Xia, X.; Moon, T.; Trudeau, V. L., Auto-regulation of estrogen receptor subtypes and gene expression profiling of 17β-estradiol action in the neuroendocrine axis of male goldfish. *Molecular and Cellular Endocrinology* **2008,** *283*, (1-2), 38-48.

121. Arukwe, A.; Kullman, S. W.; Hinton, D. E., Differential biomarker gene and protein expressions in nonylphenol and estradiol-17β treated juvenile rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **2001**, (129), 1-10.

122. Howard, G., Chemical alternatives assessment: The case of flame retardants. *Chemosphere* **2014,** *In Press - Corrected Proof.*

123. UNEP, Stockholm convention on persistent organic pollutant: "Summary of the proposal for the listing of hexabromocyclododecane (HBCDD) in Annex A to the convention.". In Committee, P. O. P. R., Ed. United Nations Environment Programme: **2009**.

124. U.S.EPA, Hexabromocyclododecane (HBCD) action plan. In EPA, U. S., Ed. U.S. EPA: **2010**.

125. U.S.EPA, Flame retardant alternatives for hexabromocyclododecane (HBCD). In Office of Pollution Prevention & Toxics, D. f. t. E., Ed. U.S. EPA: **2013**.

126. Gauthier, L. T.; Potter, D.; Hebert, C. E.; Letcher, R. J., Temporal trends and spatial distribution of non-polybrominated diphenyl ether flame retardants in the eggs of colonial populations of Great Lakes Herring Gulls. *environmental Science & Technology* **2009,** *43*, 312-317.

127. Kolic, T.; Shen, L.; MacPherson, K.; Fayez, L.; Gobran, T.; Helm, P. A.; Marvin, C.; Arsenault, G.; Reiner, E. J., The analysis of halogenated flame retardants by GC-HRMS in environmental samples. *Journal of Chromatographic Science* **2009,** *47*, 83-91.

128. Zhou, S. N.; Reiner, E. J.; Marvin, C. H.; Helm, P. A.; Brindle, I. D., Liquid chromatography/tandem mass spectrometry for analysis of 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane (TBECH) and 1,2,5,6-tetrabromocyclooctane (TBCO). *Rapid Communications in Mass Spectrometry* **2010,** *25*, 443-448.

129. Wong, F.; Kurt-Karakus, P.; Bidleman, T. F., Fate of brominated flame retardants and organochlorine pesticides in urban soil: Volatility and degradation. *Environmental Science & Technology* **2012,** *46*, (5), 2668-2674.

130. Saunders, D. M. V.; Podaima, M.; Codling, G.; Giesy, J. P.; Wiseman, S., A mixture of the novel brominated flame retardants TBPH and TBB affects fecundity and transcript profiles ofthe HPGL-axis in Japanese medaka. *Aquatic Toxicology* **2015,** *158*, 14-21.

131. Boyce-Derricott, J.; Nagler, J. J.; Cloud, J. G., Regulation of hepatic estrogen receptor isoform mRNA expression in rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* **2009,** *161*, (1), 73-78.

132. Hutchinson, T.; Ankley, G. T.; Segner, H.; Tyler, C. R., Screening and testing for endocrine disruption in fish—Biomarkers as “signposts,” not “traffic lights,” in risk assessment. *Environmental Health Perspectives* **2006,** *114*, 106-114.

133. Alaee, M.; Wenning, R. J., The significance of brominated flame retardants in the environment: Current understanding, issues and challenges. *Chemosphere* **2002,** *46*, (5), 579-582.

134. Birnbaum, L. S.; Staskal, D. F., Brominated flame retardants: Cause for concern? *Environmental Health Perspectives* **2004,** *112*, (1), 9-17.

135. Kohler, M.; Zennegg, M.; Bogdal, C.; Gerecke, A. C.; Schmid, P.; Heeb, N. V.; Sturm, M.; Vonmont, H.; Kohler, H. P. E.; Giger, W., Temporal trends, congener patterns, and sources of octa-, nona-, and decabromodiphenyl ethers (PBDE) and hexabromocyclododecanes (HBCD) in Swiss lake sediments. *Environmental Science & Technology* **2008,** *42*, (17), 6378-6384.

136. Xie, Z. Y.; Moller, A.; Ahrens, L.; Sturm, R.; Ebinghaus, R., Brominated flame retardants in seawater and atmosphere of the Atlantic and the Southern Ocean. *Environmental Science & Technology* **2011,** *45*, (5), 1820-1826.

137. Lake, I. R.; Foxall, C. D.; Fernandes, A.; Lewis, M.; Rose, M.; White, O.; Dowding, A., Effects of river flooding on polybrominated diphenyl ether (PBDE) levels in cows' milk, soil, and grass. *Environmental Science & Technology* **2011,** *45*, (11), 5017-24.

138. Boon, J. P.; Lewis, W. E.; Tjoen-A-Choy, M. R.; Allchin, C. R.; Law, R. J.; de Boer, J.; ten Hallers-Tjabbes, C. C.; Zegers, B. N., Levels of polybrominated diphenyl ether (PBDE) flame retardants in animals representing different trophic levels of the North Sea food web. *Environmental Science & Technology* **2002,** *36*, (19), 4025-4032.

139. Wan, Y.; Hu, J. Y.; Zhang, K.; An, L. H., Trophodynamics of polybrominated diphenyl ethers in the marine food web of Bohai Bay, North China. *Environmental Science & Technology* **2008,** *42*, (4), 1078-1083.

140. Wolkers, H.; Van Bavel, B.; Derocher, A. E.; Wiig, O.; Kovacs, K. M.; Lydersen, C.; Lindstrom, G., Congener-specific accumulation and food chain transfer of polybrominated diphenyl ethers in two Arctic food chains. *Environmental Science & Technology* **2004,** *38*, (6), 1667-1674.

141. Zhou, T.; Ross, D. G.; DeVito, M. J.; Crofton, K. M., Effects of short-term *in vivo* exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicological Sciences* **2001,** *61*, (1), 76-82.

142. Muirhead, E. K.; Skillman, D.; Hook, S. E.; Schultz, I. R., Oral exposure of PBDE-47 in fish: Toxicokinetics and reproductive effects in Japanese medaka (*Oryzias latipes*) and fathead minnows (*Pimephales promelas*). *Environmental Science & Technology* **2006,** *40*, (2), 523-528.

143. Alm, H.; Scholz, B.; Kultima, K.; Nilsson, A.; Andren, P. E.; Savitski, M. M.; Bergman, A.; Stigson, M.; Fex-Svenningsen, A.; Dencker, L., *In vitro* neurotoxicity of PBDE-99: Immediate and concentration-dependent effects on protein expression in cerebral cortex cells. *J. Proteome Res.* **2010,** *9*, (3), 1226-1235.

144. Ma, Y. N.; Salamova, A.; Venier, M.; Hites, R. A., Has the phase-out of PBDEs affected their atmospheric levels? Trends of PBDEs and their replacements in the Great Lakes atmosphere. *Environmental Science & Technology* **2013,** *47*, (20), 11457-11464.

145. Schmidt, J. S.; Schaedlich, K.; Fiandanese, N.; Pocar, P.; Fischer, B., Effects of di(2-ethylhexyl) phthalate (DEHP) on female fertility and adipogenesis in C3H/N mice. *Environmental Health Perspectives* **2012,** *120*, (8), 1123-1129.

146. Feige, J. N.; Gerber, A.; Casals-Casas, C.; Yang, Q.; Winkler, C.; Bedu, E.; Bueno, M.; Gelman, L.; Auwerx, J.; Gonzalez, F. J.; Desvergne, B., The pollutant diethylhexyl phthalate regulates hepatic energy metabolism via species-specific PPAR alpha-dependent mechanisms. *Environmental Health Perspectives* **2010,** *118*, (2), 234-241.

147. Chauvigne, F.; Menuet, A.; Lesne, L.; Chagnon, M. C.; Chevrier, C.; Regnier, J. F.; Angerer, J.; Jegou, B., Time- and dose-related effects of di-(2-ethylhexyl) phthalate and its main metabolites on the function of the rat fetal testis *in vitro*. *Environmental Health Perspectives* **2009,** *117*, (4), 515-521.

148. Liu, H. H.; Hu, Y. J.; Luo, P.; Bao, L. J.; Qiu, J. W.; Leung, K. M. Y.; Zeng, E. Y., Occurrence of halogenated flame retardants in sediment off an urbanized coastal zone: Association with urbanization and industrialization. *Environmental Science & Technology* **2014,** *48*, (15), 8465-8473.

149. Lam, J. C. W.; Lau, R. K. F.; Murphy, M. B.; Lam, P. K. S., Temporal trends of hexabromocyclododecanes (HBCDs) and polybrominated diphenyl ethers (PBDEs) and detection of two novel flame retardants in marine mammals from Hong Kong, South China. *Environmental Science & Technology* **2009,** *43*, (18), 6944-6949.

150. Zhou, S. N.; Buchar, A.; Siddique, S.; Takser, L.; Abdelouahab, N.; Zhu, J. P., Measurements of selected brominated flame retardants in nursing women: Implications for human exposure. *Environmental Science & Technology* **2014,** *48*, 8873-8880.

151. Cequier, E.; Ionas, A. C.; Covaci, A.; Marce, R. M.; Becher, G.; Thomsen, C., Occurrence of a broad range of legacy and emerging flame retardants in indoor environments in Norway. *Environmental Science & Technology* **2014,** *48*, (12), 6827-6835.

152. Cao, Z. G.; Xu, F. C.; Covaci, A.; Wu, M.; Wang, H. Z.; Yu, G.; Wang, B.; Deng, S. B.; Huang, J.; Wang, X. Y., Distribution patterns of brominated, chlorinated, and phosphorus flame retardants with particle size in indoor and outdoor dust and implications for human exposure. *Environmental Science & Technology* **2014,** *48*, (15), 8839-8846.

153. Allen, J.; McClean, M. D.; Stapleton, H. M.; Webster, T. F., Critical factors in assessing exposure to PBDEs via house dust. *Environment International* **2008,** *34*, (8), 1085-1091.

154. Zhou, S. N.; Reiner, E. J.; Marvin, C.; Helm, P.; Riddell, N.; Dorman, F.; Misselwitz, M.; Shen, L.; Crozier, P.; MacPherson, K.; Brindle, I. D., Development of liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry for analysis of halogenated flame retardants in wastewater. *Anal. Bioanal. Chem.* **2010,** *396*, (3), 1311-1320.

155. Hardy, M. L., The toxicology of the three commercial polybrominated diphenyl oxide (ether) flame retardants. *Chemosphere* **2002,** *46*, (5), 757-777.

156. Klosterhaus, S. L.; Stapleton, H. M.; La Guardia, M. J.; Greig, D. J., Brominated and chlorinated flame retardants in San Francisco Bay sediments and wildlife. *Environment International* **2012,** *47*, 56-65.

157. Chang-Liao, W. L.; Hou, M. L.; Chang, L. W.; Lee, C. J.; Tsai, Y. M.; Lin, L. C.; Tsai, T. H., Determination and pharmacokinetics of di-(2-ethylhexyl) phthalate in rats by ultra performance liquid chromatography with tandem mass spectrometry. *Molecules* **2013,** *18*, (9), 11452-11466.

158. Kato, Y.; Okada, S.; Atobe, K.; Endo, T.; Matsubara, F.; Oguma, T.; Haraguchi, K., Simultaneous determination by APCI-LC/MS/MS of hydroxylated and methoxylated polybrominated diphenyl ethers found in marine biota. *Analytical chemistry* **2009,** *81*, (14), 5942-5948.

159. Chang, H.; Wan, Y.; Hu, J. Y., Determination and source apportionment of five classes of steroid hormones in urban rivers. *Environmental Science & Technology* **2009,** *43*, (20), 7691-7698.

160. Stapleton, H. M.; Misenheimer, J.; Hoffman, K.; Webster, T. F., Flame retardant associations between children's handwipes and house dust. *Chemosphere* **2014,** *116*, 54-60.

161. Dingemans, M.; Van den Berg, M.; Westerink, R., Neurotoxicity of brominated flame retardants: (In)direct effects of parent and hydroxylated polybrominated diphenyl ethers on the (developing) nervous system. *Environmental Health Perspectives* **2011,** *119*, 900-907.

162. Hamers, T.; Kamstra, J. H.; Sonneveld, E.; Murk, A. J.; Visser, T. J.; Van Velzen, M. J. M.; Brouwer, A.; Bergman, A., Biotransformation of brominated flame retardants into potentially endocrine-disrupting metabolites, with special attention to 2,2',4,4 '-tetrabromodiphenyl ether (BDE-47). *Mol. Nutr. Food Res.* **2008,** *52*, (2), 284-298.

163. Peng, H.; Saunders, D. M.; Sun, J.; Codling, G.; Wiseman, S.; Jones, P. D.; Giesy, J. P., Detection, identification, and quantification of hydroxylated bis(2-ethylhexyl)-tetrabromophthalate isomers in house dust. *Environ Sci Technol* **2015,** *49*, (5), 2999-3006.

164. Peng, H.; Sun, J.; Saunders, D. M. V.; Codling, G.; Wiseman, S.; Jones, P. D.; Giesy, J. P., Hydroxylated 2-ethylhexyl tetrabromobenzoate isomers in house dust and their agonistic activities for several nuclear receptors. *Under Review, Environmental Science & Technology* **2017**.

165. Venier, M.; Audy, O.; Vojta, S.; Becanova, J.; Romanak, K.; Melymuk, L.; Kratka, M.; Kukucka, P.; Okeme, J.; Saini, A.; Diamond, M.; Klanova, J., Brominated flame retardants in the indoor environment - Comparative study of indoor contamination in three countries. *Environment International* **2016,** *94*, 150-160.

166. La Guardia, M. J.; Hale, R. C., Halogenated flame-retardant concentrations in settled dust, respirable and inhalable particulates and polyurethane foam at gymnastic training facilities and residences. *Environment International* **2015,** *79*, 106-114.

167. Lunder, S.; Hovander, L.; Athanassiadis, I.; Bergman, Å., Significantly higher polybrominated diphenyl ether levels in young U.S. children than in their mothers. *Environmental Science & Technology* **2010,** *44*, (13), 5256-5262.

168. Hoffman, K.; Fang, M.; Horman, B.; Patisaul, H. B.; Garantziotis, S.; Birnbaum, L. S.; Stapleton, H. M., Urinary tetrabromobenzoic acid (TBBA) as a biomarker of exposure to the flame retardant mixture Firemaster® 550. *Environmental Health Perspectives* **2014,** *122*, (9), 963-969.

169. Butt, C.; Congleton, J.; Hoffman, K.; Fang, M.; Stapleton, H. M., Metabolites of organosphosphate flame retardants and 2-ethylhexyl tetrabromobenzoate in urine from paired mother and toddlers. *Environmental Science & Technology* **2014,** *48*, (17), 10432-10438.

170. Sinha, M., Spotlight on Canadians: Results from the general social survey. Child care in Canada. In Canada, S., Ed. Statistics Canada: Ottawa, Canada, **2014**.

171. Chen, A.; Yolton, K.; Rauch, S. A.; Webster, G. M.; Hornung, R.; Sjodin, A.; Dietrich, K. N.; Lanphear, B. P., Prenatal polybrominated diphenyl ether exposures and neurodevelopment in U.S. children through 5 years of age: The HOME study. *Environmental Health Perspectives* **2014,** *122*, (8), 856-862.

172. Saunders, D. M. V.; Podaima, M.; Wiseman, S.; Giesy, J. P., Effects of the brominated flame retardant TBCO on fecundity and profiles of transcripts of the HPGL-axis in Japanese medaka. *Aquatic Toxicology* **2015,** *160*, 180-7.

173. Fang, M.; Stapleton, H. M., Evaluating the bioaccessbility of flame retardants in house dust using an *in vitro* Tenax bead-assisted sorptive physiologically based method. *Environmental Science & Technology* **2014,** *48*, 13323-13330.

174. Matz, C. J.; Stieb, D. M.; Davis, K.; Egyed, M.; Rose, A.; Chou, B.; Brion, O., Effects of age, season, gender and urban-rural status on time-activity: Canadian Human Activity Pattern Survey 2 (CHAPS 2). *International Journal of Environmental Research and Public Health* **2014,** *11*, 2108-2124.

175. Tilston, E.; Gibson, G. R.; Collins, C. D., Colon extended physiologically based extraction test (CE-PBET) increases bioaccessibility of soil-bound PAH. *Environmental Science & Technology* **2011,** *45*, 5301-5308.

176. Allgood, J.; Jimah, T.; McClaskey, C.; La Guardia, M. J.; Hammel, S.; Zeineddine, M.; Tang, I.; Runnderstrom, M.; Ogunseitan, O., Potential human exposure to halogenated flame-retardants in elevated surface dust and floor dust in an academic environment. *Environmental Research* **2017,** *153*, 55-62.

177. Cao, Z.; Xu, F.; Li, W.; Sun, J.; Shen, M.; Su, X.; Feng, J.; Yu, G.; Covaci, A., Seasonal and particle size-dependent variations of hexabromocyclododecanes in settled dust: Implications for sampling. *Environmental Science & Technology* **2015,** *49*, 11151-11157.

178. Mizouchi, S.; Ichiba, M.; Takigami, H.; Kajiwara, N.; Takamuku, T.; Miyajima, T.; Kodama, H.; Someya, T.; Ueno, D., Exposure assessment of organophosphorous and organobromine flame retardants via indoor dust from elementary schools and domestic houses. *Chemosphere* **2015,** *123*, 17-25.

179. Toms, L.-M.; Mazaheri, M.; Brommer, S.; Clifford, S.; Drage, D.; Mueller, J.; Thai, P.; Harrad, S.; Morawska, L.; Harden, F., Polybrominated diphenyl ethers (PBDEs) in dust from primary schools in South East Queensland, Australia. *Environmental Research* **2015,** *142*, 135-140.

180. Thuresson, K.; Awasum Bjorklund, J.; De Wit, C. A., Tri-decabrominated diphenyl ethers and hexabromocyclododecane in indoor air and dust from Stockholm microenvironments 1: Levels and profiles. *Science of The Total Environment* **2012,** *414*, 712-721.

181. Hoffman, K.; Butt, C.; Chen, A.; Limkakeng, A.; Stapleton, H. M., High exposure to organophosphate flame retardants in infants: Associations with baby products. *Environmental Science & Technology* **2015,** *49*, 14554-14559.

182. Whitehead, T.; Brown, F. R.; Metayer, C.; Park, J.-S.; Does, M.; Petreas, M.; Buffler, P.; Rappaport, S., Polybrominated diphenyl ethers in residential dust: Sources of variability. *Environment International* **2013,** *57-58*, 11-24.

183. Allen, J.; McClean, M. D.; Stapleton, H. M.; Webster, T. F., Linking PBDEs in house dust to consumer products using x-ray fluorescence. *Environmental Science & Technology* **2008,** *42*, 4222-4228.

184. Cao, Z.; Xu, F.; Covaci, A.; Wu, M.; Yu, G.; Wang, B.; Deng, S.; Huang, J., Differences in the seasonal variation of brominated and phosphorus flame retardants in office dust. *Environment International* **2014,** *65*, 100-106.

185. Rauert, C.; Harrad, S., Mass transfer of PBDEs from plastic TV casing to indoor dust via three migration pathways - A test chamber investigation. *Science of The Total Environment* **2015,** *536*, 568-574.

186. Harrad, S.; Ibarra, C.; Abdallah, M. A. E.; Boon, R.; Neels, H.; Covaci, A., Concentrations of brominated flame retardants in dust from United Kingdom cars, houses and offices: Causes of variability and implications for human exposure. *Environment International* **2008,** *34*, 1170-1175.

187. Muenhor, D.; Harrad, S., Within-room and within-building temporal and spatial variations in concentrations of polybrominated diphenyl ethers (PBDEs) in indoor dust. *Environment International* **2012,** *47*, 23-27.

188. Keimowitz, A. R.; Strunsky, N.; Wovkulich, K., Organophosphate flame retardants in household dust before and after introduction of new furniture. *Chemosphere* **2016,** *148*, 467-472.

189. Knudsen, G.; Hughes, M. F.; Sanders, J. M.; Hall, S. M.; Birnbaum, L. S., Estimation of human percutaneous bioavailability for two novel brominated flame retardants, 2-ethyhexyl 2,3,4,5-tetrabromobenzoate (EH-TBB) and bis(2-ethylhexyl) tetrabromophthalate (BEH-TEBP). *Toxicology and Applied Pharmacology* **2016,** *311*, 117-127.

190. Frederiksen, M.; Vorkamp, K.; Jensen, N. M.; Sorensen, J. A.; Knudsen, L.; Sorensen, L.; Webster, T. F.; Nielsen, J., Dermal uptake and percutaneous penetration of ten flame retardants in human skin *ex vivo* model. *Chemosphere* **2016,** *162*, 308-314.

191. Li, C.; Cui, X.-Y.; Fan, Y.-Y.; Teng, Y.; Nan, Z.-R.; Ma, L., Tenax as sorption sink for *in vitro* bioaccessibility measurement of polycyclic aromatic hydrocarbons in soils. *Environmental Pollution* **2015,** *196*, 47-52.

192. Yu, Y.; Yang, D.; Wang, X.; Huang, N.; Zhang, X.; Zhang, D.; Fu, J., Factors influencing on the bioaccessibility of polybrominated diphenyl ethers in size-specific dust from air conditioner filters. *Chemosphere* **2013,** *93*, 2603-2611.

193. Kierkegaard, A.; De Wit, C. A.; Asplund, L.; McLaughlin, M. S.; Thomas, G.; Sweetman, A.; Jones, K., A mass balance of tri-hexabrominated diphenyl ethers in lactating cows. *Environmental Science & Technology* **2009,** *43*, 2602-1607.

194. Betts, K., New flame retardants detected in indoor and outdoor environments. *Environmental Science & Technology* **2008,** *42*, 6778-6778.

195. Zhou, S. N.; Reiner, E. J.; Marvin, C. H.; Kolic, T.; Riddell, N.; Helm, P. A.; Dorman, F.; Misselwitz, M.; Brindle, I. D., Liquid chromatography-atmospheric pressure photoionization tandem mass spectrometry for analysis of 36 halogenated flame retardants in fish. *Journal of Chromatography A* **2010,** *1217*, 633-641.

196. Klopcic, I.; Skledar, D. G.; Masic, L. P.; Dolenc, M. S., Comparison of *in vitro* hormone activities of novel flame retardants TBB, TBPH and their metabolites TBBA and TBMEPH using reporter gene assays. *Chemosphere* **2016,** *160*, 244-251.

197. Fic, A.; Zegura, B.; Gramec, D.; Masic, L. P., Estrogenic and androgenic activities of TBBA and TBMEPH, metabolites of novel brominated flame retardants, and selected bisphenols, using the XenoScreen XL YES/YAS assay. *Chemosphere* **2014,** *112*, 362-369.

198. Krivoshieve, B. V.; Dardenne, F.; Covaci, A.; Blust, R.; Husson, S., Assessing *in-vitro* estrogenic effects of currently-used flame retardants. *Toxicology in Vitro* **2016,** *33*, 153-162.

199. Skledar, D. G.; Tomasic, T.; Carino, A.; Distrutti, F. S.; Masic, L. P., New brominated flame retardants and their metabolites as activators of the pregnane X receptor. *Toxicology Letters* **2016,** *259*, 116-123.

200. Belcher, S. M.; Cookman, C. J.; Patisaul, H. B.; Stapleton, H. M., *In vitro* assessment of human nuclear hormone receptor activity and cytotoxicity of the flame retardant mixture FM 550 and its triarylphosphate and brominated components. *Toxicology Letters* **2014,** *228*, (2), 93-102.

201. Pillai, H. K.; Fang, M.; Beglov, D.; Kozakov, D.; Vajda, S.; Stapleton, H. M.; Webster, T. F.; Schlezinger, J. J., Ligand binding and activation of PPARγ by Firemaster® 550: Effects on adipogenesis and osteogenesis *in vitro*. *Environ Health Perspect* **2014,** *122*, (11), 1225-32.

202. Fang, M.; Webster, T. F.; Ferguson, P. L.; Stapleton, H. M., Characterizing the peroxisome proliferator-activated receptor (PPARγ) ligand binding potential of several major flame retardants, their metabolites, and chemical mixtures in house dust. *Environmental Health Perspectives* **2015,** *123*, 166-172.

203. Watt, J.; Schlezinger, J. J., Structurally-diverse, PPARγ-activating environmental toxicants induce adipogenesis and suppress osteogenesis in bone marrow mesenchymal stromal cells. *Toxicology* **2015,** *331*, 66-77.

204. McGee, S.; Konstantinov, A.; Stapleton, H. M.; Volz, D. C., Aryl phosphate esters within a major pentaBDE replacement product induce cardiotoxicity in developing zebrafish embryos: Potential roles of the aryl hydrocarbon receptor. *Toxicological Sciences* **2013,** *133*, 144-156.

205. Bailey, J. M.; Levin, E. D., Neurotoxicity of Firemaster 550® in zebrafish (*Danio rerio*): Chronic developmental and acute adolescent exposures. *Neurotoxicol Teratol* **2015,** *52*, 210-219.

206. Howdeshell, K.; Rider, C. V.; Wilson, V. S.; Gray, J. L. E., Mechanisms of action of phthalate esters, individually and in combination, to induce abnormal reproductive development in male laboratory rats. *Environ Res* **2008,** *108*, 169-176.

207. Ward, J. M.; Peters, J. M.; Perella, C. M.; Gonzalez, F. J., Receptor and nonreceptor-mediated organ-specific toxicity of di(2-ethylhexyl)phthalate (DEHP) in peroxisome proliferator activated receptor α-null mice. *Toxicol Pathol* **1998,** *26*, 240-246.

208. Heindel, J. J.; Newbold, R.; Schug, T. T., Endocrine disruptors and obesity. *Nature reviews. Endocrinology* **2015,** *11*, (11), 653-61.

209. Sun, J.; Tang, S.; Peng, H.; Saunders, D. M.; Doering, J. A.; Hecker, M.; Jones, P. D.; Giesy, J. P.; Wiseman, S., Combined transcriptomic and proteomic approach to identify toxicity pathways in early life stages of Japanese medaka (*Oryzias latipes*) exposed to 1,2,5,6-tetrabromocyclooctane (TBCO). *Environ Sci Technol* **2016,** *50*, (14), 7781-90.

210. Linares, V.; Belles, M.; Domingo, J., Human exposure to PBDE and critical evaluation of health hazards. *Arch Toxicolo* **2015,** *89*, 335-356.

211. Lovekamp-Swan, T.; Davis, B. J., Mechanisms of phthalate ester toxicity in the female reproductive system. *Environmental Health Perspectives* **2003,** *111*, (2), 139-145.

212. Treinen KA; Dodson WC; JJ, H., Inhibition of FSH stimulated cAMP accumulation and progesterone production by mono(2-ethylhexyl) phthalate in rat granulosa cell cultures. *Toxicol Appli Pharmacol* **1990,** *106*, 334-340.

213. Meeker, J. D.; Calafat, A. M.; Hauser, R., Di(2-ethylhexyl) phthalate metabolites may alter thyroid hormone levels in men. . *Environmental Health Perspectives* **2007,** *115*, 1029-1034.

214. Erkin-Cakmak, A.; Harley, K. G.; Chevrier, J.; Bradman, A.; Kogut, K.; Huen, K.; Eskenazi, B., *In utero* and childhood polybrominated diphenyl ether exposures and body mass at age 7 years: The CHAMACOS study. *Environ Health Perspect* **2015,** *123*, (6), 636-42.

215. Meeker, J., Exposure to environmental endocrine disruptors and child development. *Arch Pediatr Adolesc Med* **2012,** *166*, 1-12.

216. Fischer, D.; Hooper, K.; Athanasiadou, M.; Athanasiadis, I.; Bergman, A., Children show highest levels of polybrominated diphenyl ethers in a California family of four: A case study. *Environmental Health Perspectives* **2006,** *114*, 1581-1584.

217. Ali, N.; Eqani, S. A. M. A.; Malik, R. N.; Heels, H.; Covaci, A., Organohalogenated contaminants (OHCs) in human serum of mothers and children from Pakistan with urban and rural residential settings. *Science of The Total Environment* **2013,** *461*, 655-662.

218. Shoeib, M.; Harner, T.; Ikonomou, M.; Kannan, K., Indoor and outdoor air concentrations and phase partitioning of perfluoroalkyl sulfonamides and polybrominated diphenyl ethers. *Environmental Science & Technology* **2004,** *38*, 1313-1320.

219. Domingo, J. L., Polybrominated diphenyl ethers in food and human dietary exposure: A review of the recent scientific literature. *Food Chem. Toxicol.* **2012,** *50*, 238-249.

220. Labunska, I.; Abdallah, M. A. E.; Eulaers, I.; Covaci, A.; Fang, T.; Wang, M.; Santillo, D.; Johnston, P.; Harrad, S., Human dietary intake of organohalogen contaminants at e-waste recycling sites in Eastern China. *Environment International* **2015,** *74*, 209-220.

221. Sahlstrom, L.; Sellström, U.; de Wit, C. A.; Lignell, S.; Darnerud, P. O., Estimated intakes of brominated flame retardants via diet and dust compared to internal concentrations in a Swedish mother–toddler cohort. *International Journal of Hygiene and Environmental Health* **2015,** *218*, 422-432.

222. Panel on Contaminants in the Food Chain, E., Scientific opinion on emerging and novel brominated flame retardants (BFRs) in food. *EFSA J* **2012,** *10*.

223. Knudsen, G.; Sanders, J. M.; Birnbaum, L., Disposition of the emerging brominated flame retardant, 2-ethylhexyl 2,3,4,5-tetrabromobenzoate, in female SD rats and male B6C3F1 mice: Effects of dose, route, and repeated administration. *Society of Toxicology* **2016,** *154*, 392-402.

224. Knudsen, G.; Sanders, J. M.; Birnbaum, L., Disposition of the emerging brominated flame retardant, bis(2-ethylhexyl) tetrabromophthalate, in female Sprague Dawley rats: Effects of dose, route and repeated administration. *Xenobiotica* **2016**, 1-10.

225. Liu, L.-Y.; He, K.; Hites, R. A.; Salamova, A., Hair and nails as noninvasive biomarkers of human exposure to brominated and organophosphate flame retardants. *Environmental Science & Technology* **2016,** *50*, 3065-3073.

226. Sahlstrom, L.; Sellström, U.; de Wit, C. A.; Lignell, S.; Darnerud, P. O., Feasibility study of feces for noninvasive biomonitoring of brominated flame retardants in toddlers. *Environmental Science & Technology* **2015,** *49*, 606-615.

227. He, S.; Mingyuan, L.; Jin, J.; Wang, Y.; Bu, Y.; Xu, M.; Yang, X.; Liu, A., Concentrations and trends of halogenated flame retardants in the pooled serum of residents of Laizhou Bay, China. *Environmental Toxicology and Chemistry* **2013,** *32*, 242-247.

228. Sahlstrom, L.; Sellström, U.; de Wit, C. A.; Lignell, S.; Darnerud, P. O., Brominated flame retardants in matched serum samples from Swedish first-time mothers and their toddlers. *Environmental Science & Technology* **2014,** *48*, 7584-7592.

229. Butt, C. M.; Miranda, M. L.; Stapleton, H. M., Development of an analytical method to quantify PBDEs, OH-BDEs, HBCDs, 2,4,6-TBP, EH-TBB, and BEH-TEBP in human serum. *Anal bioanal Chem* **2016,** *408*, 2449-2459.

230. Liu, L.; Salamova, A.; Venier, M.; Hites, R. A., Trends in the levels of halogenated flame retardants in the Great Lakes atmosphere over the period 2005-2013. *Environment International* **2016,** *92-93*, 442-449.

231. E.F.S.A., Guidance on selected default values to be used by the EFSA Scientific Committee, scientific panels and units in the absence of actual measured data. *EFSA J* **2012,** *10*, 2579.

232. Pott, U., Federal Contaminated Sites Action Plan (FCSAP): Ecological risk assessment guidance. In Canada, E., Ed. Azimuth Consulting Group: Vancouver, **2012**.

233. Abbasi, G.; Saini, A.; Goosey, E.; Diamond, M., Product screening for sources of halogenated flame retardants in Canadian house and office dust. *Science of The Total Environment* **2016,** *545-546*, 299-307.

234. de Wit, C. A.; Herzke, D.; Vorkamp, K., Brominated flame retardants in the Arctic environment - Trends and new candidates. *Science of The Total Environment* **2010,** *408*, (15), 2885-2918.

235. Hong, H.; Shen, R.; Liu, W.; Li, D.; Huang, L.; Shi, D., Developmental toxicity of three hexabromocyclododecane diastereoisomers in embryos of the marine medaka *Oryzias melastigma*. *Marine Pollution Bulletin* **2015,** *101*, 110-118.

236. Suhring, R.; Busch, F.; Fricke, N.; Kotke, D.; Wolschke, H.; Ebinghaus, R., Distribution of brominated flame retardants and dechloranes between sediments and benthic fish - A comparison of a freshwater and marine habitat. *Science of The Total Environment* **2016,** *542*, 578-585.

237. Fire-Administration, U. S., U.S. fire statistics website. In https://www.usfa.fema.gov/data/statistics/, **2016**.

APPENDIX[1]

[1] Supplementary data are included in this chapter. The figure or table number is presented as Cx.Sy, format, where ‘Cx’ indicates chapter number; ‘Sy’ indicates figure or table number.

Table C2.S1 Physical-chemical properties of 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB), *Bis*(2-ethylhexyl)-2,3,4,5-tetrabromophtalate (TBPH), and 1,2,5,6-tetrabromocyclooctane (TBCO)

	TBPH	TBB	TBCO
Molecular Weight	706.15	549.93	427.80
Solubility (mg/L)	1.98×10^{-9} [b]	1.14×10^{-5} [c]	0.06915[b]
Log K_{OW}	11.95[a]	8.8[c]	5.24[a]

[a] Estimated from: KowWIN v1.68 (U.S. EPA)
[b] Estimated from: WSKow v1.42 (U.S. EPA)
[c] Bearr et al., 2010.[29]

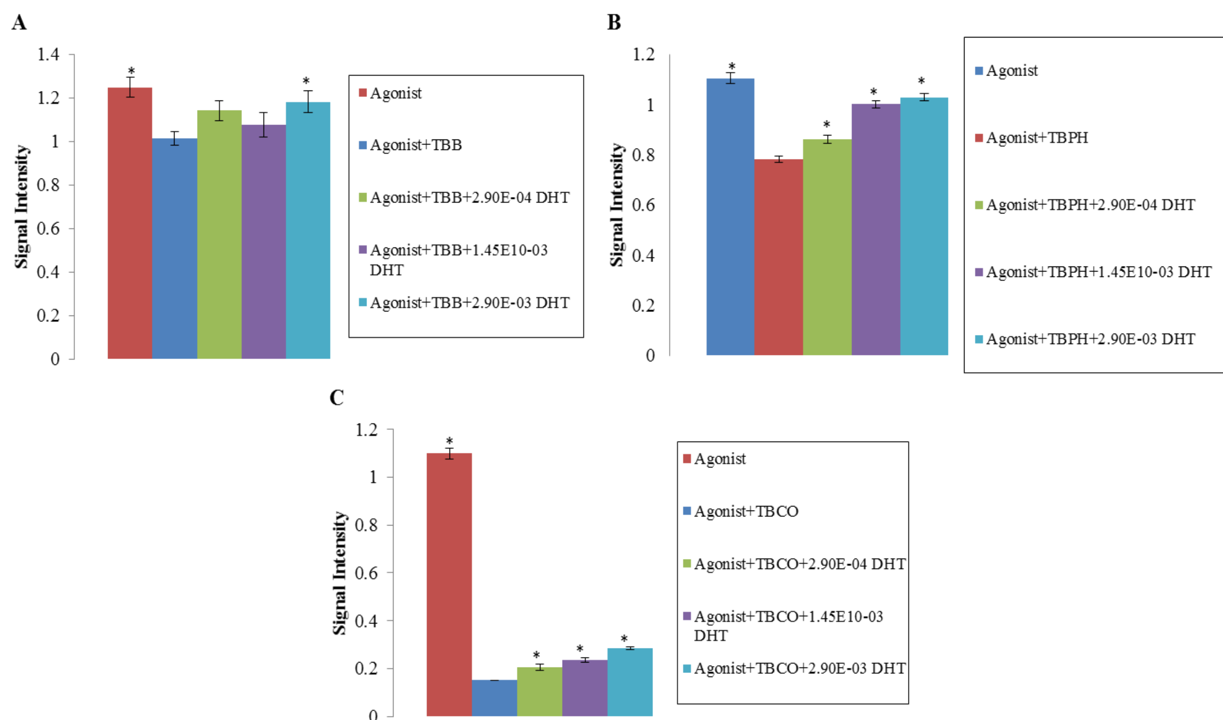

Figure C2.S1. The control for recovery of signal activity of (A) TBB at $5x10^{-01}$ mg/L, (B) TBPH at 1000 mg/L, and (C) TBCO at 300 mg/L measured by the yeast androgen screen (YAS). A baseline agonist (DHT) concentration of $1.45x\ 10^{-3}$ mg/L was added to each well with increasing concentrations added to demonstrate the recovery of signal activity. Activity is presented as mean± SE. Each assay contained four wells per NBFR exposure concentration. Exposures that resulted in effects that were significantly different than inhibition controls (agonist + NBFR) are indicated by asterisks (*p<0.05).

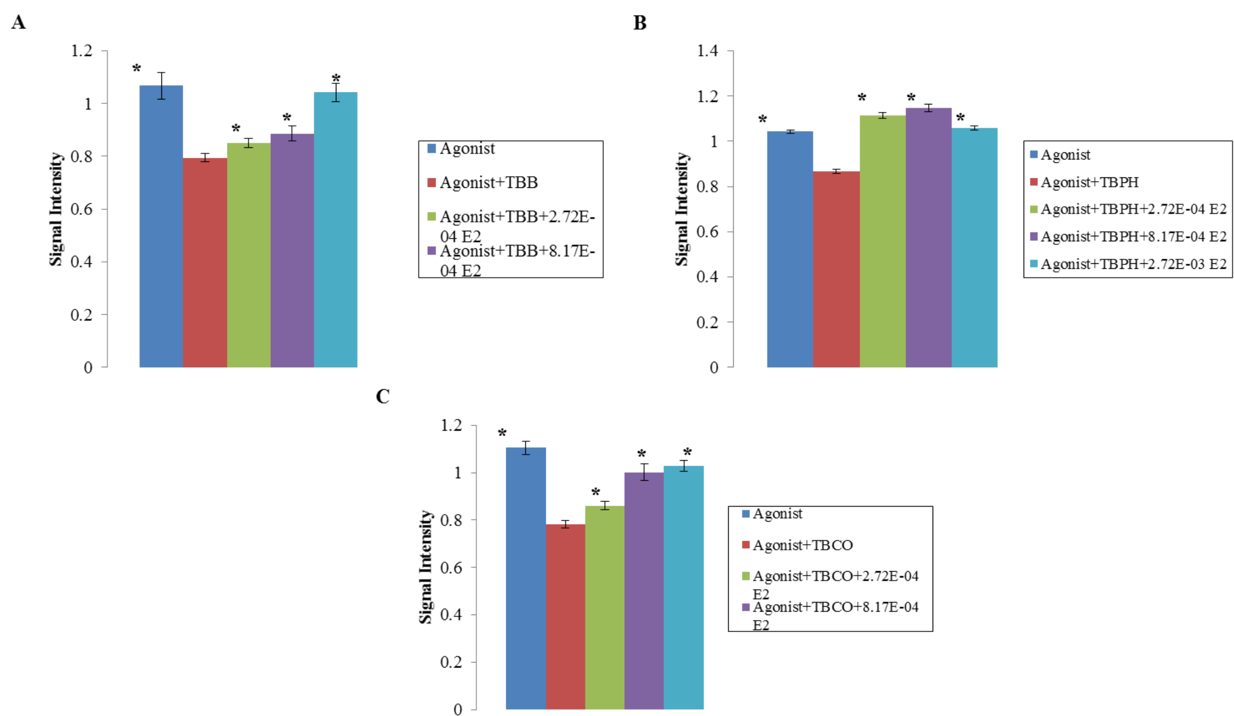

Figure C2.S2. The control for recovery of signal activity of (A) TBB at 5×10^{-01} mg/L, (B) TBPH at 0.03 mg/L, and (C) TBCO at 30 mg/L measured by the yeast estrogen screen (YES). A baseline agonist (E2) concentration of 8.17×10^{-4} mg/L was added to each well with increasing concentrations added to demonstrate the recovery of signal activity. Activity is presented as mean± SE. Each assay contained four wells per NBFR exposure concentration. Exposures that resulted in effects that were significantly different than inhibition controls (agonist + NBFR) are indicated by asterisks (*p<0.05).

Table C3.S1. Target gene, primer sequence, and efficiency of 35 genes across the HPGL axis of Japanese medaka.

Target gene	Primer sequence (5' - 3') Forward	 Reverse	Efficiency (%)
ERα	CGGACCAGCACTCAGATCCA	CAGGGGAGCAGAGTAGTAGC	110
ERβ	GCTGGAGGTGCTGATGATGG	CGAAGCCCTGGACACAACTG	110
ARα	ACCTGGCTCACTTCGGACAC	TCTGACGCCGTACTGCTCTG	98
Neuropep Y	CTTCCACAGTCAAGTTACAAC	TGATCTGCAAGGACGAATG	95
cGnRH II	TGTCTCGGCTGGTTCTAC	GAGTCTAGCTCCCTCTTCC	95
mfGnRH	GTGTCGCAGCTCTGTGTTC	GTGTCGCAGCTCTGTGTTC	105
sGnRH	GATGATGGGCACAGGAAGAGT	GGGCACTTGCATCTTCAGGA	106
GnRH RI	CTGCGCTGCTCAAAGAACAA	GTGGAAGCGAGTGGTGAAGA	104
GnRH RII	GCAGCGGCACAGACATCATC	GGACAGCACAATGACCACAGA	100
GnRH RIII	ACTTCCAGAGGAGCCAGTTGAG	GCCAGCCAAGAGTCGTTGTC	110
GTHa	GCAGAACGGAGGGATGAAGGA	ATTGGAGTAGGTGTCGGCTGTG	104
LH- β	GCCAGCCAGTCAAGCAGAAG	GCCAGCCAGTCAAGCAGAAG	90
CYP19B	TCCTGATAACCCTGCTGTCTCG	TCCTGATAACCCTGCTGTCTCG	106
FSHR	TTCAGGCCACTGATGATGTTAT	CCTTCGTGGGTTCCAGTGAGT	96
LHR	GTCCTGGTCATCCTGCTCGTT	AACCGGGAGATGGTCAGTTTGT	98
HDLR	TCTGCCGAACTGTCACTGTC	CCACCTGGTCGTCGATGATG	109
LDLR	GTGCTACGAAGGCTACGAGAT	AGGTCAATGCGGCGGATTTC	108
HMGR	CCAGCTCGCAGGATGAAGT	GTAGTTGGCCAGCACAGACA	108
StAR	TGACAGGTTTGAGAAAGAATG	CAATGCGAGAACTTAGAAGG	96
CYP11A	GCTGCATCCAGAACATCTATCG	GACAGCTTGTCCAACATCAGGA	108
CYP17	CGACCACCACCGTACTCAAA	CACATGGGGGATGAGCAGAG	102
CYP19A	CTCTTCCTGGGTGTTCCTGTTG	GCTGCTGTCTTGTGCCTCTG	89
20β-HSD	TGATCTTGGCTCGTCGTCTG	CACGGCTGGACTTCCTTCTC	100
3β-HSD	GGGCGGGACGAAACTCAG	GGAGGCGGTGTGGAAGAC	110
Inhibin A	CGTTTCCCTTCCAGCCTTC	AAGAGCGTTGCGGATGAG	109
Activin BA	GATGGTGGAAGCAGTGAAG	TTCTTGATGGCGTTGAGTAG	110
Activin BB	GGCTAATCGGCTGGAATG	CATGCGGTACTGGTTCAC	104
VTG I	ACTCTGCTGCTGTGGCTGTAG	AAGGCGTGGGAGAGGAAAGTC	101
VTG II	TCGCCGCAAGAGCAAGAC	CTGGAGGAGCTGGAAGAACTG	99
CHG H	TGGCAAGGCACTGGAGTATCAC	CTGAGGCTTCGGCTGTGGATAG	95
CHG HM	GGAGCCATTACCAGGGACAG	AAGTTCCACACGCAAGATTCC	98
CHG L	TCCTGTCTCTGACTCTGAATGG	GCTTGGCTCGTCCTCACC	105
CYP3A	GAGATAGACGCCACCTTCC	ACCTCCACAGTTGCCTTG	99
Annexin max2	CTGATCGTGGCTCTGATGAC	CTGCTGAGGTGTTCTGGAAG	96
RPL-7	GTCGCCTCCCTCCACAAAG	AACTTCAAGCCTGCCAACAAC	94

Table C3.S2. Toxicant-induced effects on medaka gonadal-somatic index (GSI) and hepatic-somatic index (HSI). GSI and HSI are presented as mean ± standard error.

Treatment	Female		Male
	GSI	HSI	HSI
Control	15.7±1.89	2.72±0.20	2.44±0.38
924 µg/g food	18.1±3.27	4.87±1.00	3.69±0.29
85 µg/g food	19.7±3.44	3.52±0.36	2.63±0.50

n = 4, **p*-Value < 0.05

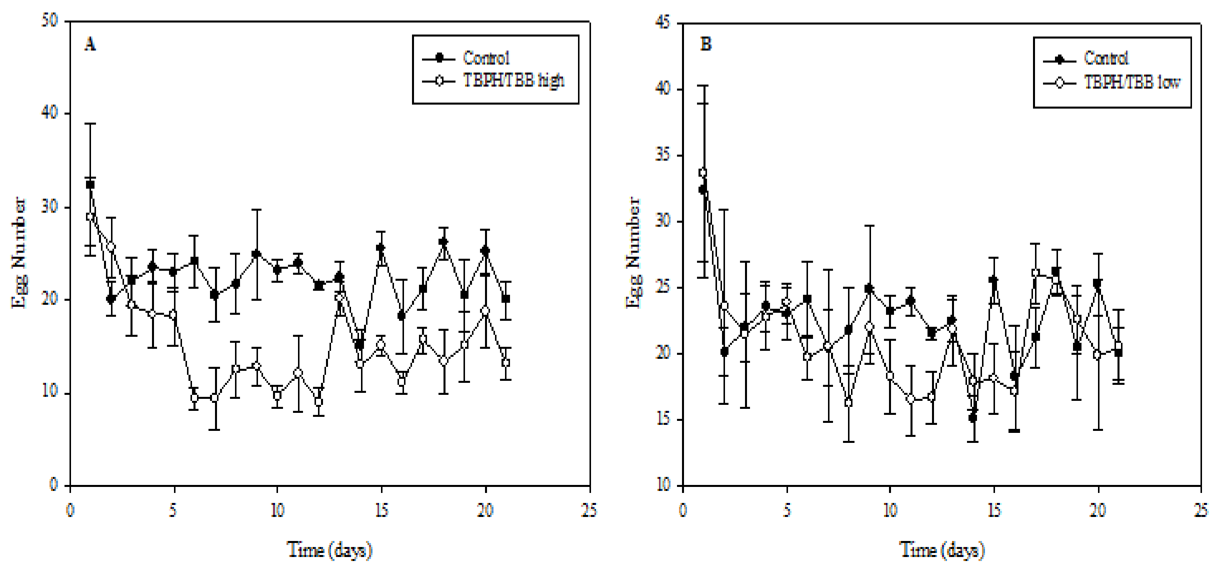

Figure C3.S1. Profile analysis of daily fecundity of (A) solvent control vs. the greatest dose of the TBPH/TBB mixture and (B) solvent control vs. the low dose of the TBPH/TBB mixture. The experiment included 4 replicate tanks, and each contained 8 female fish. The profile (parallelism) of TBPH/TBB high was statistically different than solvent control. Significant differences of parallelism were set at $p < 0.05$.

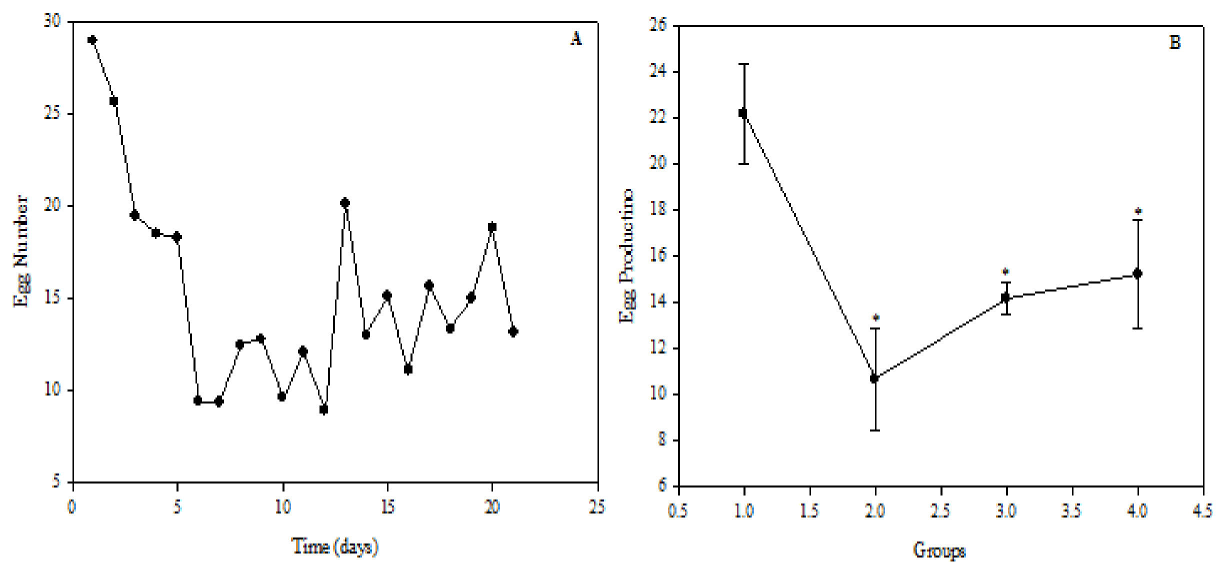

Figure C3.S2. Within-group repeated measures analysis of variance of (A) daily egg production and (B) pooled time-points of fish exposed to the greatest dose of the TBPH/TBB mixture. Time-points were pooled to preserve significant differences after Bonferroni adjustments. Asterisks indicate significant differences ($p < 0.05$) when compared to 100% fecundity (group 1). Significant within-group main effects were also observed in daily egg production.

Table C4.S1 Target gene, primer sequence and efficiency of 35 genes across the HPGL axis of Japanese medaka.

Target gene	Primer sequence (5' - 3') Forward	Reverse	Efficiency (%)
ERα	CGGACCAGCACTCAGATCCA	CAGGGGAGCAGAGTAGTAGC	110
ERβ	GCTGGAGGTGCTGATGATGG	CGAAGCCCTGGACACAACTG	110
ARα	ACCTGGCTCACTTCGGACAC	TCTGACGCCGTACTGCTCTG	98
Neuropep Y	CTTCCACAGTCAAGTTACAAC	TGATCTGCAAGGACGAATG	95
cGnRH II	TGTCTCGGCTGGTTCTAC	GAGTCTAGCTCCCTCTTCC	95
mfGnRH	GTGTCGCAGCTCTGTGTTC	GTGTCGCAGCTCTGTGTTC	105
sGnRH	GATGATGGGCACAGGAAGAGT	GGGCACTTGCATCTTCAGGA	106
GnRH RI	CTGCGCTGCTCAAAGAACAA	GTGGAAGCGAGTGGTGAAGA	104
GnRH RII	GCAGCGGCACAGACATCATC	GGACAGCACAATGACCACAGA	100
GnRH RIII	ACTTCCAGAGGAGCCAGTTGAG	GCCAGCCAAGAGTCGTTGTC	110
GTHa	GCAGAACGGAGGGATGAAGGA	ATTGGAGTAGGTGTCGGCTGTG	104
LH- β	GCCAGCCAGTCAAGCAGAAG	GCCAGCCAGTCAAGCAGAAG	90
CYP19B	TCCTGATAACCCTGCTGTCTCG	TCCTGATAACCCTGCTGTCTCG	106
FSHR	TTCAGGCCACTGATGATGTTAT	CCTTCGTGGGTTCCAGTGAGT	96
LHR	GTCCTGGTCATCCTGCTCGTT	AACCGGGAGATGGTCAGTTTGT	98
HDLR	TCTGCCGAACTGTCACTGTC	CCACCTGGTCGTCGATGATG	109
LDLR	GTGCTACGAAGGCTACGAGAT	AGGTCAATGCGGCGGATTTC	108
HMGR	CCAGCTCGCAGGATGAAGT	GTAGTTGGCCAGCACAGACA	108
StAR	TGACAGGTTTGAGAAAGAATG	CAATGCGAGAACTTAGAAGG	96
CYP11A	GCTGCATCCAGAACATCTATCG	GACAGCTTGTCCAACATCAGGA	108
CYP17	CGACCACCACCGTACTCAAA	CACATGGGGGATGAGCAGAG	102
CYP19A	CTCTTCCTGGGTGTTCCTGTTG	GCTGCTGTCTTGTGCCTCTG	89
20β-HSD	TGATCTTGGCTCGTCGTCTG	CACGGCTGGACTTCCTTCTC	100
3β-HSD	GGGCGGGACGAAACTCAG	GGAGGCGGTGTGGAAGAC	110
Inhibin A	CGTTTCCCTTCCAGCCTTC	AAGAGCGTTGCGGATGAG	109
Activin BA	GATGGTGGAAGCAGTGAAG	TTCTTGATGGCGTTGAGTAG	110
Activin BB	GGCTAATCGGCTGGAATG	CATGCGGTACTGGTTCAC	104
VTG I	ACTCTGCTGCTGTGGCTGTAG	AAGGCGTGGGAGAGGAAAGTC	101
VTG II	TCGCCGCAAGAGCAAGAC	CTGGAGGAGCTGGAAGAACTG	99
CHG H	TGGCAAGGCACTGGAGTATCAC	CTGAGGCTTCGGCTGTGGATAG	95
CHG HM	GGAGCCATTACCAGGGACAG	AAGTTCCACACGCAAGATTCC	98
CHG L	TCCTGTCTCTGACTCTGAATGG	GCTTGGCTCGTCCTCACC	105
CYP3A	GAGATAGACGCCACCTTCC	ACCTCCACAGTTGCCTTG	99
Annexin max2	CTGATCGTGGCTCTGATGAC	CTGCTGAGGTGTTCTGGAAG	96
RPL-7	GTCGCCTCCCTCCACAAAG	AACTTCAAGCCTGCCAACAAC	94

Table C4.S2. Toxicant-induced effects on medaka gonadal-somatic index (GSI) and hepatic-somatic index (HSI). GSI and HIS are presented as mean ± standard error.

Treatment	Female		Male
	GSI	HSI	HSI
Control	15.69 ± 1.89	2.72 ± 0.20	2.44 ± 0.38
607 µg/g	15.11 ± 1.84	3.57 ± 0.43	2.56 ± 0.25
58 µg/g	12.97 ± 1.34	3.77 ± 0.84	1.95 ± 0.13

n = 4, $*p < 0.05$

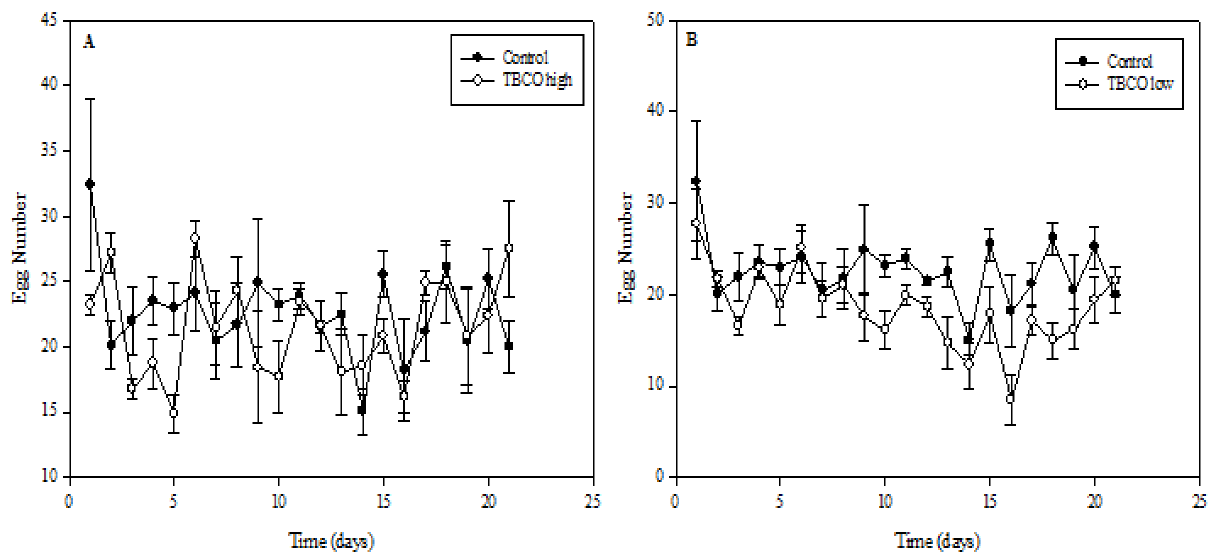

Figure C4.S1 Profile analysis of daily fecundity of (A) solvent control vs. the high dose of TBCO and (B) solvent control vs. the low dose of TBCO. The experiment included 4 replicate tanks, and each contained 8 female fish. The profile (parallelism) of TBCO low was statistically different than solvent control. Significant differences of parallelism were set at $p < 0.05$.

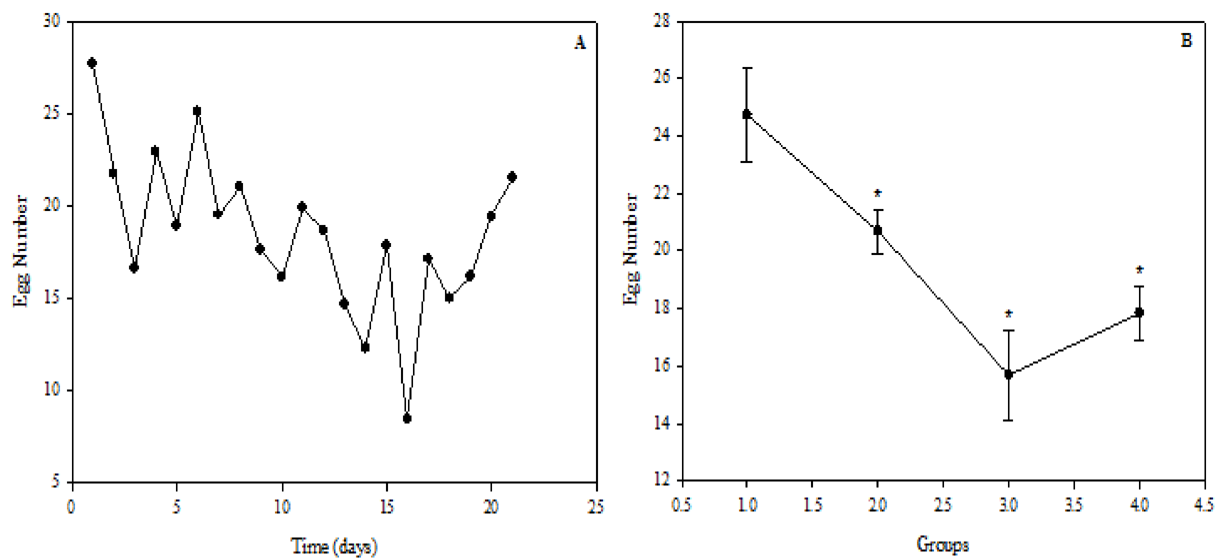

Figure C4.S2. Within-group repeated measures analysis of variance of (A) daily deposition of eggs and (B) pooled time-points of fish exposed to the lesser concentration of TBCO. Time-points were pooled to preserve significant differences after Bonferroni adjustments. Asterisks indicate significant differences ($p < 0.05$) when compared to 100% fecundity (group 1). Significant within-group main effects were also observed in daily egg production.

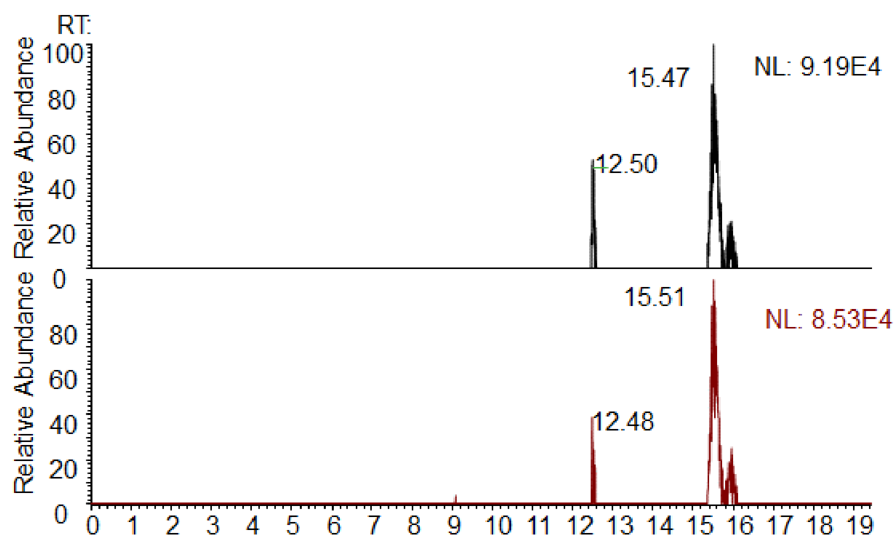

Figure C5.S1. Chromatogram of extracted ions with *m/z* 640.9946 (10 ppm window) in negative ion mode for commercial standard using pure methanol as mobile phase.

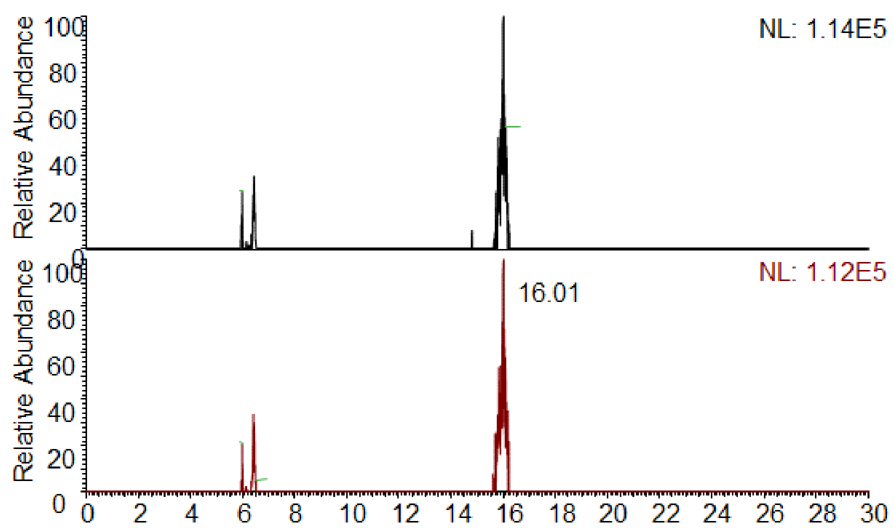

Figure C5.S2. Chromatogram of extracted ions with *m/z* 640.9946 (10 ppm window) in negative ion mode for highly purified standard (AccuStandard, Connecticut, U.S.).

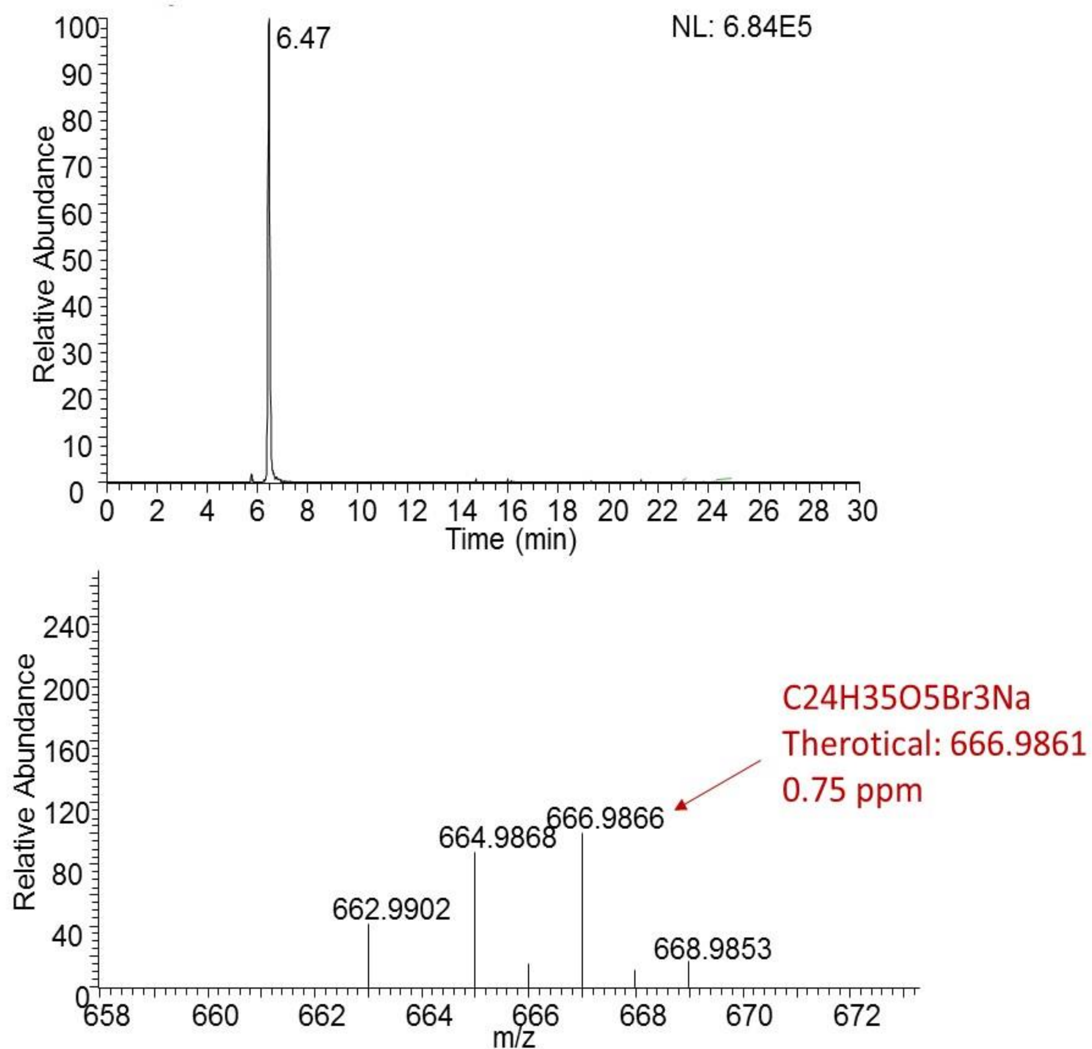

Figure C5.S3 (A) Chromatogram of extracted ions with *m/z* 666.9861 (10 ppm window) in positive ion mode for BZ-54 standard. (B) Mass spectra of OH-TBPH in positive ion mode with mass error of 0.75 ppm to sodium adduct.

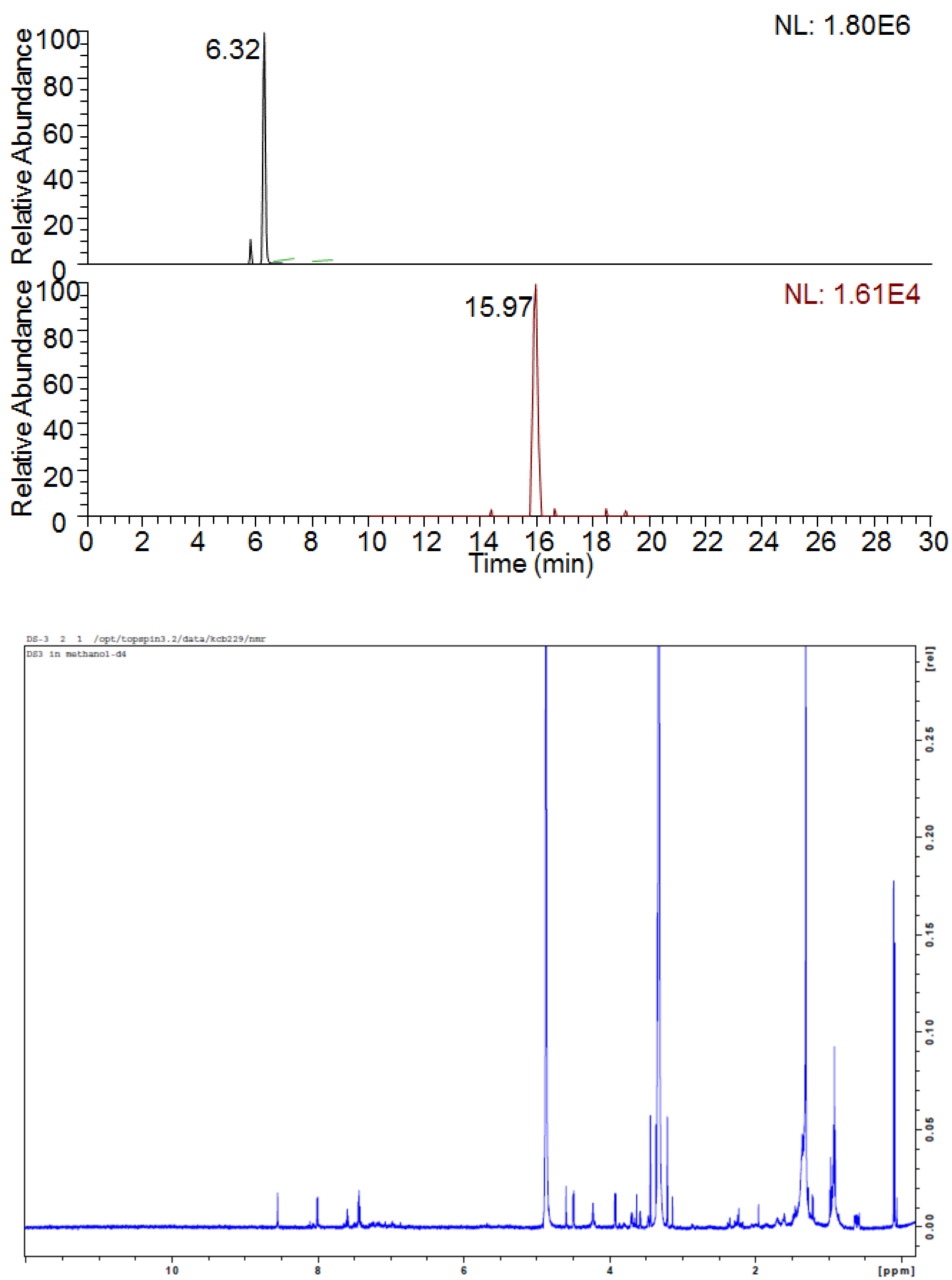

Figure C5.S4. Ultra-High Resolution LC/mass spectrometry (above) and ^{1}H NMR (bottom) analysis of purified OH-TBPH standards. The impurity of TBPH was 100-fold lower than OH-TBPH2 in purified standard.

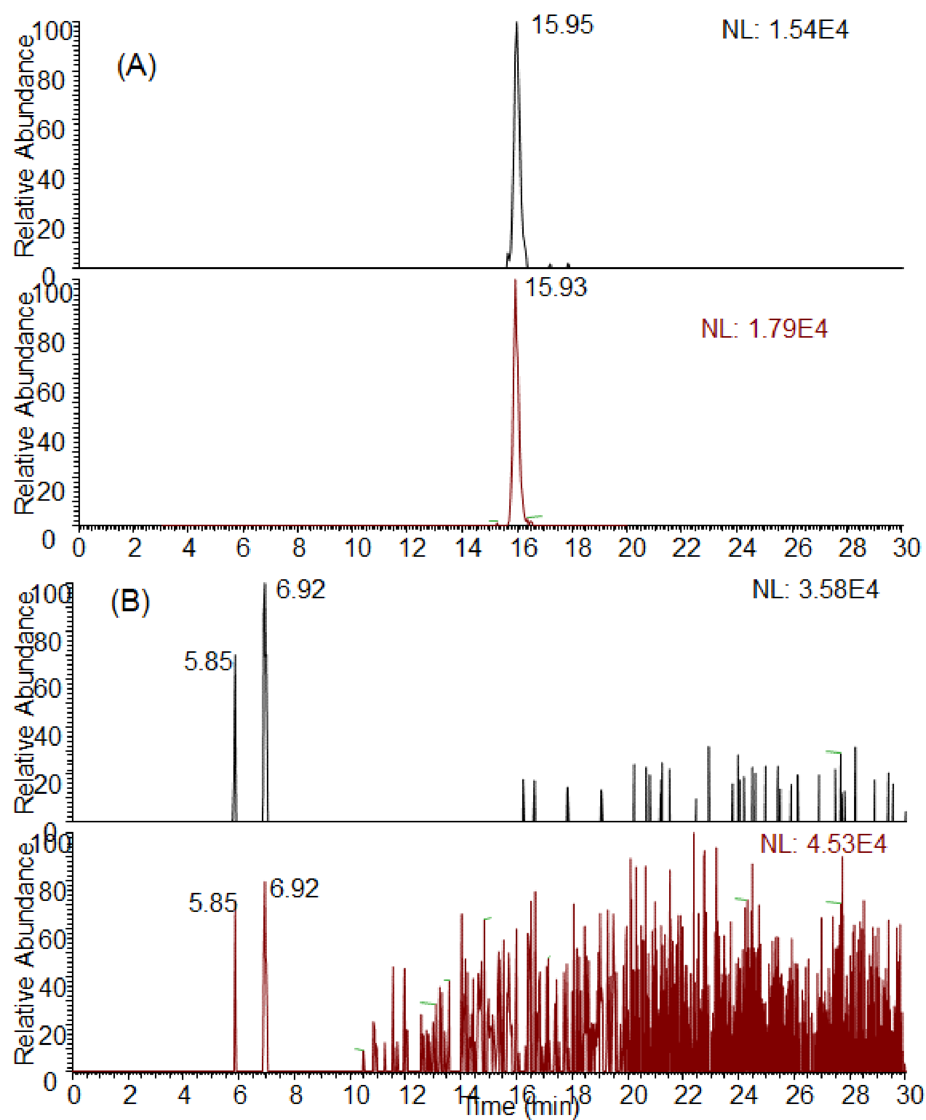

Figure C5.S5. (A) TBPH was eluted in the first fraction from Florisil cartridges using DCM; (B) TBPH isomers were eluted in the third fraction from Florisil cartridges using a mixture of methanol:DCM (v/v, 1:1).

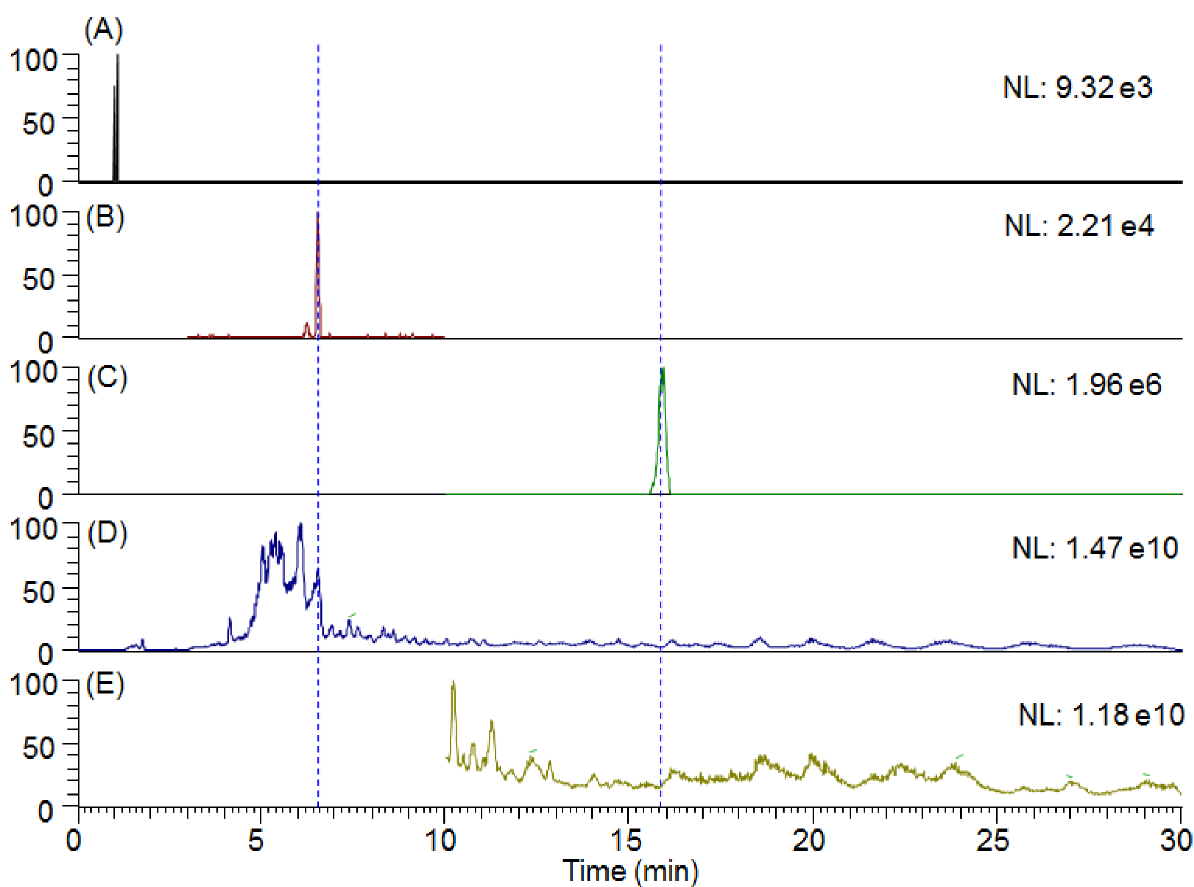

Figure C5.S6. Comparison of the SIM mode and full scan mode for OH-TBPH analysis in dust samples. (A) OH-TBPH isomers could not be detected under full scan mode when ions were extracted in a 10 ppm window. (B) Two OH-TBPH isomers were successfully detected using SIM mode when ions were extracted in a 10 ppm window. (C) TBPH was observed in full scan mode. (D) The total ion intensity in negative ion mode was much greater than those of OH-TBPH at the similar elution time. (E) Total ion intensity in positive ion mode and comparison to TBPH intensity.

Table C6.S1. Ionization sources, ions, and instrumental detection limits for the analysis of TBPH, TBB, and their OH-isomers.

	ESI			APCI		
	Ion mode	*m/z*	IDL[a]	Ion Mode	*m/z*	IDL[a]
TBPH	Positive	723.9486	0.01	-	-	-
OH-TBPHs	Negative	640.9946	0.005	-	-	-
TBB	-	-	-	Negative	484.8789	0.83
OH-TBBs	Negative	484.8789	0.008	-	-	-

[a]Instrumental detection limit (ug/L). IDLs have been reported in our previous articles[163, 164].

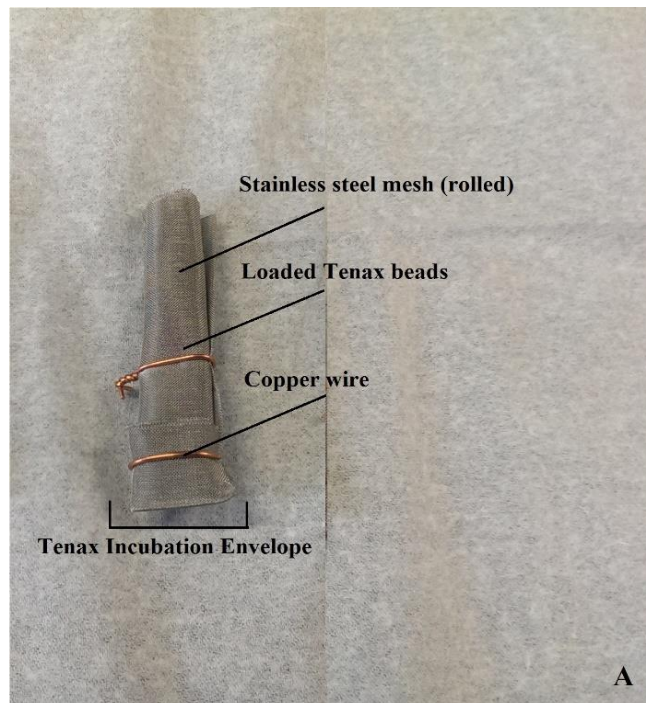

Figure C6.S1. Schematic depicting (A) a pre-loaded Tenax incubation envelope, and (B) Tenax loaded (sealed) incubation envelopes.

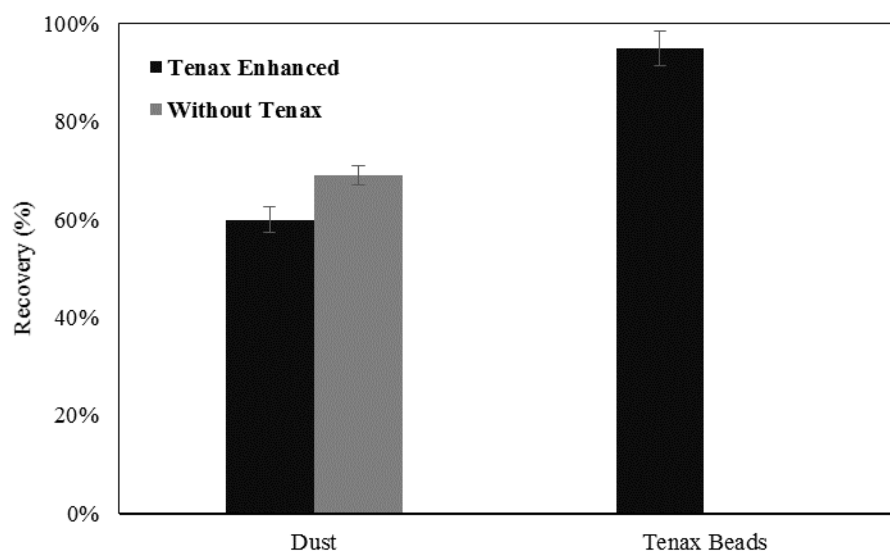

Figure C6.S2. Recovery of Tenax and dust (NIST) following incubation in CE-PBET (n=6). Error bars represent standard deviation.

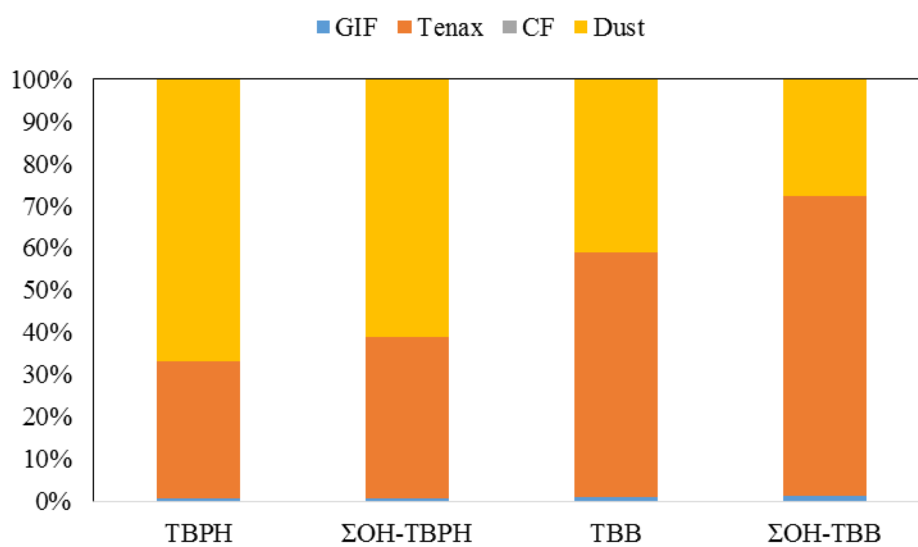

Figure C6.S3. Distribution of TBPH, TBB or their OH-isomers in gastro-intestinal fluid, Tenax, colon fluid, and dust.

Table C6.S2. Measurements of bioaccessibility for TBPH, TBB and their OH-isomers in dust samples (DS) (n = 14).

		TBPH	ΣOH-TBPHs	TBB	ΣOH-TBBs
Summer	DS-1	22%	26%	44%	84%
	DS-2	39%	9%	33%	34%
	DS-3	32%	45%	56%	86%
	DS-4	15%	43%	67%	71%
	DS-5	8%	21%	61%	66%
	DS-6	11%	12%	53%	78%
	DS-7	23%	11%	60%	67%
Winter	DS-8	25%	35%	70%	81%
	DS-9	9%	40%	74%	71%
	DS-10	43%	55%	19%	35%
	DS-11	22%	21%	25%	47%
	DS-12	13%	11%	72%	69%
	DS-13	34%	18%	32%	66%
	DS-14	19%	32%	61%	85%

DS-1 to DS-7 were collected in summer, 2013 and DS-8 to DS-14 were collected in winter, 2014.

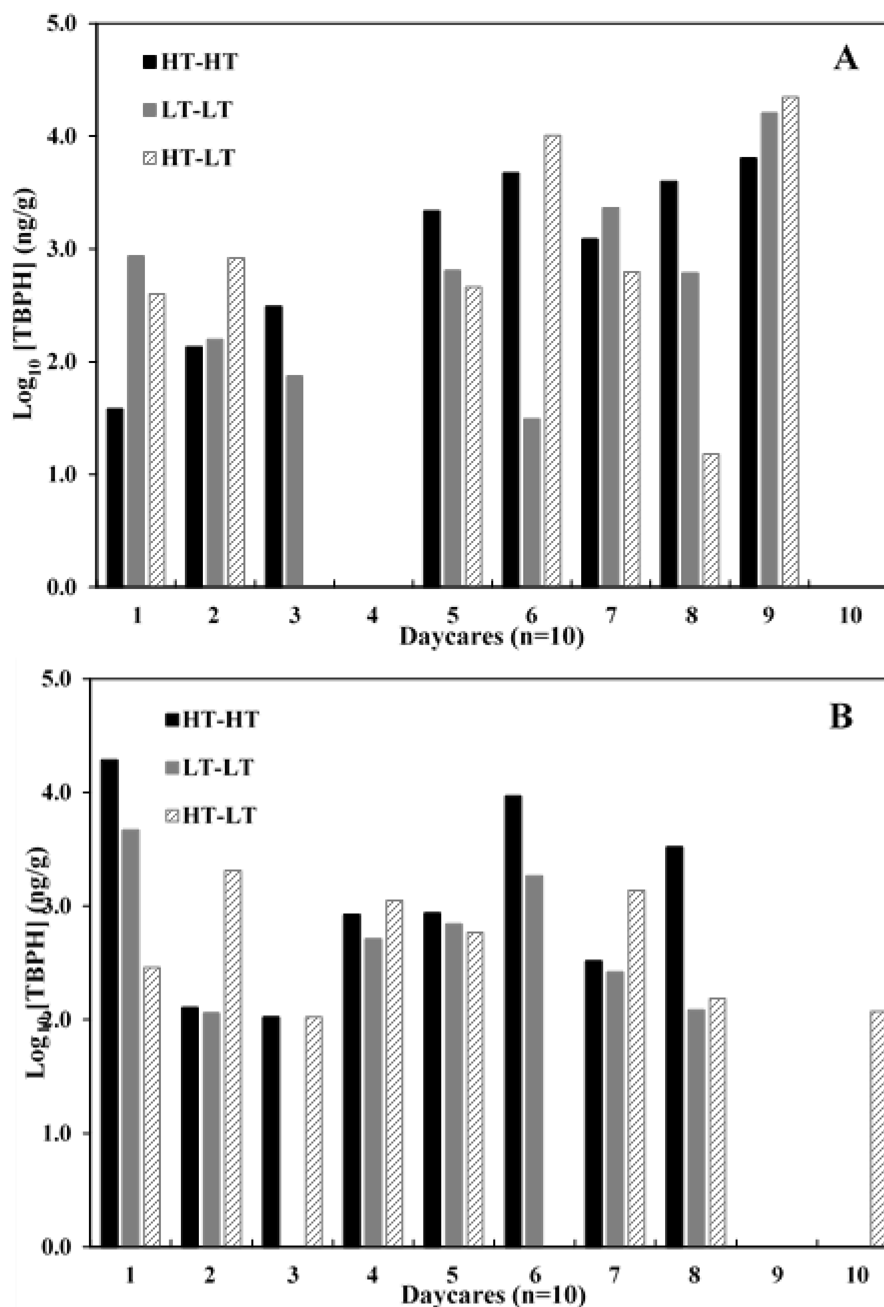

Figure C6.S4. Log transformed concentration of TBPH in higher traffic-higher toy environments (HT-HT), lower traffic-lower toy environments (LT-LT), and higher traffic-lower toy environments (HT-LT). Dust was collected from each of these environments in ten daycares across Saskatoon, SK, Canada in summer (A), and winter (B).

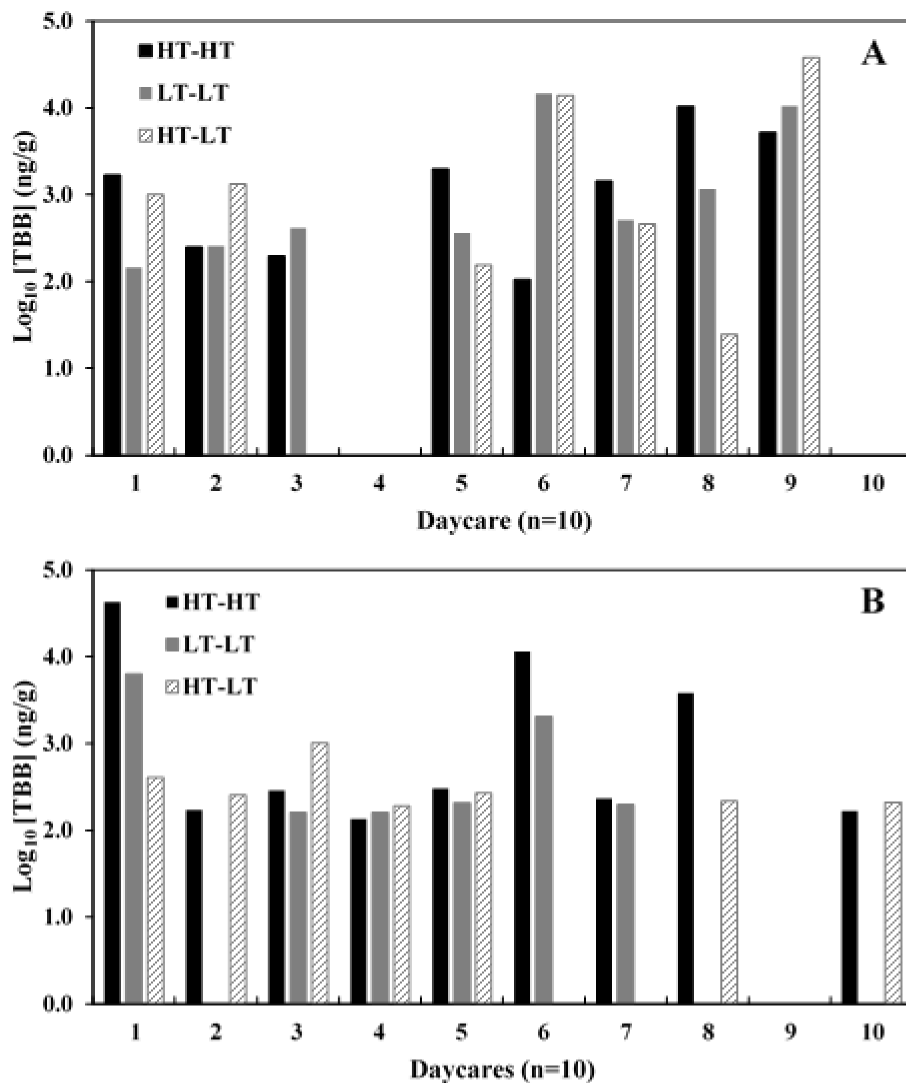

Figure C6.S5. Log transformed concentration of TBB in higher traffic-higher toy environments (HT-HT), lower traffic-lower toy environments (LT-LT), and higher traffic-lower toy environments (HT-LT). Dust was collected from each of these environments in ten daycares across Saskatoon, SK, Canada in summer (A), and winter (B).

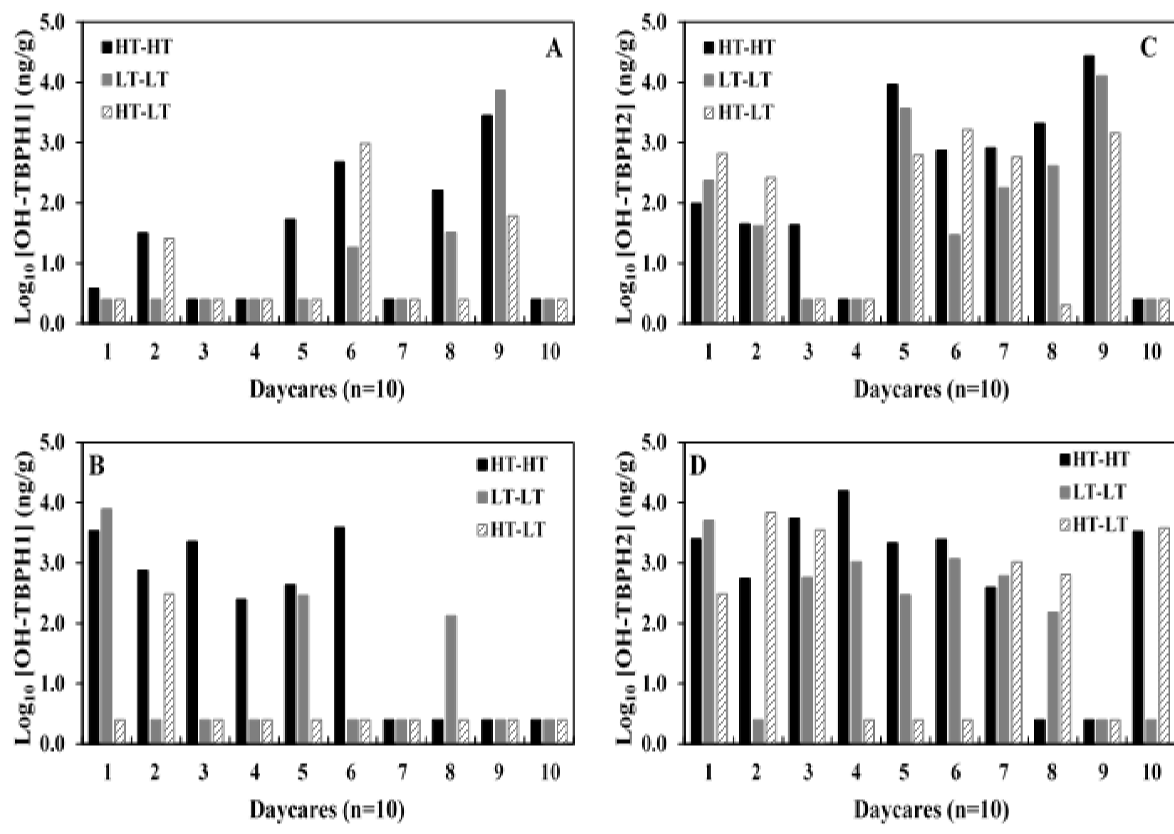

Figure C6.S6. Log transformed concentration of OH-TBPH1 (A,B) and OH-TBPH2 (C,D) in higher traffic-higher toy environments (HT-HT), lower traffic-lower toy environments(LT-LT), and higher traffic-lower toy environments (HT-LT). Dust was collected from each of these environments in ten daycares across Saskatoon, SK, Canada in summer (A,C), and winter (B,D).

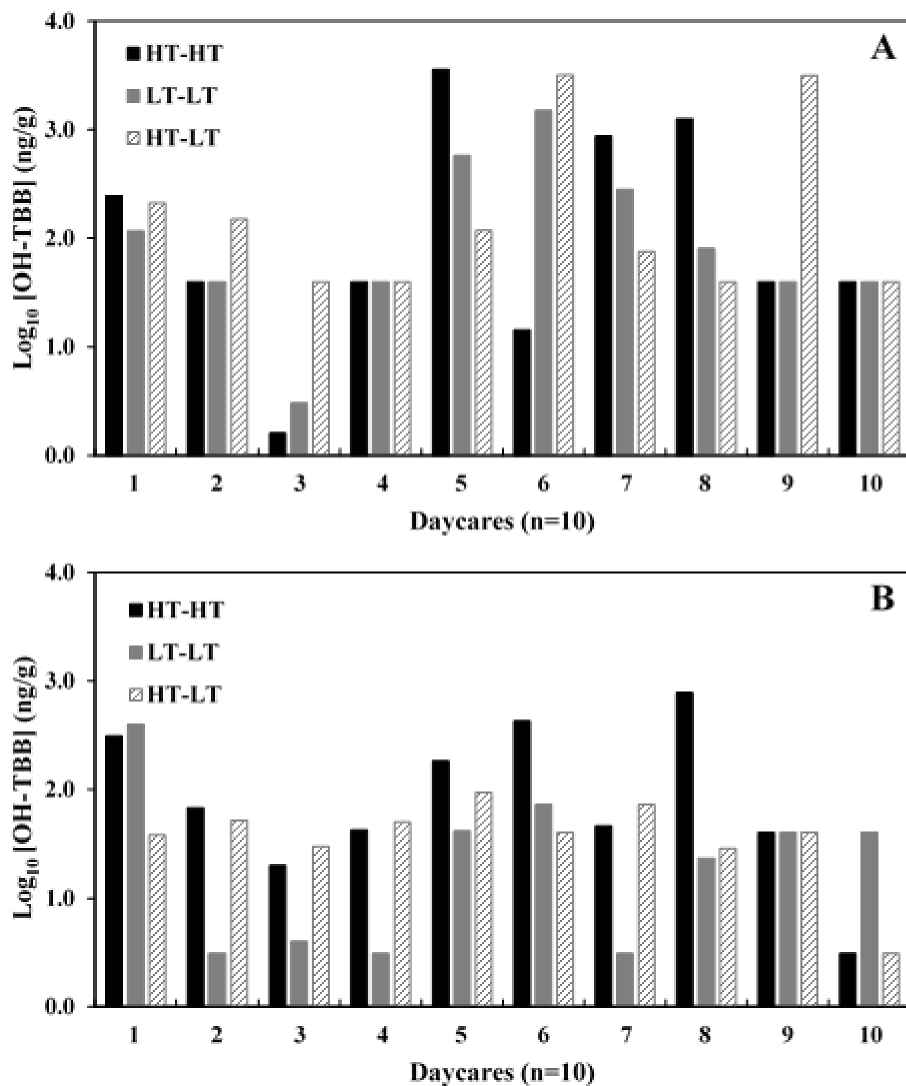

Figure C6.S7. Log transformed concentration of and ΣOH-TBB1/2/3 in higher traffic-higher toy environments (HT-HT), lower traffic-lower toy environments (LT-LT), and higher traffic-lower toy environments (HT-LT). Dust was collected from each of these environments in ten daycares across Saskatoon, SK, Canada in summer (A), and winter (B).