The persistence, bioaccumulation, and inherent toxicity of two organophosphate flame retardants tris (2-butoxyethyl) phosphate and tris (1-chloro-2-propyl) phosphate in juvenile rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Organophosphate flame retardants (OPFRs) are additive flame retardants that have been used for many decades in a variety of different products to suppress or delay the combustion of the treated materials in order to meet flammability standards. Due to the recent ban of several polybrominated diphenyl ether (PBDE) congeners, the use of alternative or replacement FRs has significantly increased, including OPFRs. Tris (2-butoxyethyl) phosphate (TBOEP) and tris (1-chloro-2-propyl) phosphate (TCIPP) are two OPFRs that have been identified as potential replacements for PBDEs. The increase in production volumes of OPFRs, their ubiquitous presence in the environment, and little known toxicological effects was the impetus for our current study. The purpose of this study was to assess the persistence, bioaccumulation, and inherent toxicity (PB&iT) of these two compounds in juvenile rainbow trout (*Oncorhynchus mykiss*). The fish were exposed to either TBOEP or TCIPP, at environmentally relevant concentrations, via their diet to determine the bioaccumulation potential and endocrine effects. The environmentally relevant concentrations used in the food were determined by the levels found in Lake Ontario lake trout. The exposure period was 49 days, where fish were fed the OPFR fortified food in two tanks and a reference diet in the third, which was followed by 98 days depuration phase in which all fish were fed a reference diet. Tissue and plasma samples were taken to measure various endpoints, including liver and gonad somatic indices, sex, cortisol, and thyroid hormone

production, and accumulation of the OPFRs. TBOEP did not show any appreciable bioaccumulation in muscle tissue and concentrations were similar to the method detection limit, and therefore bioaccumulation parameters could not be calculated. Bioaccumulation of TCIPP was linear during the uptake phase and reached a maximum concentration of 3.6 ± 1.3 pmole/g ww. TCIPP followed first-order depuration kinetics, from which the half-life ($t_{1/2}$) was calculated (50.2 ± 19.3 days). The biomagnification factor (BMF) was calculated to be 0.8 ± 0.4, indicating that TCIPP has a low probability of biomagnifying in aquatic food webs. Neither parent nor suspected metabolites were measureable in the liver on day 49 of the uptake phase. There were some significant differences in the molar amounts of estradiol (E2), testosterone (T), cortisol (CT) and thyroid (T4 and T3) hormones in the plasma of fish from the treated and untreated groups; however, there were no consistent trends among any of the hormones. There was a significant decrease ($p < 0.05$) in total T4 and T at day 49 in TCIPP exposed fish relative to the reference fish. We conclude that TCIPP and not TBOEP fulfill the bioaccumulative and persistent criteria in rainbow trout, and further study of the effects on hormones is necessary to draw any conclusions.

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ca.	about, around
α	assimilation efficiencies
µL	microlitre
µg	micrograms
^{125}I	labelled iodine
$^{13}C_x$	labelled carbon 13
ACN	acetonitrile
AChE	acetylcholinesterase enzyme
ADL	analytical detection limit
B	bioaccumulative
BAF	bioaccumulation factor
BCF	bioconcentration factor
BFRs	brominated flame retardants
CEPA	Canadian Environmental Protection Act
CT	cortisol
d^{-1}	growth rate
DCM	dichloromethane
DDT	dichlorodiphenyltrichloroethane
d_x	deuterium
E2	17β-estradiol
EDCs	endocrine disrupting chemicals
FM 550	Firemaster 550
FRs	flame retardants
g	grams
GC	gas chromatography
GSI	gonad somatic index
HBCD	hexabromocyclododecane
HPLC	high performance liquid chromatography
IPS	internal precision standard
iT	inherent toxicity
k_d	depuration rates
L	litre
LC	liquid chromatography
log K_{ow}	octanol-water partition coefficient

LSI	liver somatic index
lw	lipid weight
m/z	mass to charge ratio
MDL	method detection limit
MeOH	methanol
mg	milligrams
mL	millilitre
mol	mole
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometer
MS-222	Tricaine methanesulfonate
n	Sample size
ng	nanogram
n/a	not available
NPD	nitrogen-phosphorous detector
OH-BDEs	hydroxylated metabolites of PBDEs
OP	organophosphate
OPFRs	organophosphorus flame retardant
P	persistent
PAHs	polycyclic aromatic hydrocarbons
PB&iT	persistence, bioaccumulation, and inherent toxicity
PBDEs	polybrominated diphenyl ethers
PCBs	polychlorinated biphenyls
POPs	persistent organic pollutants
RIA	radioimmunoassay kits
RIS	recovery internal standard
REACH	Registration, Evaluation, and Authorisation of Chemicals
S/N	signal to noise ratio
SC	Stockholm Convention
SEM	standard error of the mean
SPE	solid phase extraction
STPs	sewage treatment plants
T	testosterone
$t_{1/2}$	half-life
T3	triiodothyronine

T4	thyroxine
TBECH	tetrabromoethylcyclohexane
TBOEP	tris (2-butoxyethyl) phosphate
TBP	tri-n-butyl phosphate
TCEP	tris (chloro-ethyl) phosphate
TCIPP	tris (1-chloro-2-propyl) phosphate
THs	thyroid hormones
TTR	transthyretin
TPhP	triphenyl phosphate
TSH	thyroid stimulating hormone
ww	wet weight

1. Literature Review

1.1 The Stockholm Convention on Persistent Organic Pollutants

The Stockholm Convention (SC) came into effect in 2004 and is a global treaty designed to protect human health and the environment from persistent organic pollutants (POPs) (Lambert *et al*., 2011). POPs are chemicals that are persistent in the environment (P), undergo long-range transport, bioaccumulate through the food web (B), and are inherently toxic (iT). If chemicals meet the criteria for P, B, and iT they are further subject to a more demanding, time-consuming, and costly risk assessment to determine whether they are hazardous (Gobas *et al*., 2009). Chemicals listed by the SC as POPs are either completely phased out, or restrictions are made to limit their production and emissions into the environment (Lambert *et al*., 2011). The original twelve chemicals listed under the SC, known as the dirty dozen, consisted of pesticides (e.g., dichlorodiphenyltrichloroethane, DDT), industrial chemicals (e.g., polychlorinated biphenyls, PCBs), and unintentional by-products of industrial processes (e.g., dioxins and furans). Several flame retardants (FRs) have since been added; in 2010, two formulations of the polybrominated diphenyl ethers (PBDEs), the penta- and octa-BDE congeners, and in 2014, hexabromocyclododecane (HBCD). These bans have led to an increase in the use of previously existing FRs and the development of new, alternative FRs in order to

meet flammability standards, most of which have very little information available on their fate in the environment and their toxicological effects.

1.1.1 Persistence, bioaccumulation and inherent toxicity (PB&iT)

The persistence of a chemical is measured by its half-life ($t_{1/2}$) in various matrices including water, soil, sediments, and air. More recently, biological $t_{1/2}$ have gained acceptance by the SC. The bioaccumulative properties of chemicals are typically determined by the bioconcentration factor (BCF) or the bioaccumulation factor (BAF) in aquatic species, or by its octanol-water partition coefficient (log K_{ow}). The BCF and BAF values are defined as the ratio of the concentration of the chemical in the organism to the concentration of the chemical in the water (BAF includes food uptake). The log K_{ow} of a chemical is determined by the distribution of the chemical between equal volumes of n-octanol and water. A higher affinity for n-octanol indicates the chemical is more likely to accumulate in biota. Inherent toxicity is assessed by the possible adverse effects, such as reproductive toxicity, neurotoxicity, developmental effects, or endocrine disruption in biota and humans (Lambert *et al.*, 2011). Table 1.1 lists the criteria used for screening chemicals under the Canadian Environmental Protection Act (CEPA), European Union (REACH), and Annex D of the Stockholm Convention on POPs (UNEP) for PB&iT assessments.

Table 1.1: Screening criteria for determining PB&iT compounds according to CEPA, REACH, and UNEP (EC, 1999; Lambert *et al*., 2011; Muir & Howard, 2006; UNEP, 2001).

	Criteria		
	CEPA [a]	REACH [b]	Stockholm Convention [c]
Persistence	Half-life (days): - air ≥ 2 - water ≥ 182 - sediments ≥ 365 - soil ≥ 182	Half-life (days): - water ≥ 180 - sediments ≥ 120 - soil ≥ 120	Half-life (months): - air ≥ 2 days - water ≥ 2 - sediment ≥ 6 - soil ≥ 6
Bioaccumulation	- BCF/BAF ≥ 5000 (log BCF/BAF ≥ 3.7) - log K_{ow} ≥ 5	- BCF/BAF ≥ 2000 (log BCF/BAF ≥ 3.3)	- BCF/BAF ≥ 5000 (log BCF/BAF ≥ 3.7) - log K_{ow} ≥ 5
Toxicity	- Have or may have an immediate or long-term harmful effect on the environment or its biological diversity - Constitute or may constitute a danger to the environment on which life depends - Constitute or may constitute a danger in Canada to human life or health	- Long-term EC50 or LC50 aquatic toxicity < 0.01 mg/L - Short-term EC50 or LC50 aquatic toxicity < 0.1 mg/L	- Evidence of effects adverse to human health or the environment that justify its inclusion - Toxicity and ecotoxicity data indicating potential damages to human health or to the environment

[a] CEPA – Canadian Environmental Protection Act (1999)

[b] REACH – Registration, Evaluation, and Authorisation of Chemicals Annex XII (European Commission 2001)

[c] Annex D of the Stockholm Convention on Persistent Organic Pollutants (UNEP 2001)

1.1.2 Example: Polybrominated diphenyl ethers (PBDEs)

PBDEs used to be one of the most widely used brominated flame retardants (BFRs) (Birnbaum & Staskal, 2004). There are three technical mixtures of PBDEs available: penta-, octa- and deca-BDE. PBDEs are used in a wide range of products such as polyurethane foam, plastics in electronic equipment, printed circuit boards, expanded and extruded plastic, textiles, as well as tubes for coating wire (de Wit *et al.*, 2010). The high use of these compounds has resulted in their wide spread contamination in most environmental compartments, including biota and humans. The log K_{ow} of PBDEs ranges from 5.9 to 9.9, indicating that these chemicals are highly lipophilic and have a high potential to bioaccumulate in biota (de Wit, 2002). PBDEs, along with their hydroxylated metabolites, have a potential to elicit toxicological effects on humans and biota including developmental, neurological, reproductive, and endocrine toxicity (de Wit *et al.*, 2010). As a result of this the penta- and octa-BDE congeners have recently been added to the list of POPs as defined by the SC.

1.2 Organophosphorus Flame Retardant (OPFRs)

Flame retardants (FRs) are anthropogenic chemicals used as additives in a variety of different products such as plastics, foams, textiles, and furniture to suppress or delay the combustion of the treated materials and to meet regulatory flammability standards (Chen *et al.*, 2012; Stapleton *et al.*, 2009). There are two types of FRs:

reactive and additive. Reactive FRs are chemically bound to the polymer, whereas additive FRs are mixed into the polymer and they are not chemically bound to the host product. Additive FRs are more readily available to diffuse out into the surrounding environment by volatilization, leaching, or abrasion processes (Campone *et al.*, 2010; de Wit, 2002; Marklund Sundkvist *et al.*, 2010; Reemtsma *et al.*, 2008). There are more than 175 classified FRs which can be sorted into four main groups: organic halogen, organophosphorus, nitrogen-based organic, and inorganic compounds (Birnbaum & Staskal, 2004).

The total consumption of FRs in Europe in 2006 was 465,000 tonnes, of which 20% were organophosphorus FRs (OPFRs) (Figure 1.1) (van der Veen & de Boer, 2012).

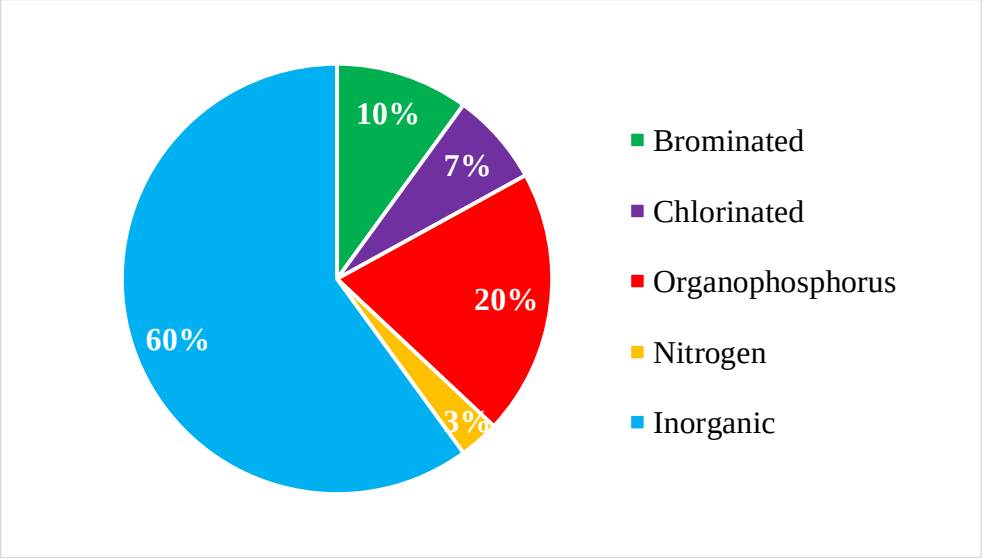
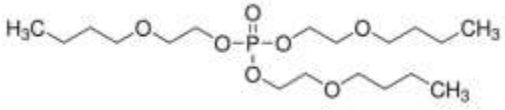
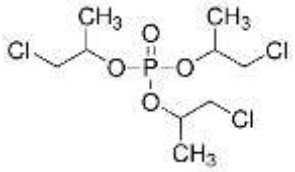


Figure 1.1: Industry estimate of total consumption of flame retardants in Europe in 2006 (CEFIC, 2013; van der Veen & de Boer, 2012).

OPFRs have been used for many decades, and their use dates back to the 1970's (Reemtsma *et al.*, 2008). The first studies on the fate, bioaccumulation and biodegradation of OPFRs were conducted by Muir *et al.* (1980; 1981; 1983) in the 1980's, but further studies on OPFRs were suspended in the 1990's as most OPFRs were found to be degradable in the environment (Reemtsma *et al.*, 2008). However, their use has increased greatly due to the recent ban and voluntary phase out of several of the PBDE congeners (Reemtsma *et al.*, 2008). Increasing their use has led to their detection in many environmental compartments including surface, ground, and drinking water, wastewater, air, sediment, dust, human biological samples, and biota, both in Europe and North America (Campone *et al.*, 2010; Nacher-Mestre *et al.*, 2011). Considering these FRs are now ubiquitous, there is very little information on their fate in the environment and their effects on wildlife and humans. As a result, research on these chemicals is now re-emerging. Tris (2-butoxyethyl) phosphate (TBOEP) and tris (1-chloro-2-propyl) phosphate (TCIPP) are two examples of additive OPFRs that are still in use today (Figure 1.2) and were examined in my thesis.



Tris (2-butoxyethyl) phosphate (TBOEP)



Tris (1-chloro-2-propyl) phosphate (TCIPP)

Figure 1.2: Chemical structures of TBOEP and TCIPP.

1.2.1 How Flame Retardants Work

The basic mechanisms of FRs vary depending on their chemical makeup. In general, FRs interact at different stages of the fire cycle in order to prevent or suppress the combustion of the material. Combustion of a material requires three key components: oxygen, heat, and fuel (Figure 1.3) (CEFIC, 2013). Effective FRs will interfere with one of these three elements, in order to prevent or extinguish the fire.

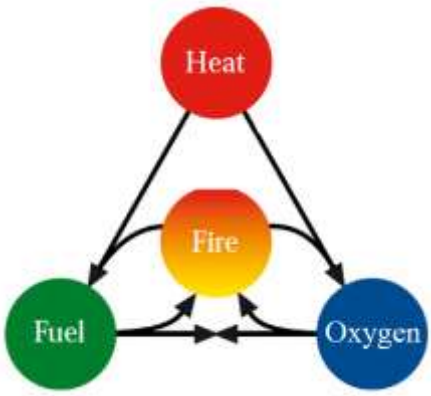


Figure 1.3: Fire triangle (CEFIC, 2013).

When a polymer is heated, it undergoes a degradation process known as pyrolysis. This degrades the material's long-chain molecules into smaller molecules, and emits flammable gases. Combustion occurs when these flammable gases mix with the oxygen from the air via exothermic chemical reactions. The energy released by the exothermic reactions is conveyed back to the polymer, reinforcing pyrolysis, enabling the fire to sustain itself (CEFIC, 2013).

The most effective mechanisms of FRs are reactions that occur either in the condensed phase or in the gas phase of the fire. Halogenated FRs (e.g. BFRs) act in the gas phase, while non-halogenated OPFRs act in the condensed phase. In the gas phase, the halogenated FR removes the free radicals, •H and •OH, from the flammable gases by reacting with the halogen atoms that have been released from the FR upon heating (van der Veen & de Boer, 2012). Removal of the •H and •OH radicals results in a cooling of the system and slows down the burning process. In the condensed phase, the FR builds up a layer of char, which smothers the material and inhibits the oxygen supply, preventing formation of flammable gases (CEFIC, 2013).When heated, the phosphorous is converted to give a polymeric form of phosphoric acid by thermal decomposition. Water is then released from the pyrolysing substrate, causing the substrate to char (CEFIC, 2013). OPFRs act mainly through the condensed phase to contain fires, but some OPFRs, such as TCIPP, contain both phosphorous and chlorine or bromine atoms, thus combining different FR mechanisms to increase their effectiveness (CEFIC, 2013).

1.2.2 Industrial Synthesis of TBOEP and TCIPP

OPFRs are industrially produced by reacting phosphorous oxychloride ($POCl_3$) with various other reactants (Marklund, 2005). TBOEP is produced by the reaction of phosphorous oxychloride ($POCl_3$) and butoxyethanol ($C_6H_{14}O_2$) and stripping

the hydrochloric acid and the excess butoxyethanol (Verbruggen *et al.*, 2005) (Figure 1.4).

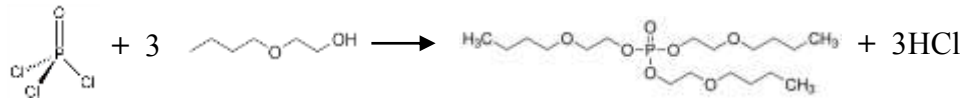


Figure 1.4: Industrial synthesis of TBOEP

TCIPP is produced by the reaction of phosphorous oxychloride ($POCl_3$) and propylene oxide (C_3H_6O) (Verbruggen *et al.*, 2005) (Figure 1.5). The entire process (from reaction to packaging) is carried out in a closed system (EU, 2008). The final commercial product of TCIPP consists of a mixture of four isomers: tris (2-chloro-1-methylethyl) phosphate (50 - 85%), bis (2-chloro-1-methylethyl)-2-chloro-1-propyl phosphate (15 - 40%), bis (2-chloro-1-propyl)-2-chloro-1-methylethylphosphate (< 15%), and tris (2-chloro-1-propyl) phosphate (< 1%) (Verbruggen *et al.*, 2005). The individual isomers are not separated or produced separately.

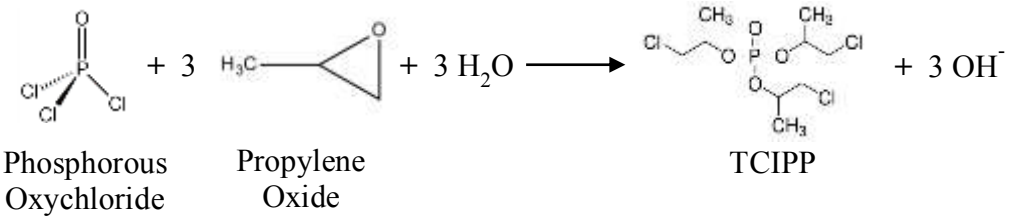


Figure 1.5: Industrial synthesis of TCIPP

1.2.3 Uses and Applications of TBOEP and TCIPP

TBOEP is used mainly as an antifoaming agent, plasticizer in rubber and plastics, and in floor polishes and to a lesser extent as a flame retardant (Andresen *et al.*, 2004; WHO, 2000). The worldwide production volume was estimated to range from 500 to 5000 tons in 2010 (Todd *et al.*, 2012). TCIPP is used as a flame retardant mostly in polyurethane foam used in furniture and as a plasticizer (Brandsma *et al.*, 2014). TCIPP is used as one of the substitute FRs for the banned penta-BDE congener and is also the main replacement product of the tris (chloro-ethyl) phosphate (TCEP) FR, which has been phased out due to its carcinogenic and neurotoxic properties in rats and mice (Brandsma *et al.*, 2014; Leonards *et al.*, 2011). In 2010, it was estimated that the production of TCIPP was in the range of 5000 to 25000 tons (Todd *et al.*, 2012).

1.2.4 Physical and Chemical Properties of TBOEP and TCIPP

The physicochemical properties of TBOEP and TCIPP are listed in Table 1.2. The physicochemical properties of OPFRs are particularly variable and are dependent on the alcohol moieties esterified to the phosphoric acid core of the OPFR being considered (Reemtsma *et al.*, 2008). The potential for OPFRs to biodegrade decreases with chain length and the chlorinated OPFRs are more resistant to degradation in comparison to alkyl or aryl compounds (Marklund, 2005). The half-lives of TCIPP in water, soil, and sediment are greater than the criteria set by CEPA,

REACH, and the SC (Table 1.1), indicating that TCIPP is expected to be persistent in the environment and it does not biodegrade readily. The octanol-water partition coefficient (log K_{ow}) of a chemical is used to predict its potential to bioaccumulate. Generally, the higher the log K_{ow} value, the higher the chemicals ability to bioaccumulate in biota (Evenset *et al.*, 2009). A chemical is considered potentially bioaccumulative as defined by the SC if it has a BCF greater than 5000 or a log K_{ow} greater than 5 (Table 1.1). Therefore, based solely on their log K_{ow} it can be predicted that TBOEP and TCIPP would not bioaccumulate in biota.

Table 1.2: Physicochemical properties of TBOEP and TCIPP.

Property	**TBOEP**	**TCIPP**	**Reference**
Physical state	Liquid, colourless	Liquid, colourless	(WHO, 1998; 2000)
Molecular mass (g/mol)	398.47	327.57	(WHO, 1998; 2000)
Chemical formula	$C_{18}H_{39}O_7P$	$C_9H_{18}Cl_3O_4P$	(WHO, 1998; 2000)
Boiling point (°C)	200-230	235-248	(WHO, 1998; 2000)
Vapour Pressure (Pa)	3.33×10^{-6}	2.69×10^{-3}	(Marklund, 2005)
Octanol-water partition coefficient (log K_{ow})	3.75	2.59	(Marklund, 2005)
Water solubility (mg/L at 25^0C)	1100	1200	(Marklund, 2005)
Henry's Law Constant (Pa.m^3/mol at 25^0C)	1.22×10^{-6}	4.25×10^{-4}	(van der Veen & de Boer, 2012)
Half-life in water (days) (estimated)	n/a	150	(Lambert *et al.*, 2011)
Half-life in soil (days) (estimated)	n/a	300	(Lambert *et al.*, 2011)
Half-life in sediment (days) (estimated)	n/a	3000	(Lambert *et al.*, 2011)
Bioconcentration Factor (BCF)	< 5.8 in carp	0.8 – 2.8 in carp	(Lambert *et al.*, 2011)

n/a: data not available

1.2.5. Environmental Levels and Toxicology

TBOEP and TCIPP are ubiquitous global contaminants that have been detected in ground water, surface water, wastewater and sewage sludge, air and dust, soils and sediments, human biological samples including human breast milk and serum, and biota (including birds and various aquatic organisms) (Campone *et al.*, 2010; Chen *et al*., 2012; Marklund Sundkvist *et al*., 2010; Reemtsma *et al.*, 2008).

TBOEP and TCIPP may be released during manufacturing, during incorporation into polymers, and during the entire lifecycle of the FR products into the environment (Leisewitz *et al*., 2000). Given that both TBOEP and TICPP are applied as additive FRs and are not chemically bonded to the final products, they can leach from the material and into the environment. The summary of the environmental distribution of TBOEP and TCIPP are listed in Table 1.3.

Water

TBOEP and TCIPP have been quantified in the influents, effluents, and sludge from sewage treatment plants (STPs) (Andresen *et al.*, 2004). TBOEP was generally the most prevalent OPFR found in influents and effluents, followed by the chlorinated OPFRs, including TCIPP (Marklund *et al*., 2005). TBOEP concentrations in STP influents and effluents ranged from 5.2 to 20.0 µg/L and 1.6 to 11.0 µg/L, respectively (Green *et al*., 2008; Marklund *et al*., 2005); while TCIPP concentrations in Swedish and Norwegian STP influents and effluents were in the

range of 1.1 to 3.4 µg/L and 1.5 to 2.4 µg/L, respectively (Green *et al*., 2008; Marklund *et al*., 2005). Both TBOEP and TCIPP were poorly removed from the wastewater. Marklund *et al*. (2005) showed that the percent elimination of TBOEP and TCIPP from the water were between 15 - 60% and 0 - 40%, respectively. Another study showed that TBOEP had an average removal of 80 - 90%, whereas TCIPP showed no significant removal at all (Meyer & Bester, 2004).

The high concentrations of TBOEP and TCIPP in STP effluent water raise some concern on concentrations in surface and ground waters, as well as aquatic biota. The levels of TBOEP and TCIPP detected in rivers and lakes greatly depend on the local emissions and dilutions, making it difficult to determine typical concentrations (Reemtsma *et al*., 2008). Levels of TBOEP and TCIPP reported in Lake Ontario ranged from < 2 to 1487 ng/L, with the higher concentrations found within urban areas (Lee *et al*., 2012). Similar concentrations of TBOEP and TCIPP were found along the River Ruhr in Germany ranging from 10 to 200 ng/L, with the higher levels occurring downstream of STP discharges (Andresen *et al*., 2004).

Air and dust

TBOEP and TCIPP have been detected in indoor air and dust samples. TCIPP is more volatile than TBOEP, and therefore is found at higher concentrations in air samples. The global mean concentrations of TBOEP and TCIPP, according to Reemtsma *et al*. (2008) are 15 and 84 ng/m^3, respectively. The distribution patterns

of OPs have a tendency to reflect the materials and products used in that environment (Marklund *et al*., 2003). The average concentrations of TBOEP and TCIPP in house dust in Belgium is 6.58 and 4.82 µg/g, respectively (Van den Eede *et al*., 2011). These levels can be compared to the ΣPBDEs at a concentration of 0.70 µg/g and ΣHBCDs at a concentration of 1.74 µg/g (Van den Eede *et al*., 2011). Salamova *et al.* (2014) measured atmospheric concentrations (in the particle phase) of 12 OPFRs at five sites in the North American Great Lakes basin. The urban sites dominated in chlorinated compounds, including TCIPP, whereas the non-halogenated compounds (TBOEP) were present in large amounts at the remote sites. TCIPP ranged from 25 to 850 pg/m^3 and TBOEP ranged from 67 to 330 pg/m^3 at the five sampling sites (Salamova *et al.*, 2014). These levels, in combination with the other ten OPFRs sampled, are 2 - 3 orders of magnitude higher than BFRs.

Biota and Human

TBOEP and TCIPP have been detected in various biota including herring gull eggs, perch, and human breast milk samples. Pooled samples of herring gull eggs from Channel-Shelter Island colony (Lake Huron) contained levels of TBOEP and TCIPP ranging from < 0.15 to 20.4 and 3.7 to 55.4 ng/g lipid weight (lw), respectively (Chen *et al.*, 2012). Fresh water perch in Sweden ranged from 240 to 1000 ng/g lw for TBOEP and 170 to 770 ng/g lw for TCIPP (Marklund *et al*., 2010). The concentrations of TBOEP and TCIPP in lake trout from the Lake Ontario food

web study ranged from 0.32 to 9.81 and 0.06 to 0.32 ng/g wet weight (ww), respectively (Table 3.1). The levels detected in breast milk in Sweden range from non-detect (n.d.) to 63 ng/g lw for TBOEP and 22 to 82 ng/g lw for TCIPP (Letcher & Chen, 2012).

Table 1.3: Summary of the environmental distribution of TBOEP and TCIPP.

Environmental Compartment	**TBOEP**	**TCIPP**
STP influents (Sweden)	5.2 - 20.0 µg/L	1.1 - 3.4 µg/L
STP effluents (Sweden)	1.6 - 11.0 µg/L	1.5 - 2.4 µg/L
Water (Lake Ontario)	<2 -325 ng/L	12 - 487 ng/L
Water (River Ruhr, Germany)	10 - 200 ng/L	10 - 200 ng/L
Atmosphere (Great Lakes)	67 - 330 pg/m^3	25 - 850 pg/m^3
House dust (Belgium)	6.58 µg/g	4.82 µg/g
Herring gull eggs (Lake Huron)	< 0.15 - 20.4 ng/g lw	3.7 - 55.4 ng/g lw
Fresh water perch (Sweden)	240 - 1000 ng/g lw	170 - 770 ng/g lw
Lake trout (Lake Ontario)	0.32 - 9.81 ng/g ww	0.06 - 0.32 ng/g ww
Breast milk (Sweden)	n.d. - 63 ng/g lw	22 - 82 ng/g lw

Toxicology

There is very little information available on the toxicological effects of OPFRs, and TBOEP and TCIPP in particular. OPFRs may possess possible carcinogenic, neurotoxic, and endocrine disrupting properties and they may also interfere with liver toxicity and growth during long term exposure (Ali *et al.*, 2012; Brommer *et al.*, 2012). Further research is required to identify and determine the possible effects

of the various OPFRs in humans and wildlife. According to Lambert *et al.* (2011), TBOEP and TCIPP are both considered as toxic compounds (Table 1.4).

Metabolism

OPFRs can be metabolized to phosphoric acid diesters and monoesters through phase I biotransformation mechanisms (Chu *et al*., 2011). There is very limited data on the metabolized forms of the OPFRs or their occurrence and behaviour in the environment. The metabolized forms may be used as biomarkers for their parent triester OPFRs in biota samples (Chu *et al*., 2011). The major metabolite of TBOEP is bis (2-butoxyethyl) phosphate (BBOEP), as well as various other metabolites and the major metabolite of TCIPP is bis (1-chloro-2-propyl) 1-carboxy phosphate (BCIPP) (van den Eede *et al*., 2013). The biotransformation products of OPFRs requires further examination because it may be a major factor determining the *in vivo* metabolism and toxicity of the compounds.

Table 1.4: Possible toxicological effects of TBOEP and TCIPP (Lambert *et al*., 2011; WHO, 1998, 2000).

Adverse Effect	**TBOEP**	**TCIPP**
96 hr LC_{50} fathead minnow	11.2 mg/L (not toxic to aquatic organisms)	51 mg/L (not toxic to aquatic organisms)
Carcinogenicity	n/a	- high potential due to the fact that it is structurally similar to TDCPP and TCEP which are considered carcinogens
Mutagenicity	- results from *in vitro* studies do not indicated that compound is genotoxic	- not bacterial mutagen - non-mutagenic in fungi - shows clastogenic activity *in vitro* in presence of metabolic activation
Reproductive/ Developmental Toxicity	n/a	- results of fertility and developmental studies show that TCIPP may cause adverse effects to reproduction and development
Neurotoxicity	- 70% decrease in plasma butyrylcholinesterase (BuChE) activity - 45% inhibition of acetylcholinesterase (AChE) in brain - TBOEP is neurotoxic but large doses are required to cause the damage and the lessening of the nerve conduction velocity	n/a
Endocrine effects	n/a	n/a

n/a: data not available

1.2.6 Analytical Methods for OPFR Analysis

There are no standardized analytical techniques for sample preparation and instrumental analysis of OPFRs. Brandsma *et al*. (2013) designed an interlaboratory study in order to determine the various techniques of OPFR analyses in various abiotic and biotic samples (i.e., standard solutions, dust, fish oil, and sediment samples), as well as to improve the quality of OPFR data. The study outlines the various extraction and clean-up methods and the analytical instruments used for quantification (Brandsma *et al*., 2013). The most commonly used techniques for analysis of OPFRs in environmental samples are gas chromatography coupled to mass spectrometry (GC-MS), a GC coupled to a nitrogen-phosphorous detector (GC-NPD), and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Brandsma *et al*., 2013). A lack of adequate labelled standards and blank contamination are major concerns in the analysis of OPFRs. In the interlaboratory study, TBOEP and TCIPP were among the most predominant OPFRs reported in the blanks (Brandsma *et al*., 2013). In order to minimize the blank contamination in my samples, I used methods described in Brandsma *et al*. (2013), including pre-cleaning all glassware, equipment, and SPE columns and silica with solvent, covering samples and glassware with aluminum foil, and minimizing the surface contact of the sampling.

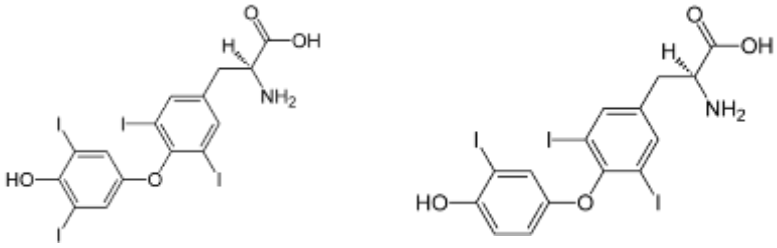
1.3 The Endocrine System

In general terms, the endocrine system can be described as a series of cells, glands, and tissues that synthesize and secrete various hormones into the bloodstream to regulate biological processes and homeostasis (Pait & Nelson, 2002). Hormones are required to regulate various functions in fish including metabolism, growth and development, tissue function, reproduction, mineral and water balance, and immune response (Pait & Nelson, 2002). Hormones can be used as biomarkers or indicators of exposure to pollutants and other environmental stressors, and in many cases provide information on the specific toxic mechanisms of contaminants. Deviations in the various hormone levels may disrupt the proper functioning of the endocrine system and may exert negative effects on the target tissues. Endocrine disrupting chemicals (EDCs) in fish may interfere with hormones in various ways *inter alia* including mimicking the effects of the endogenous hormones, antagonistic effects of the endogenous hormones, alteration in the synthesis and metabolism of the hormones, variation of hormone receptor levels, and they may cause interference with the binding proteins that transport the hormones (Pait & Nelson, 2002).

My thesis examined the effects on the thyroid system, the stress response, and the reproductive system.

1.3.1 The Thyroid System

The thyroid hormones (THs), thyroxine (T4) and triiodothyronine (T3), play a critical role in regulating development and growth, metabolism, osmoregulation, and reproduction in fish (Blanton & Specker, 2007; Eales & Brown, 1993) (Figure 1.6). Synthesis of T4 occurs in the thyroid follicle and is controlled by the brain-pituitary-thyroid axis with the release of the thyroid stimulating hormone (TSH) (Brown *et al*., 2004) (Figure 1.7). TSH regulates T4 secretion and the uptake of iodide by the thyroid follicles. T4 is the primary hormone secreted, but has very few direct actions. T4 essentially acts as a precursor for T3, which is the biologically active form of the hormone. The conversion of T4 to T3 occurs in peripheral tissues and requires the removal of one iodide unit of the outer ring of T4 by a deiodinase enzyme (Brown *et al*., 2004). T3 and T4 circulate in the plasma in either free (unbound) form (FT3, FT4) or bound to transport proteins. Total T3 and T4 (TT3, TT4) refer to the combination of free and bound hormones. However, the free forms represent the most active forms of the hormone in tissues and in biofeedback mechanisms. EDCs can interfere with the thyroid hormone system in a number of ways, including the production and release of TSH, interferences with TH binding and transport proteins, the activity of the deiodinase enzymes, iodide uptake into the cells, and metabolism processes. A full suite of targeted assays would be required to explain which of these mechanisms may play a role in thyroid axis disruption.



Thyroxine, T4 Triiodothyronine, T3

Figure 1.6: The chemical structures of thyroxine, T4 and triiodothyronine, T3.

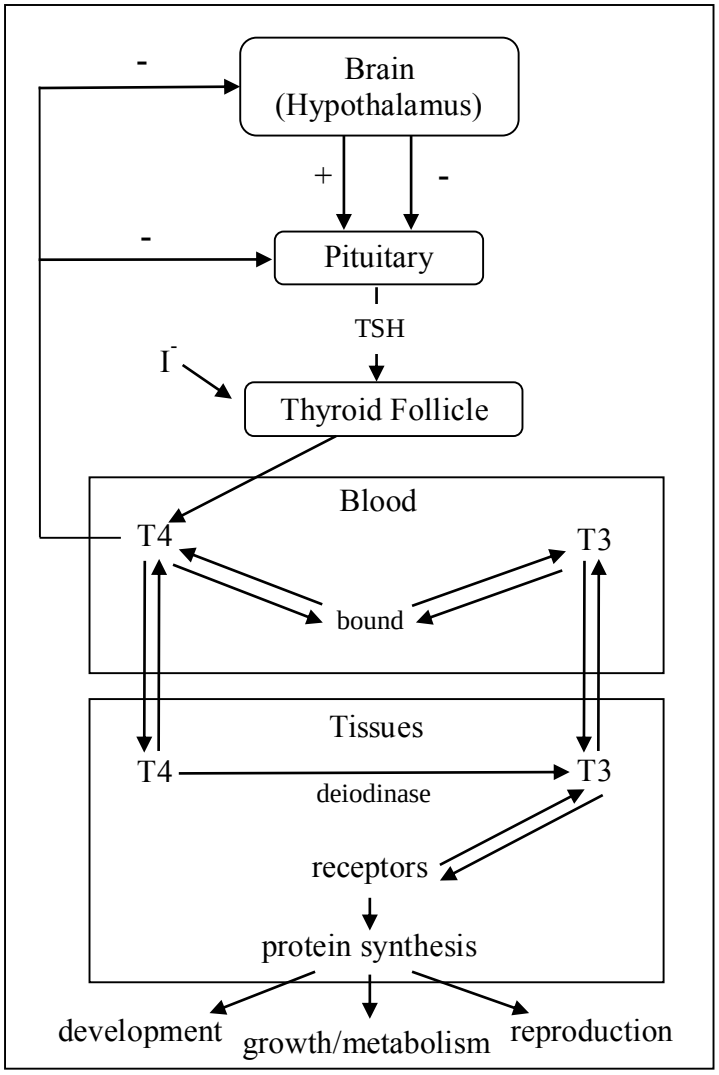


Figure 1.7: Outline of the brain-hypothalamus-pituitary-thyroid axis in fish (Blanton *et al*., 2007).

1.3.2 Steroidogenesis

Steroid hormones play a key role in the endocrine systems of fish, regulating mineral and water balance, immune response, and reproduction (Pankhurst, 2008). Steroid hormones are synthesized from cholesterol via an enzymatically mediated cleavage pathway that involves sequential loss of carbon atoms and the loss or addition of various active groups (Figure 1.8) (Pankhurst, 2008).

Cortisol (CT) is the principle glucocorticoid in teleost fish that is plays a role in metabolism, osmoregulation, growth and reproduction, and the stress response (Mommsen *et al.*, 1999). CT is a good indicator of stress in fish, given that there is a significant rise in plasma CT levels during periods of stress. Stress is caused by a chemical or physical factor that induces physiological changes in an organism, and more specifically, it is an altered state that increases the bodies' energy demands. The physiological changes in the organism may compromise its integrity and ultimately induce negative impacts on the fish such as reduced immune response, increase susceptibility to disease or predation, changes in ion regulation, reduced reproduction, and reduced feeding and growth (Martinez-Porchas *et al*., 2009; Wagner *et al.*, 2002). The stress response in fish activates the hypothalamus-pituitary interrenal (HPI) axis, stimulating the release of adrenocorticotrophic hormone (ACTH) from the pituitary, which activates the secretion of CT in the interrenal tissue, and into the bloodstream. CT is frequently used to evaluate the stress levels in fish because of its responsiveness to acute stressors, its ease of

measurement, and its functional significance in physiological processes affecting fish health (Barton & Iwama, 1991).

Androgen and estrogen hormones are produced by the hypothalamus-pituitary-gonadal axis in response to a series of environmental cues. The hypothalamus releases gonadotropin releasing hormone (GnRH), which results in the production and release of gonadotropin hormones (GTH) from the pituitary (Pait & Nelson, 2002). The gonadotropins stimulate the gonads to produce androgens and estrogens.

Androgens are the primary reproductive steroids in male fish. Testosterone (T) is produced in the testes and is converted to 11- ketotestosterone (11-KT) which is the dominant circulating androgen in male teleost fish (Pottinger *et al*., 1996). T and 11-KT are responsible for gonadal growth, initiation of spermatogenesis, and development of the secondary sex characteristics (Pankhurst, 2008). T is also produced in female oocytes and is converted to estrogen steroids by the activity of aromatase enzymes. 17β-estradiol (E2) is the primary steroid that is involved in regulation of ovarian growth, the development of female secondary sex characteristics, and stimulates hepatic synthesis of egg yolk protein-precursor vitellogenin and its deposition into the maturing oocytes (Pankhurst, 2008).

Metabolism of androgens and estrogens involves conjugation of the parent hormone with glucuronide or sulphate enzymes, UDP-glucuronosyltransferase (UGT) and sulfotransferase, respectively (Thibaut & Porte, 2004). This increases

their solubility and facilitates excretion via the urine and bile. Typical conjugated steroids of T and E2 include T-G, T-S, E2-3S, and E2-3G. Variations of these metabolic pathways may greatly affect the levels of the parent hormones in the fish. Environmental contaminants, including FRs, may disrupt the proper functioning of the endocrine system and may exert negative effects on development, growth and metabolism, and reproduction. Some examples of FRs that have shown evidence of endocrine disruption in fish include PBDEs (Birnbaum & Staskal, 2004; Pait & Nelson, 2002), HBCD (Palace *et al*., 2008, 2010), β-tetrabromoethylcyclohexane (β-TBECH) (Park *et al*., 2011), and the brominated components of Firemaster 550 (FM 550) (Mankidy *et al*., 2014; Saunders *et al*., 2013). The interferences of FRs with the synthesis and clearance of plasma hormones in fish can be used as a biomarker of exposure to pollutants and other environmental stressors.

1.3.3 Endocrine Disruption with Exposure to PBDEs

PBDEs have been found to be endocrine disruptors both *in vitro* and *in vivo*, with many studies on their effects on thyroid hormones and fewer on the reproductive hormones (Darnerud, 2008). PBDE exposure has been correlated with decreased TH levels, particularly T4, in many organisms. Effects of PBDEs on THs could be a result of alterations in the transport and metabolism or deactivation of THs or by ligand binding to TH receptors (Darnerud, 2008). OH-BDEs have the ability to bind to the plasma TH transport protein transthyretin (TTR), disrupting TH homeostasis

and affecting plasma T4 levels (Darnerud, 2008). This binding of OH-BDEs to TTR is thought to be due to the similarity in structures between the OH-BDEs and T4 (Yu *et al*., 2010). Serum T4 levels may also be reduced due to an increase in activity of phase II metabolism enzymes, inducing glucuronidation of T4, resulting in increased elimination of the hormone (Yu *et al*., 2010).

PBDEs and their metabolites also have the ability to affect the sex hormone receptor affinity *in vitro*, including androgen receptor antagonistic and estrogen receptor agonistic properties (Darnerud, 2008). Two similar studies in which rats were exposed to either BDE-99 or BDE-47 during pregnancy resulted in significant decreases in E2 levels in both male and female offspring, as well as significantly reduced T levels in male offspring (Darnerud, 2008).

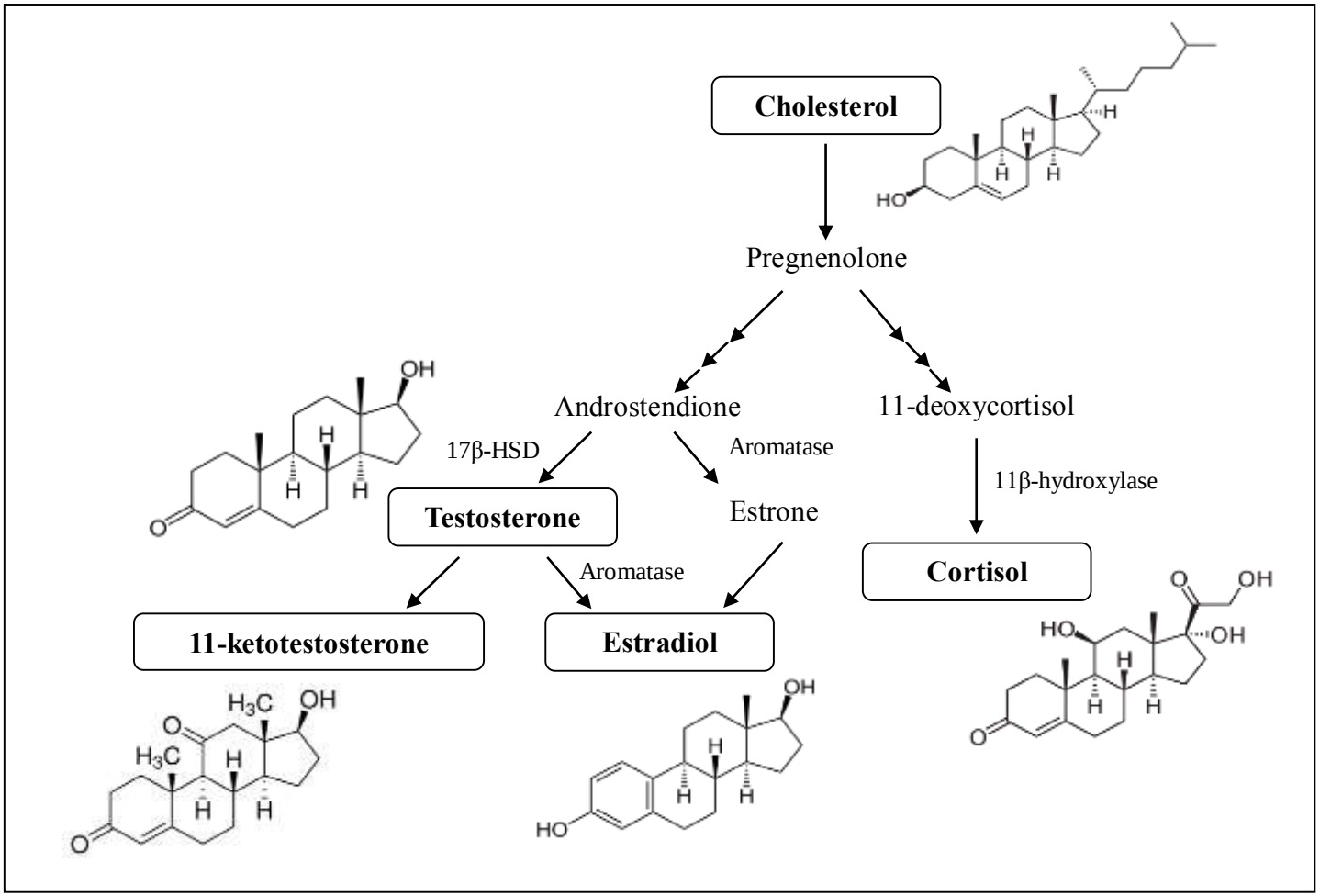


Figure 1.8: General schematic for steroid synthesis in teleost fish.

1.4 Objectives of Thesis

The ban of several of the PBDE congeners has led to an increase in the use of previously existing FRs and the development of new, alternative FRs (Stapleton *et al.*, 2009). OPFRs are one of the alternative FRs being used as replacements in order to meet the flammability standards which has increased their detection in the environment. The increase in production volumes of TBOEP and TCIPP, their ubiquitous presence in the environment, and paucity of information regarding toxicological effects provided the impetus for the current study.

The first objective of this study was to determine the concentrations of TBOEP and TCIPP found in the Lake Ontario lake trout, in order to use them as target concentrations for an exposure study. The second objective was to determine if TBOEP and TCIPP fulfill the PB&iT criteria defined by the SC in an exposure study with juvenile rainbow trout (*Oncorhynchus mykiss*) at the environmentally relevant concentrations determined by lake trout in the Lake Ontario food web study. The extent of bioaccumulation was assessed by feeding fish with known amounts of each OPFR for a prescribed duration. Persistence was assessed by comparing the experimental determined half-life ($t_{1/2}$) to those of other known P compounds (i.e., several PBDE congeners). The T criterion was determined by measuring circulating plasma concentrations of sex, stress and thyroid hormones at various time points in the exposed fish and comparing them to those in reference fish. Because of the general lack of effects based studies on these compounds, we

embarked on a range-finding study to look at what effects, if any, these two compounds might have on targeted biological endpoints that we have already developed in the laboratory (i.e., thyroid, stress, and sex hormones).

The results of this study will provide new information to assess PB&iT of these two OPFRs.

It was hypothesized that TBOEP and TCIPP would bioaccumulation the fish during the exposure period, that there would be transient effects on the hormones levels during this period, and that the compounds would be rapidly cleared by biotransformation processes during the depuration phase.

2. Methods

2.1 Chemicals

TBOEP (94% purity) and TCIPP were purchased from Sigma-Aldrich (Oakville, ON, Canada). Tris (2-butoxy-[$^{13}C_2$]-ethyl) phosphate ($^{13}C_2$-TBOEP), tris (2-chloroethyl) phosphate-d_{12}, (d_{12}-TCEP), tri-n-butyl phosphate-d_{27} (d_{27}-TBP), and $^{13}C_{18}$-triphenyl phosphate ($^{13}C_{18}$-TPhP) were obtained from Wellington Laboratories (Guelph, ON, Canada). Testosterone (T), ketotestosterone (11-KT), 17β-estradiol (E2), 17β-estradiol-3 sulfate (E2-3S), cortisol (CT), deuterium mass-labelled 17β-estradiol (d_4-E2), deuterium mass-labelled cortisol (d_4-CT), deuterium mass-labelled 17β-estradiol-3 sulfate (d_4-E2-3S), deuterium mass-labelled testosterone (d_2-T) were attained from Cambridge Isotope Laboratories (Andover, MA). HPLC optima grade methanol and water were acquired from Fisher Scientific (Nepean, ON, Canada). Distilled in glass hexane, dichloromethane (DCM), and ethyl acetate were obtained from Caledon Laboratories (Georgetown, ON, Canada). Corn oil, gelatin, and tricaine methanesulfonate (MS-222) were obtained from Sigma-Aldrich (Oakville, ON, Canada). The thyroid radioimmunoassay kits (RIA) were obtained from MP Biomedical (Santa Ana, CA, USA).

2.2 Food Preparation

1.8 kg of commercial starter fish food (ProForm Aquaculture Feed; Early's Farm and Garden Centre, Saskatoon, SK) was added to a pre-cleaned (soap and water, then methanol rinsed) Hobart blender. To prepare the reference diet, corn oil (20 mL) was added to the feed and mixed at low speed for 20 min. To prepare the OPFR enriched diets, a known amount of TBOEP (90 mg) or TCIPP (90 mg) was added to the corn oil prior to mixing. The amount of OP added to the food was approximated by the levels of TBOEP and TCIPP detected in the lake trout from Lake Ontario (Section 3.1). A gelatin binder was prepared by vigorously stirring 40 g gelatin to 1.5 L Milli-Q water (heated to 37 °C). The aqueous gelatin was added to the food slowly and was mixed until it had a firm consistency (~20 min). The food was air dried for *ca*. 2 hr and then extruded through a 4mm die. The noodles were then air dried at 25 °C for 3-4 days in the dark in low humidity conditions (30%). Once dry, the noodles were broken into pellets and stored at -20 °C. The mean lipid content of the food was determined gravimetrically and was found to be 12.9%. The average concentrations at day 28U and 49U of TBOEP and TCIPP in the OPFR enriched diets were determined to be 148.6 and 74.1 pmoles/g (wet weight), respectively. The concentrations were determined with the same method as whole fish extractions (section 2.5.1). The amounts of TBOEP and TCIPP in the fortified food were significantly greater than in the reference food at concentrations of 35.4 and 11.7 pmoles/g (ww), respectively ($p < 0.001$).

2.3 Lake Ontario Lake Trout Samples

Pelagic fish from Lake Ontario, including lake trout (*Salvelinus namaycush*, n = 13), round goby (*Neogobius melanostomus*, n = 4), alewife (*Alosa pseudoharengus*, n = 5), rainbow smelt (*Osmerus mordax*, n = 5), slimy sculpin (*Cottus cognatus*, n = 5), and deepwater sculpin (*Myoxocephalus thompsonii*, n = 5) were provided by Tim Johnson of the Ontario Ministry of Natural Resources. Samples were collected by nylon gillnets at a depth between 30 and 100 m from offshore stations in Lake Ontario between June and July 2011.

2.4 Exposure Experiment

Two hundred juvenile rainbow trout (*Oncorhynchus mykiss*, mean weight *ca*. 90 g) obtained from Grand Rapids Fish Hatchery (Grand Rapids, MB, Canada) were randomly divided into three 800 L fibreglass tanks. The fish were acclimatized in their tanks for 2 weeks prior to beginning the experimental feeding stages and fed a diet of reference food for this time. Each tank received dechlorinated Winnipeg City tap water (12 ± 2°C) at a flow rate of 2.0 L/min. The pH was between 7.6 and 8.4 and the dissolved oxygen was always > 90% saturation. Each group was fed 1.5% of its body weight three times per week of either the reference diet or the OPFR enriched diets. The experiment consisted of two phases: (*i*) an uptake phase of 49 days where the fish in two tanks were fed the OPFR enriched foods; fish in the third tank were fed reference food and (*ii*) a depuration phase of 98 days where

all fish were fed reference food. Five fish from each tank were randomly sampled on days 0, 7, 14, 28, and 49 of uptake phase and days 7, 14, 21, 35, 56, and 98 of depuration phase. Fish were sacrificed 48 hrs after previous feeding by an overdose of a pH buffered solution of tricaine methanesulfonate (MS-222) (300 mg/L) until opercular movement ceased (< 3 min) (Tomy, 2012). Fork lengths and weights were recorded and 2-5 mL of blood was removed from the caudal vein using a heparinized syringe. The liver and gonad was removed and each was weighed. Blood from each fish was centrifuged (Beckman Allegra X-15R) for 6 min at 3000 x *g* at 8°C to obtain plasma. Plasma and liver were immediately frozen and stored at -80°C until analyzed. The liver was used to determine the extent of accumulation and also to screen for potential metabolites.

2.5 Sample Extractions

2.5.1. Whole Fish (Exposure Study)

Figure 2.1 shows a schematic of my approach to extracting OPFRs from whole fish. Whole fish (minus liver and plasma) were partially thawed and homogenized whole in a blender (Knife Mill Grindomix GM 200, Retsch, Haan, Germany) and the OPFR extraction was achieved using a Bullet Blender 50 (Ideal Scientific, Ancaster, ON). A 5 g sample was weighed out into 50 mL skirted centrifuge tubes with about 5 g 4.8 mm stainless steel beads (~30 beads). The tubes were spiked

with 10 ng of d_{27}-TBP (10 µL of a 1.0 ng/µL solution) acting as recovery internal standard (RIS) and extraction was achieved using 10 mL of 50:50 DCM:hexane. The samples were homogenized for 12 min, centrifuged (Thermo IEC), and then the supernatant was transferred to a clean glass tube. The samples were rinsed once more with another 10 mL 50:50 DCM:hexane, homogenized, centrifuged, and transferred to the same glass tube. The samples were reduced in volume by a gentle stream of ultra-high purity N_2 to 11 mL. Lipids were determined gravimetrically by a 1 mL portion of the extract. Lipids were then removed from the extract using an isolute aminopropyl silica gel SPE column (3 g; 50 µm particle size; 54 Å pore diameter; Biotage, Charlotte, NC, USA) (Chen *et al*., 2012). The column was washed and conditioned prior to loading the sample. The OPFRs were eluted with 6 mL 20% DCM:hexane followed by 35 mL DCM. The extracts were reduced in volume to 1mL by rotary evaporation (Heidolph , Fisher Scientific, Pittsburgh, PA), followed by N_2-evaporation to a final volume of 200 µL in 2-propanol. The sample was spiked with 10 ng $^{13}C_{18}$-TPhP, $^{13}C_2$-TBOEP, and d_{12}-TCEP (10 µL of 1.0 ng/µL) prior to HPLC-MS/MS analysis. All of the glassware used for extractions were baked at 300°C for a minimum of 12 hrs prior to use. The mass-labelled TBOEP and TCEP were not added as RIS because they were not commercially available until after all my extractions were completed.

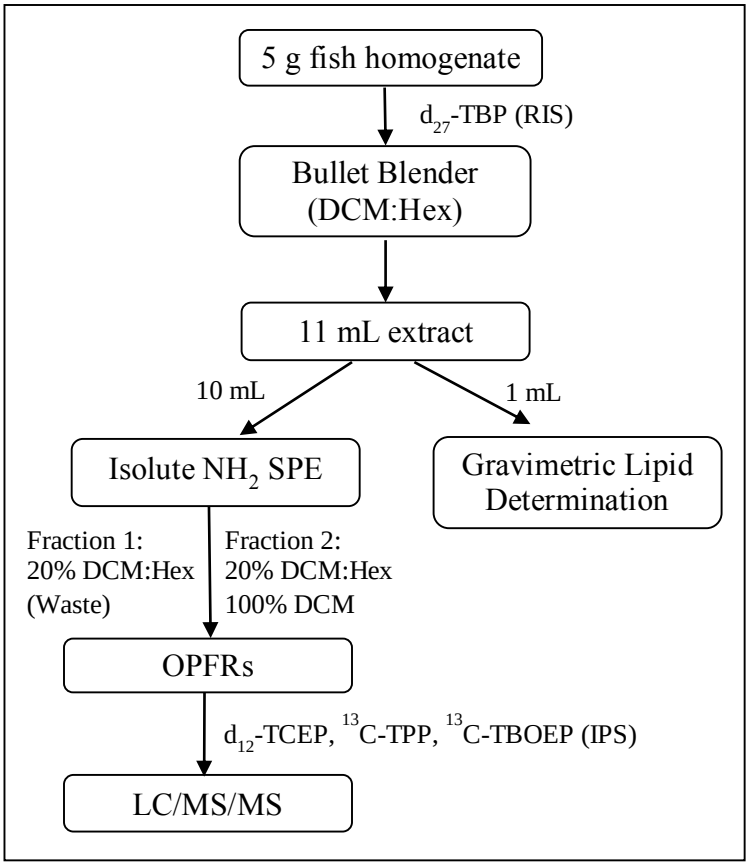


Figure 2.1 Schematic of OPFR extraction from whole fish for the exposure study.

2.5.2. Liver

Liver samples were thawed, weighed and transferred to a 7 mL Precellys lysing tube pre-filled with 50 preps of 1.4 mm ceramic (zirconium oxide) beads (Bertin Technologies, MD, USA). The tubes were spiked with 10 ng of $^{13}C_2$-TBOEP and d_{12}-TCEP (10 µL of a 1.0 ng/µL solution) and extracted using 3 mL of 50:50 DCM:hexane. The samples were homogenized on a Precellys 24 Dual (Bertin Technologies, MD, USA) for 15 sec at a speed of 5000 rpm, centrifuged, and the

supernatant was transferred to a clean glass tube. Another 3 mL of 50:50 DCM:hexane was added, the sample homogenized, centrifuged (Thermo IEC), and the supernatant was transferred to the same glass tube. The sample volume was adjusted to 6 mL and lipids were determined gravimetrically by removing a 1 mL portion of the extract. Lipids were then removed by an isolute aminopropyl silica gel SPE column (1 g; 50 μm particle size; 54 Å pore diameter; Biotage, Charlotte, NC, USA). The column was washed and conditioned prior to sample addition. The OPFRs were eluted using 3 mL of 20:80 DCM:hexane followed by 8 mL of DCM. The extracts were reduced in volume to 1 mL by N_2-evaporation to a final volume of 200 μL in 2-propanol. The sample was spiked with 10 ng d_{27}-TBP and $^{13}C_{18}$-TPhP (10 μL of 1.0 ng/μL) prior to HPLC-MS/MS analysis. All of the glassware used for extractions were baked at 300°C for a minimum of 12 hrs prior to use.

2.5.3. Whole Fish (Lake Ontario)

Whole fish were homogenized by Knife Mill Grindomix GM 200 blender (Retsch, Haan, Germany) and invertebrates were homogenized by mortar and pestle. OPFR extraction from the samples was carried out using a Dionex accelerated solvent extractor (ASE-300, Dionex Canada Ltd., Oakville, ON, Canada). Samples (15 g) were mixed with heat-treated (600°C for 6hr) pelleted diatomaceous earth and added to the cells. Heat-treated (600°C for 6hr) Ottawa sand was used to fill any voids. Cells were spiked with 10μL of 10ng/μL ^{13}C-TPP. The ASE parameters

were: Solvent 50:50 DCM:Hexane, temperature 100°C, pressure 2000 psi, heat up time 5 min, static time 5 min, flush volume 60%, purge time 80 sec, and one cycle. The extracts were dried with heat-treated (600°C for 6h) anhydrous sodium sulphate (10-60 mesh size) and reduced in volume to 11 mL by rotary evaporation (Heidolph, Fisher Scientific, Pittsburgh, PA). Lipids were determined gravimetrically using 1 mL of extract. Extracts were filtered using 1.0µm acrodisc glass syringe filters (Waters, Milford, Massachusetts). Samples were then reduced to either 2.5 mL or 5.0 mL volume by a gentle stream of ultra-high purity N_2, depending on lipid content (2.5 mL if lipid < 0.1 g, 5.0 mL if lipid > 0.1 g) and made up with addition of 50% ethyl acetate. Lipids were removed from extract by an automated gel permeation chromatograph (GPC, Knauer, Advanced Scientific Instruments, Berlin, Germany) on column packed with 60g (dry weight) of 200-400 mesh S-X3 Bio-beads (Bio-Rad Laboratories, Mississauga, ON, Canada) with a mobile phase of 50:50 cyclohexane:ethyl acetate. The lipid free extracts were then reduced in volume by rotary evaporation and further by N_2 evaporation to 200 µL in 2-propanol and spiked with 10 ng d_{27}-TBP prior to (HPLC-MS-MS) analysis.

2.5.4. Plasma

2.5.4.1. Thyroid

TT4, FT4, TT3, and FT3 in plasma were determined using commercially available RIA kits (MP Biomedical; Santa Ana, CA, USA) according to the manufacturer's directions. In brief, plasma samples were thawed and 25, 50, 100, and 100 µL was pipetted into the respective RIA tubes for total T3 (TT3) and T4 (TT4), and free T3 (FT3) and T4 (FT4). Plasma standards that were provided with the RIA kits were run with each batch. 1 mL of ^{125}I tracer, specific for each hormone, was added to each tube. The TT3, FT4, and FT3 were incubated at 37°C for 1, 1.5, and 2.5 hrs respectively, while TT4 was incubated at room temperature for 1 hr. Following incubation, the tubes were decanted and rinsed with 1 mL distilled water and decanted once more. The samples were analyzed by a gamma counter (Perkin Elmer 2480 Automatic gamma counter Wizard^2, Waltham, Massachusetts, USA). Due to limited plasma volumes, not all parameters could be measured on all sampling dates.

2.5.4.2. Sex Hormones and Cortisol

Figure 2.2 shows a schematic of my approach to extracting hormones from plasma. Plasma samples were thawed and 400 µL was pipetted into a 15 mL disposable glass tube. The samples were spiked with 5 ng of d_4-E2, d_4-E2-3S, d_2-T, and d_4-C

(10 µL of 0.5 ng/µL solution). 3 mL of 9:1 hexane:ethyl acetate was added to each of the samples, they were vortexed for 60 s, centrifuged for 5 min at 4000 x *g*, and frozen for 5 min in -80 °C freezer. The top layer was removed and transferred to a clean glass tube. The steps were repeated using 3:2 hexane:ethyl acetate, followed by 100% acetonitrile (ACN) and the upper layer was removed and combine with the first extract. The samples were blown to dryness using N_2 and reconstituted in 45 µL methanol, vortex mixed, and transferred to a 2 mL glass autosampler vial with 250 µL glass insert. The samples were spiked with 2.5 ng d_4-E1 (5 µL of a 0.5 ng/µL solution). The samples were analyzed by HPLC-MS/MS (Table 2.1 and Appendix A).

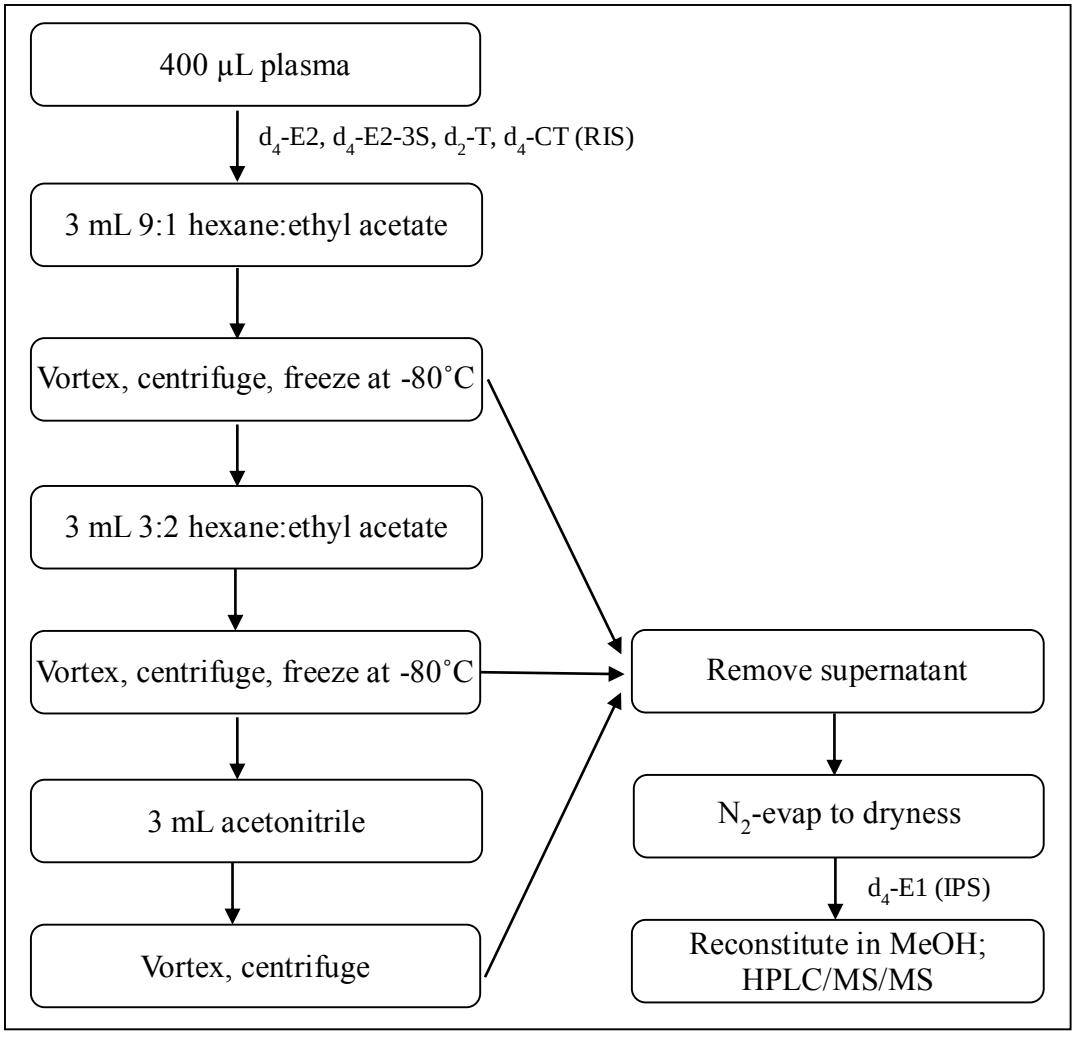


Figure 2.2: Schematic of extraction of sex hormones and cortisol from plasma.

2.6 High Performance Liquid Chromatography Tandem Mass Spectrometry (HPLC/MS/MS)

An Agilent 1100 series HPLC system (Agilent Technologies, Palo, Ca, USA) coupled to a Sciex 2000 triple quadrupole mass spectrometer (Applied Biosystems,

Foster City, CA, USA) was used for the identification and quantification of OPFRs, E2, T and CT. It was equipped with an Agilent 1100 series vacuum degasser, a binary pump, and an autosampler (Agilent Technologies, Palo, Ca, USA). A C_{18} analytical column (Grace; 50 mm x 2.1 mm i.d., 4 µm particle size; Chromatographic Specialties Inc, Brockville, ON, Canada) was used to separate both the hormones and the OPFRs. The mobile phase consisted of a gradient of water and methanol at a flow rate of 300 µL/min. For the targeted hormones, the initial composition was 80:20 water:MeOH (v/v), held for 1 min, ramped linearly to 100% MeOH in 9 min, and held for 6 min. The column was equilibrated between runs for 7 min. For the targeted OPFRs, the initial composition was 60:40 water/MeOH (v/v), held for 1 min, ramped linearly to 100% MeOH in 2 min, held for 9 min. The column was equilibrated for 7 min between runs. E2 and CT were monitored in the negative ion mode, and T and the OPFRs were monitored in the positive ion mode under multiple reaction monitoring (MRM) conditions. The mass to charge (*m/z*) values, product ion transitions for quantification and confirmation for all the compounds are listed in Table 2.1. The optimized MS/MS parameters in ESI –ve and +ve ion MRM mode are shown in Appendix A.

Table 2.1: MRM and ions monitored.

Hormone	Mass (m/z)	Transitions Monitored		Ion Mode
		Quantification	Confirmation	
Estradiol (E2)	271.2	145.2	183.1	-
d4-estradiol (d_4-E2)	275.2	147.1	187.1	-
Estradiol – 3 sulfate (E2-3S)	351.2	271.0	80.0	-
d_4-estradiol-3 sulfate (d_4-E2-3S)	355.2	275.1	80.0	-
d_4-estrone (d_4-E1)	273.1	147.1	145.1	-
Testosterone (T)	289.2	97.1	109.1	+
d_2-testosterone (d_2-T)	291.2	99.1	111.1	+
11-ketotestosterone (11-KT)	303.3	121.1	256.2	+
Cortisol (CT)	361.1	331.0	282.0	-
d_4-cortisol (d_4-C)	365.0	335.0	301.0	-
TBOEP	399.0	298.9	198.9	+
TCIPP	328.8	98.9	174.8	+
d_{27}-TBP	294.2	101.9	166.0	+
^{13}C-TPhP	344.9	83.0	163.9	+
^{13}C-TBOEP	405.9	303.5	201.2	+
d_{12}-TCEP	297.5	67.1	102.1	+

2.7 Quality Assurance/Quality Control

Exposure Study

Procedural blanks for whole fish extractions were analyzed every 8 samples and followed the same extraction and cleanup method as the fish. These blanks were used to monitor the contamination that may occur during the extraction and cleanup. OPFRs were detectable in the blanks and blank correction was necessary. Measured concentrations of TBOEP and TCIPP in reference fish were also used to correct the concentrations measured in the exposed fish (Appendix B). The measured concentrations were also corrected for growth. Recovery correction was determined by addition of d_{27}-TBP at the point of sample extraction and ^{13}C-TPP, ^{13}C-TBOEP, and d_{12}-TCEP prior to injection into HPLC-MS/MS. Duplicate samples were analyzed with every 5 samples to verify the repeatability of the analytical methods. As TBEOP and TCIPP concentrations were not correlated with lipid, no correction for lipid content was applied to the data (Spearman's rank correlation, -0.182, $p > 0.05$).

Lake Ontario

Procedural blanks for whole fish extractions were analyzed every 4 samples and followed the same extraction and cleanup method as the fish. These blanks were used to monitor the contamination that may occur during the extraction and

cleanup. OPFRs were detectable in the blanks and blank correction was necessary. Recovery correction was determined by addition of ^{13}C-TPP at the point of sample extraction and d_{27}-TBP prior to injection into HPLC/MS/MS. As TBEOP and TCIPP concentrations were not correlated with lipid, no correction for lipid content was applied to the data (Spearman's rank correlation, -0.182, $p > 0.05$).

Plasma

For plasma, procedural blanks were analyzed every 15 samples. The native hormones were not detected in our blanks, so blank correction was not necessary. Recovery correction was determined by addition of d_4-E2, d_4-CT and d_2-T at the point of sample extraction. Duplicate samples were analyzed with every 5 samples to verify the repeatability of the analytical methods.

Injections of methanol (3 μL) were used as instrument injection blanks for HPLC/MS/MS, and were run every 6 samples. Recovery percentages are listed in Table 2.2 for OPFRs and plasma hormones.

Table 2.2: Percent recoveries of OPFRs and plasma hormones.

Hormone	**% recovery ± SEM**
d_4-Estradiol (d_4-E2)	36.7 ± 1.2 % (n = 87)
d_4-cortisol (d_4-CT)	52.1 ± 0.9 % (n = 195)
d_2-Testosterone (d_2-T)	28.5 ± 0.8 % (n = 195)
^{13}C-TBOEP	52.7 ± 2.0 % (n = 110)
d_{12}-TCEP	47.9 ± 1.9 % (n = 120)
^{13}C-TPP (Lake Ontario)	40.8 ± 1.8 % (n = 38)

2.8 Analytical Detection Limits and Method Detection Limits

Analytical and method detection limits are listed in Table 2.3. Analytical detection limits (ADLs) were determined by injecting a known amount of compound and suppressing the signal to noise (S/N) ratio to a 5:1 value. Method detection limits (MDLs) are defined as the amount of analyte in the procedural blanks plus 3 x standard deviation and were normalized to mass or volume of sample extracted (Winefordner & Long, 1983). The signals of the analytes in the blanks were adjusted to estimate concentrations that would give an S/N ratio of 5:1. For those samples that had undetectable amounts of the compounds in the blanks, the MDLs were determined by adding a known amount of each compound to the blank extract and suppressing the S/N value to 5:1. In the cases where compounds were below the MDLs, a concentration of ½ MDL was assumed.

Table 2.3: ADLs and MDLS for hormones and OPFRs.

Hormone	Analytical Detection Limit (ADL)	Method Detection Limit (MDL)
Estradiol (E2)	2.4 pg	0.03 pmole
Estradiol – 3 Sulfate (E2-3S)	0.5 pg	0.003 pmole
Testosterone (T)	0.4 pg	0.02 pmole
11-Ketotestosterone (11-KT)	2.3 pg	0.05 pmole
Cortisol (CT)	5.9 pg	0.01 pmole
TBOEP	4.00 pg	1.31 pmole/g
TCIPP	0.38 pg	0.28 pmole/g

2.9 Data Analysis

The effects of TBOEP and TCIPP on the hormone levels, LSI, and GSI, relative to the controls, were tested statistically using a 2-tailed Student's *t*-test at each sampling time point. Significance was determined at $p < 0.05$. Outliers were determined by the Q-test at a confidence level of 95%.The Spearman rank analysis was used to test for correlation of concentration of OPFR and % lipid in sample, as OPFRs seem not to be as lipid associated as other environmental pollutants (Marklund Sundkvist *et al.*, 2010). All data was tested for normality prior to the statistical test and the data that failed were normalized by a log transformation.

Growth rates of the fish were estimated by plotting the ratio of the average fish weight at each sampling point (W_t) to the fish weight at the start of experiment (W_0) versus time:

$$W_t = W_0 [1 + b \text{ x } t]$$

Where b is the growth rate, and t is time in days. The statistical differences of the slope of the regression line for growth and % lipid were determined by the z-test within a 95% confidence interval. The growth was corrected throughout the experiment by multiplying the concentrations by a factor (1+ b x time).

Statistical analyses were conducted using SigmaPlot 12.0 (Systat Software).

Bioaccumulation Parameter Calculations

Growth rates (d^{-1}), depuration rates (k_d), half-life ($t_{1/2}$), assimilation efficiencies (α), and BMFs were calculated using the equations described in Tomy *et al.* (2004).

The depuration rates, k_d, were calculated by fitting the depuration data to a first-order decay curve:

$$\ln \text{concentration} = a + b \text{ x time (day)}$$

where a is a constant and b is the depuration rate. The depuration half-lives ($t_{1/2}$) were calculated with the following equation:

$$t_{1/2} = \frac{\ln(2)}{k_d}$$

The assimilation efficiencies (α) were calculated by the equation

$$\alpha = \frac{(control\ corrected\ concentration\ in\ fish)\ x\ (mass\ of\ fish)}{(control\ concentration\ in\ food) x\ (mass\ of\ food\ eaten)}$$

The biomagnification factor (BMF) was estimated from the equation

$$BMF = \frac{\alpha \; x \; F}{k_d}$$

Where *F* is the feeding rate of 1.5% of the average body weight of the fish.

The assumption was made that all the food was eaten by the fish and the TBOEP and TCIPP did not enter into the water column and subsequently stayed in the food.

3. Results and Discussion

3.1 Lake Ontario fish

The concentrations of TBOEP and TCIPP in the lake trout collected from Lake Ontario were 6.46 ± 2.03 and 0.60 ± 0.16 pmole/g ww. TBOEP was detected in 100% of the samples, whereas TCIPP was only detected in 31% of the samples. The concentrations of TBOEP and TCIPP in the all the species collected from Lake Ontario are listed in Table 3.1.

3.2 Growth Rates

The growth rates of the fish were estimated by plotting the ratio of the average fish weight at each sampling point (W_t) to the fish weight at the start of experiment (W_o) versus time. Regression analysis indicates that fish grew linearly throughout the study period. The growth rates (d^{-1}) for the fish in reference, TBOEP, and TCIPP treatment tanks were: 0.0145, 0.0164, and 0.0116 per day, respectively (Figure 3.1). Whole body growth rates did not vary between the exposed fish and the reference fish (z-test with 95% confidence interval) and as a result TBOEP and TCIPP did not have any negative effect on growth of the fish.

3.3 Liver and gonad somatic indices

The liver and gonad somatic indices (LSI and GSI) are both indicators of the overall metabolic health and reproductive status of fish. The LSI and GSI are calculated as a percentage of the ratio of liver/gonad weight to total body weight. The LSI provides an estimation of the energy status of the fish, whereas the GSI provides an estimation on the reproductive condition of the fish. Both can be used as biomarkers for exposures to environmental contaminants. There were no significant differences among the LSI or GSI in the treated and reference fish at any sampling point ($p > 0.05$) (Figure 3.2 and Figure 3.3). These results indicate that TBOEP and TCIPP did not have any measurable adverse effects on liver or gonad development during the exposure. This could be due to fast elimination of TCIPP, the minor bioaccumulation of TBOEP, the short duration of the exposure study, or TBOEP and TCIPP are just not toxic at these environmentally relevant concentrations.

Table 3.1: Concentrations of TBOEP and TCIPP in the various fish from a Lake Ontario food web.

Species	**TBOEP (pmoles/g ww)**	**TCIPP (pmoles/g ww)**
Lake Trout (*Salvelinus namaycush*)	6.46 ± 2.03	0.60 ± 0.16
Round Goby (*Neogobius melanostomus*)	4.24 ± 1.09	0.86 ± 0.21
Alewife (*Alosa pseudoharengus*)	5.46 ± 1.17	0.29 ± 0.09
Rainbow Smelt (*Osmerus mordax*)	7.49 ± 2.95	0.27 ±0.17
Slimy Sculpin (*Cottus cognatus*)	13.62 ± 3.24	2.17 ± 0.85
Deepwater Sculpin (*Myoxocephalus thompsonii*)	13.61 ± 5.34	3.69 ± 1.20

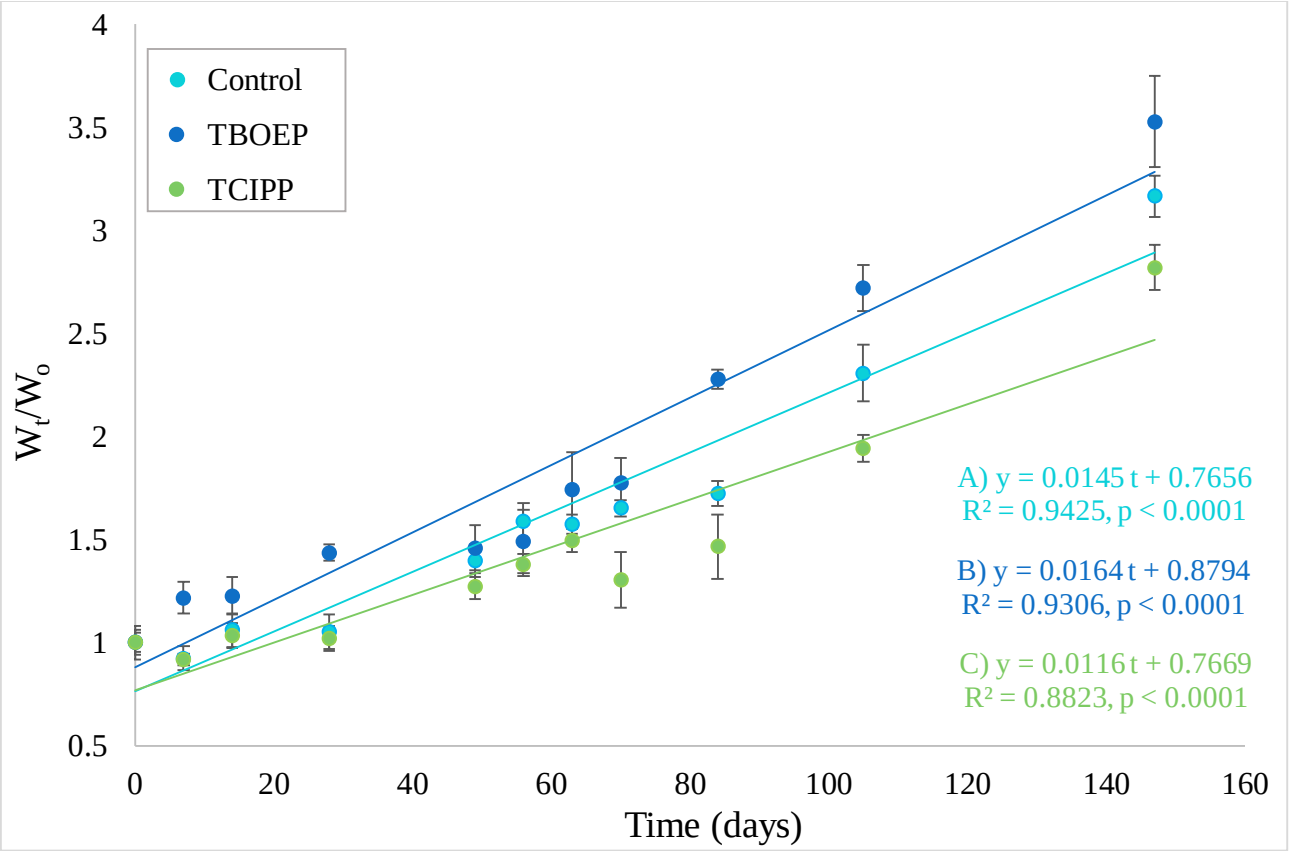


Figure 3.1 Growth rates (W_t/W_o) of juvenile rainbow trout exposed to food fortified with (A) no OPFR, (B) TBOEP, and (C) TCIPP. Linear regression analysis is shown in each plot. Each data point represents the mean of five fish.

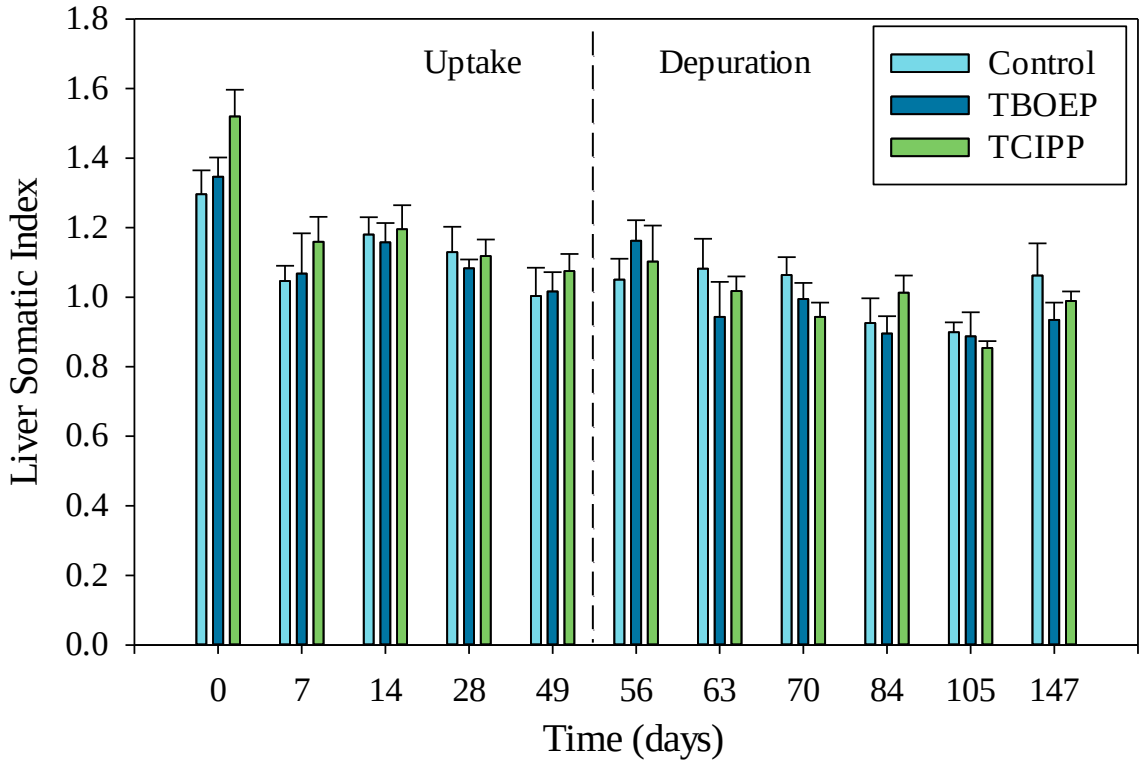


Figure 3.2: Plot of the average LSI values for reference, TBOEP, and TCIPP exposed fish for 147 day exposure. Data are presented as arithmetic mean ± standard error (n=5).

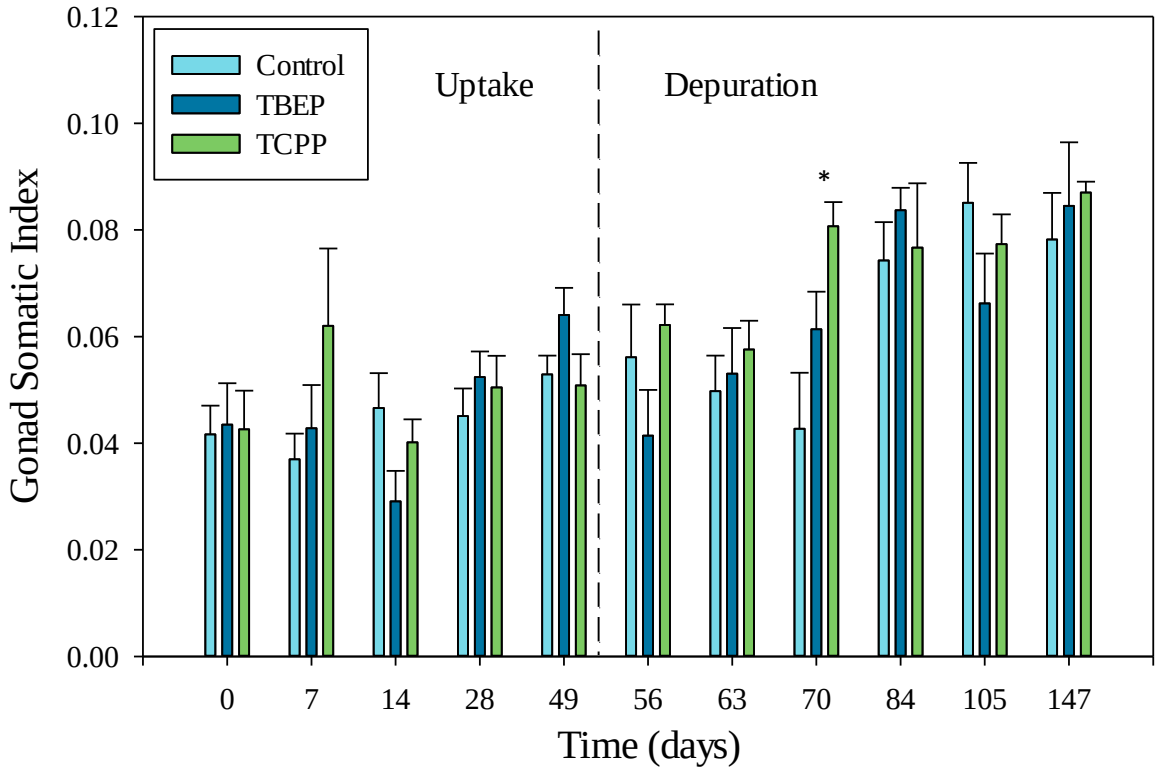


Figure 3.3: Plot of the average GSI values for reference, TBOEP, and TCIPP exposed fish for 147 day exposure. Data are presented as arithmetic mean ± standard error (n=5). (* indicates p-value < 0.05).

3.4 Toxicokinetics of TBOEP and TCIPP

3.4.1. Bioaccumulation

The bioaccumulation and depuration plots of TBOEP and TCIPP are shown in Figure 3.4 and 3.5. There were two phases to the exposure, the uptake phase of 49 days and the depuration phase of 98 days. TBOEP showed negligible bioaccumulation in fish throughout our exposure study with measured amounts close to the MDLs (1.3 pmoles/g ww). Possible reasons for this include rapid elimination, biotransformation, or poor assimilation efficiency of TBOEP. As such, I was unable to determine any bioaccumulation metrics for TBOEP.

Data analysis for the uptake phase for TCIPP suggested that this compound did bioaccumulate. TCIPP was detectable after day 7 (0.62 ± 0.29 pmole/g ww) of the uptake period and the highest concentrations were detected on day 49 (3.59 ± 1.26 pmole/g ww). Measured concentrations of TCIPP at day 49 were significantly greater ($p = 0.03$) than concentrations at day 7. Based on the analysis of the regression (Figure 3.6) with the profile being linear throughout the uptake period, TCIPP did not reach steady state before the end of the uptake phase.

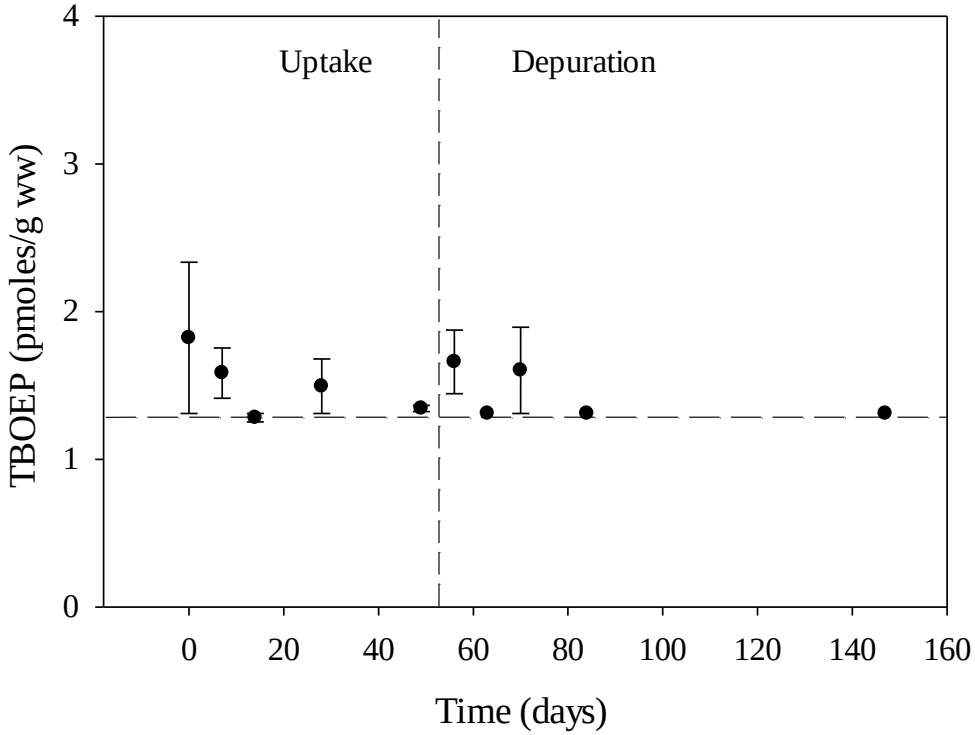


Figure 3.4: Uptake and depuration of juvenile rainbow trout exposed to TBOEP. Each point represents the arithmetic mean ± standard error for five fish. The vertical dashed line separates the uptake period (0 to 49 day) and the depuration period (49 to 147 day), while the horizontal line indicates the MDL (1.31 pmole/g).

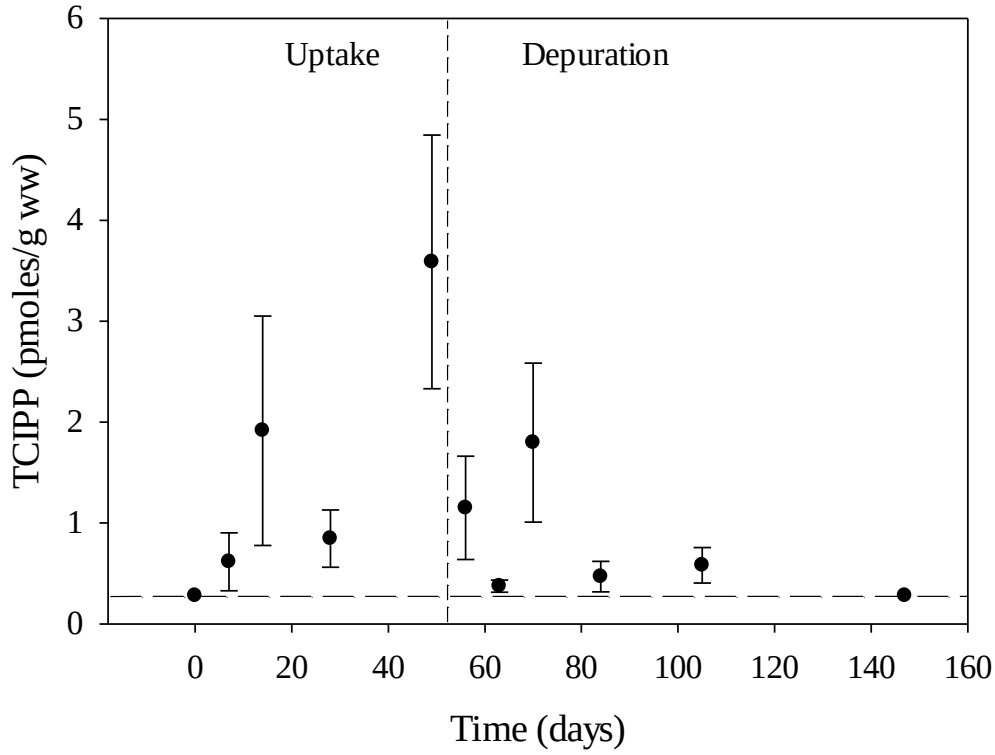


Figure 3.5: Uptake and depuration of juvenile rainbow trout exposed to TCIPP. Each point represents the arithmetic mean ± standard error for five fish. The vertical dashed line separates the uptake period (0- 49 day) and the depuration period (49- 147 day), while the horizontal line indicates the MDL (0.28 pmole/g).

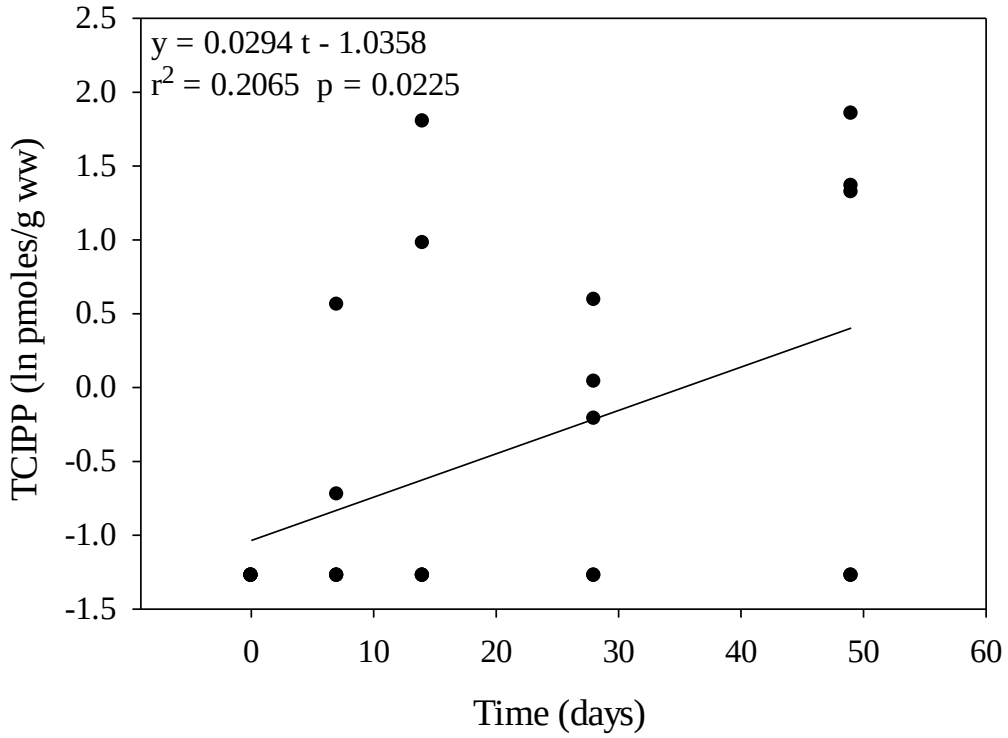


Figure 3.6: Plot of the log transformed concentration of TCIPP in juvenile rainbow trout versus time (days) during the uptake phase of the exposure (day 0 to 49). Regression analysis is given in the plot.

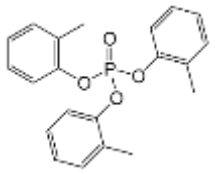
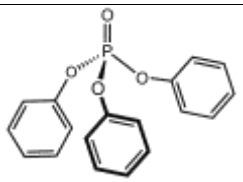
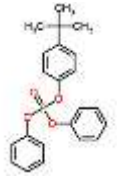
3.4.2 Depuration

The elimination of TCIPP fitted both zero- and first-order elimination kinetics equally well (Appendix C). However, elimination of chemicals from fish at low concentrations as those used in my study are best described by first-order kinetics (Ballantyne *et al*., 1995; Di Giulio & Hinton, 2008).The depuration rate constant (k_d) based on the slope of the plot was calculated to be 0.0138 ± 0.0053 day^{-1} (Figure 3.7). Muir *et al.* (1983) measured k_d on a suite of OPFRs in rainbow trout. Table 3.2 shows the structures and some physicochemical properties of the compounds investigated by Muir *et al.* (1983). Overall, the k_d of TCIPP in my study was *ca*. 3 orders of magnitude smaller than those measured by Muir *et al*. (1983). Reasons for the discrepancies between the two studies could include *inter alia* differences in chemical structure and physicochemical properties including log K_{ow}, BCF and water solubility. All these intrinsic properties are known to affect the partitioning, distribution and biotransformation of chemicals in biological systems.

The depuration half-life ($t_{1/2}$) is inversely proportional to k_d and was calculated to be 50.23 ± 19.29 days. The α for TCIPP was calculated as 0.77 ± 0.21 %. Using α we were able to calculate a BMF of 0.81 ± 0.39. A BMF value less than 1 suggests that a chemical has a low potential to biomagnify in aquatic food webs (Tomy *et al*., 2004). The BMF value from the current study is consistent with my field data from Lake Ontario where TCIPP was measureable in biota from all trophic levels

in that system. The highest BMF value determined in Lake Ontario was for the lake trout to rainbow smelt predator prey relationship at 2.73 ± 1.75 and the lowest BMF value was for the lake trout to deepwater sculpin predator prey relationship at 0.20 ± 0.07. The BMF determined in the exposure study for TCIPP fits within this range. To assess the P of TCIPP I compared the biological $t_{1/2}$ calculated from this study to $t_{1/2}$ values of other known POPs that have been regulated by the SC. Tomy *et al.* (2004) determined the $t_{1/2}$ values of various BDEs congeners in juvenile lake trout. For the congeners present in the penta-BDE, experimentally measured $t_{1/2}$'s of BDE-28, -47, -85, -99 and -100 were 58 ± 10, 39 ± 8, 43 ± 11, 87 ± 11 and 63 ± 17 days, respectively. Respective biological $t_{1/2}$'s of two known congeners in the octa-BDE mixture, BDE-138, and -183, were 58 ± 14 and 69 ± 14 days. These values are very similar to the $t_{1/2}$ of TCIPP determined in my study. As the chemical structures, as well as the physicochemical properties of PBDEs and OPFRs are quite different, it is hard to compare why TCIPP may be acting similar to PBDEs. The targeted exposure amounts agreed well with those measured in a Lake Ontario lake trout. TBOEP was measured in lake trout to be 6.46 pmole/g ww, while in the exposure study, at the peak of uptake, we measured TBOEP to be 1.31 pmoles/g ww. Similarly, the target TCIPP amounts based on what was measured in lake trout from Lake Ontario was 0.60 pmole/g ww, while the measured amount in the rainbow trout exposure study was 3.59 pmoles/g ww.

Table 3.2: Chemical structures and some physicochemical properties of the compounds investigated by Muir *et al.* (1983).

	Tri-cresyl phosphate (TCP)	Tripenyl phosphate (TPhP)	Tert-butylphenyl diphenyl phosphate (tBPDP)
Chemical Structure			
Water Solubility (mg/L)	0.36	1.9	0.009
log K_{ow} (estimated)	5.11	4.59	5.12
BCF (estimated)	8560	113	778

3.4.3 Bioaccumulation in liver and metabolite formation

The liver is a tissue where biotransformation enzymes are produced, and functions in detoxification, protein synthesis, and metabolism. The liver of the fish was used to determine if the OPFRs accumulated in a more protein-based tissue, compared to the more fatty muscle tissue and to determine if there were any OPFR metabolites. I was unable to measure either TBOEP or TCIPP in exposed fish livers at concentrations greater than those in the reference fish liver at day 49 of the uptake phase. This suggests that there was negligible bioaccumulation in the liver. I also screened the liver for possible metabolites using the MRM transition values for TBOEP and TCIPP from van den Eede *et al.* (2013), but was unable to detect any of the proposed metabolites.

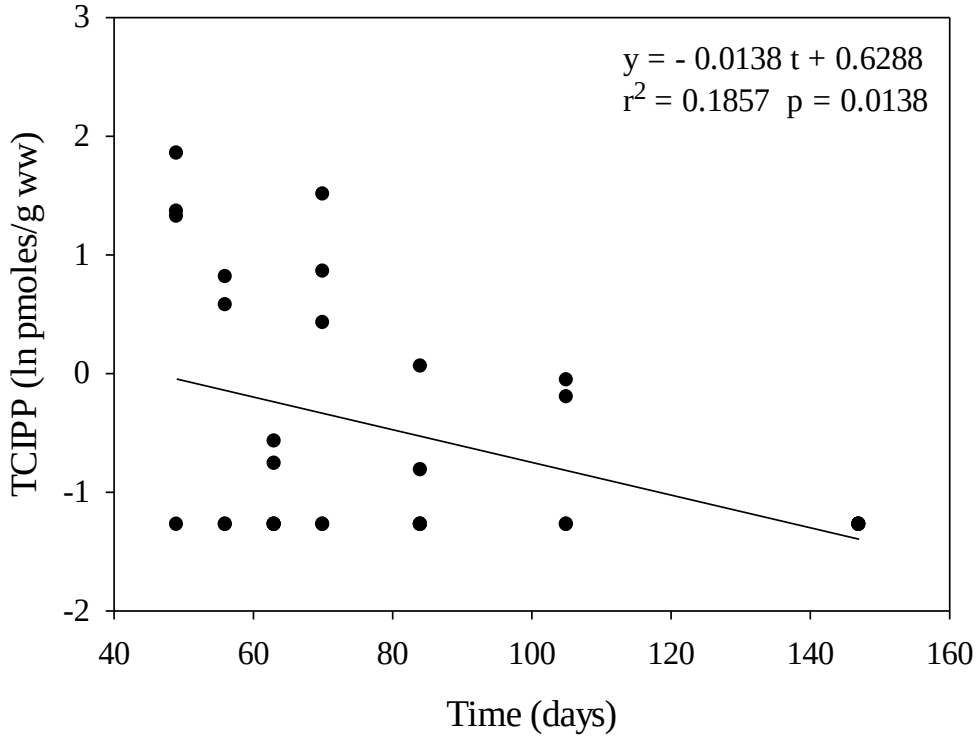


Figure 3.7: Plot of the log transformed concentration of TCIPP in juvenile rainbow trout versus time (days) during the depuration phase of the exposure (day 56to 147). Regression analysis is given in the plot.

3.5 Biochemical Effects

3.5.1. Thyroid

In my exposure experiment, there were no significant differences among treatments in TT3 or FT3 ($p > 0.05$) (Figure 3.8). TT4 was significantly reduced in the TCIPP dose group relative to reference fish by *ca.* 20% during the uptake phase at day 49 ($p < 0.05$, Figure 3.9) and FT4 was significantly increased at uptake day 28 for TCIPP by *ca.* 30% ($p < 0.05$). The differences in T4 levels may be a result of chemical interference with iodide uptake, peroxidase activity, secretion from thyroid gland, plasma binding proteins, peripheral metabolism, or natural variability (Park *et al.*, 2011). More tests would have to be conducted in order to validate the test results. A study by Farhat *et al*. (2013) in which domestic chicken eggs were injected with various doses of TCIPP (90 ng TCIPP/g egg to 51,600 ng TCIPP/g egg) and the plasma T3 and T4 levels were measured after hatching saw similar results to my study. TCIPP did not cause any significant changes in plasma FT3 levels in the exposed chicken embryos at any of the doses applied, which was similar to that seen in the rainbow trout. Although, Farhat *et al*. (2013) did not see a significant difference in TT4 or FT4 levels in the chicken embryo, there was significant differences in T4 levels in the rainbow trout. There was a significant decrease in TT4 at day 49U and a significant increase in FT4 at day 28U.

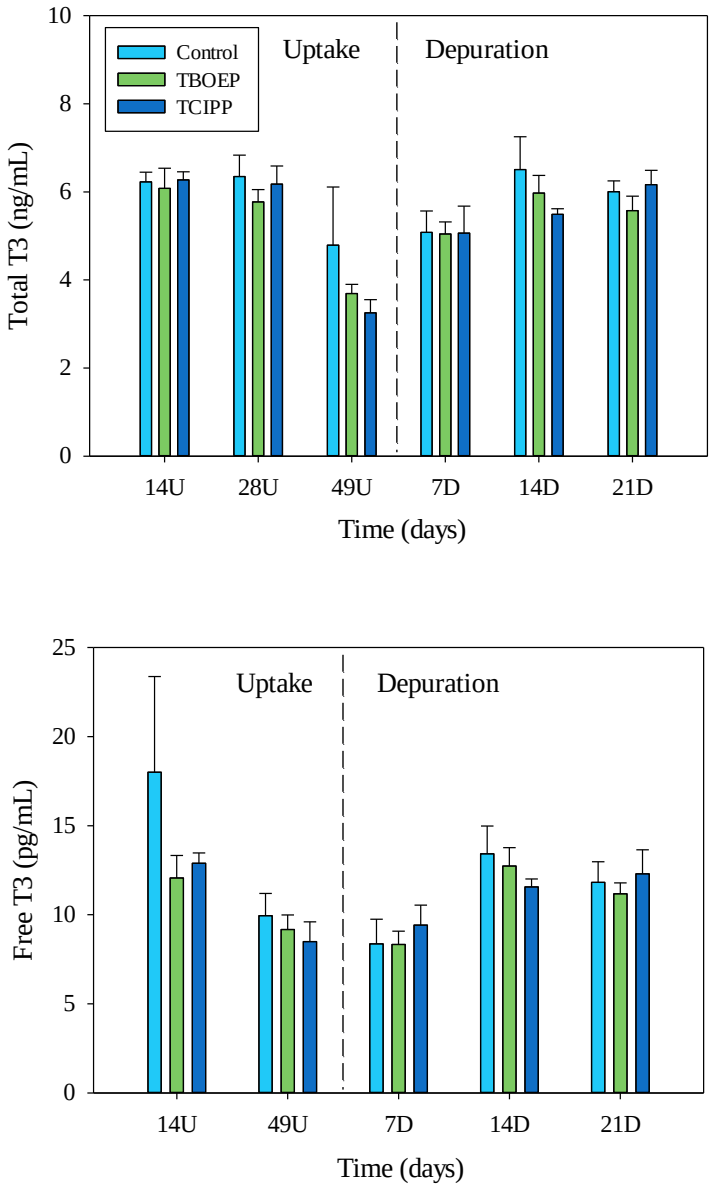


Figure 3.8: Plasma concentrations of free and total triiodothyronine (T3) of fish fed reference diets or diets enriched with TBOEP or TCIPP for 49 days (uptake), followed by reference diet for 98 days (depuration). Data are presented as arithmetic mean ± standard error.

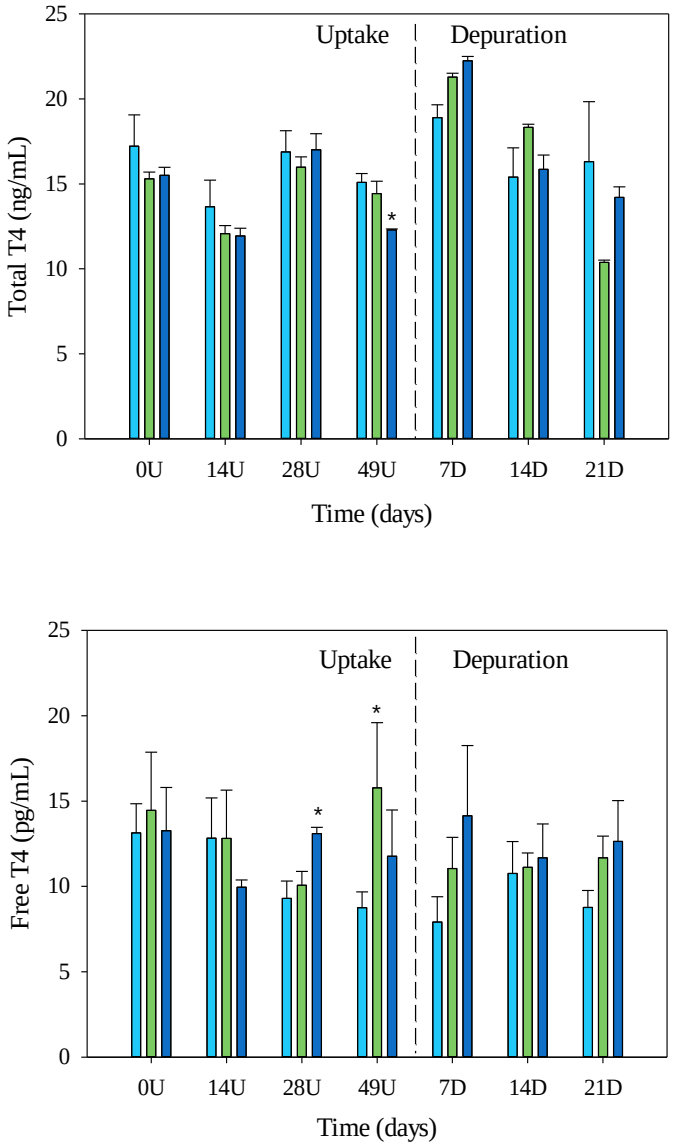


Figure 3.9: Plasma concentrations of free and total thyroxine (T4) of fish fed reference diets or diets enriched with TBOEP or TCIPP for 49 days (uptake), followed by reference diet for 98 days (depuration). Data are presented as arithmetic mean ± standard error. * indicates p-value < 0.05.

3.5.2. Steroid Hormones

Figure 3.10 shows the change in plasma CT levels in pmoles (normalized to 400 µL of plasma) in fish from the two exposure groups compared to the reference group. CT levels of the exposed fish at various time points were lower than in the reference fish. TBOEP exposed fish had CT levels that were significantly smaller at exposure day 28U, 70D, and 105D. At day 28 of the uptake phase CT levels in the reference group were 51.0 ± 7.1 pmoles, while the TBOEP exposed group had CT levels of 34.1 ± 5.0 pmoles, which is *ca.* 33% lower. TCIPP CT levels were significantly reduced at exposure days 49U, 63D, 70D, and 105D. At day 49 of the uptake phase CT levels in the reference group were 44.1 ± 5.2 pmoles, while the TCIPP exposed groups had CT levels of 18.2 ± 1.5 pmoles, which is *ca.* 59% lower. There appears to be a lack of CT response in fish exposed to both TBOEP and TCIPP. Other studies have investigated the effects of organic contaminants (e.g., polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and mercury) on the CT dynamics in fish, and have also failed to show a CT response to an acute stressors compared with fish from reference sites (Mommsen *et al*., 1999). This could be due to the prolonged hyperactivity of the CT-producing cells followed by an exhaustion of the HPI axis, or increased metabolism and elimination of CT (Mommsen *et al*., 1999).

The typical plasma levels of cortisol in resting or unstressed rainbow trout range from < 5.5 to 44.3 pmoles and post-stress levels increase to the range of 44.3 to

221.5 pmoles (Barton & Iwama, 1991). The plasma CT levels in the rainbow trout in this exposure experiment fell within these ranges.

The anaesthetic used in the exposure study to sacrifice fish was able to control for some of the sampling stress. The lethal dose of MS-222 (100 mg/L) does not appear to produce a plasma CT response compared to the lower, immobilizing doses (Carter *et al*., 2011). Although, there were still some effects seen due to the sampling procedures. Statistically, the measured amount of CT in the first fish sampled for each treatment was always significantly lower (Student's *t*-test, $p < 0.05$) than the other four fish. Therefore, for statistically purposes, I removed the CT data for the first fish sampled and used the average of the other four fish. While the overall trend of CT levels in the other four fish appeared to increase with the time it took to be sampled, that trend was not statistically significant (Student's *t*-test, $p > 0.05$).

Figure 3.11 and 3.12 show the amounts of E2 and T concentrations (normalized to 400 uL plasma) in the rainbow trout over the duration of the exposure. E2 levels remained relatively unchanged throughout the uptake phase of the exposure. There were no differences in E2 levels between the reference and exposed fish, except at day 28 where the fish exposed to TBOEP had reduced E2 levels compared to the reference fish ($p = 0.008$). The T levels remained relatively consistent, except for day 49 in the uptake phase, where the T levels are reduced for both TBOEP and TCIPP exposure compared to the reference fish ($p < 0.05$). The plasma T levels in

the reference fish at day 49 was 0.77 ± 0.02 pmole, while the TBOEP and TCIPP T concentrations were 0.54 ± 0.06 (*ca*. 30% lower) and 0.46 ± 0.04 pmole (*ca*. 40% lower), respectively. The increased T levels over the duration of experiment is partially due to maturation of fish during study period. Liu *et al.* (2012) recently demonstrated that TBOEP, TCIPP, and other OPFRs have the potential to alter sex hormone balance through steroidogenesis or estrogen metabolism (Liu *et al.*, 2012). Human cell lines (H295R cells) were exposed to TBOEP and TCIPP at concentrations between 0.01 to 10 and 0.1 to 100 mg/L, respectively (Liu *et al.*, 2012). H295R cells are used to measure sex hormone synthesis. There were significant differences in E2 and T levels for both compounds at the higher OPFR doses, but unlike my study where there was a decrease in the hormones levels, they observed a significant increase in hormones levels ($p < 0.05$). 11-KT and E2-3S were not detected in our fish plasma. The significance values (p-value) for each hormone compared to the reference fish is listed in Appendix C.

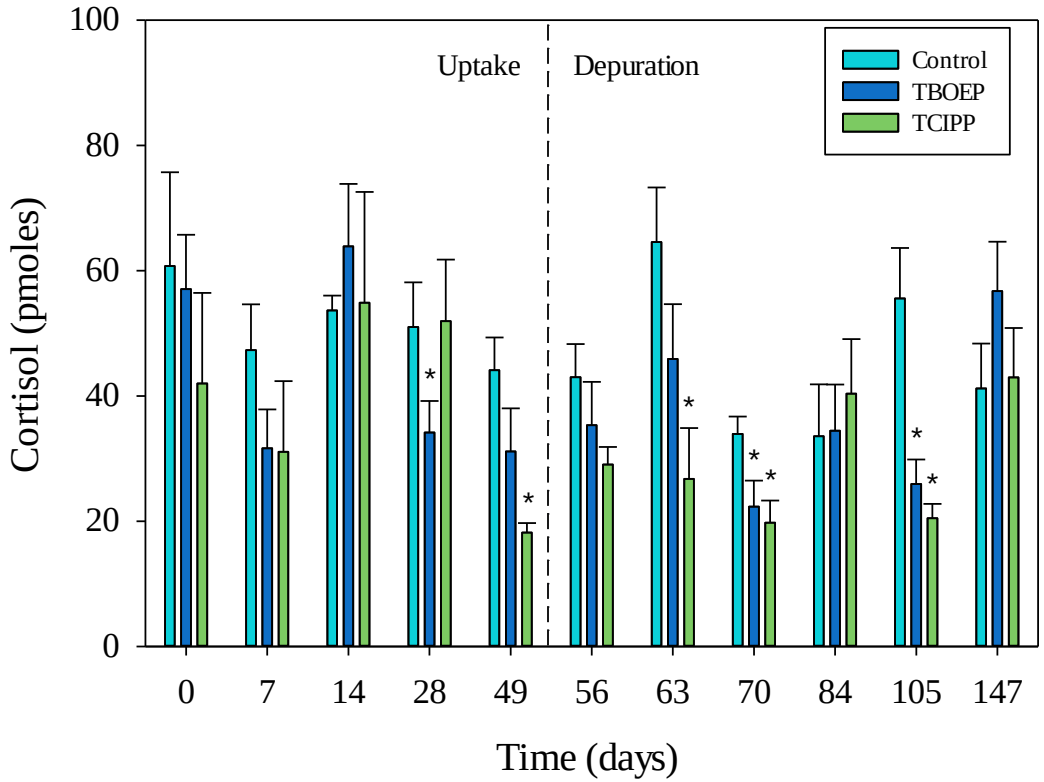


Figure 3.10: Plasma concentrations (pmoles) of cortisol in fish fed reference diets or diets enriched with TBOEP or TCIPP for 49 days (uptake), followed by reference diet for 98 days (depuration). Data are presented as arithmetic mean ± standard error. * indicates p-value < 0.05.

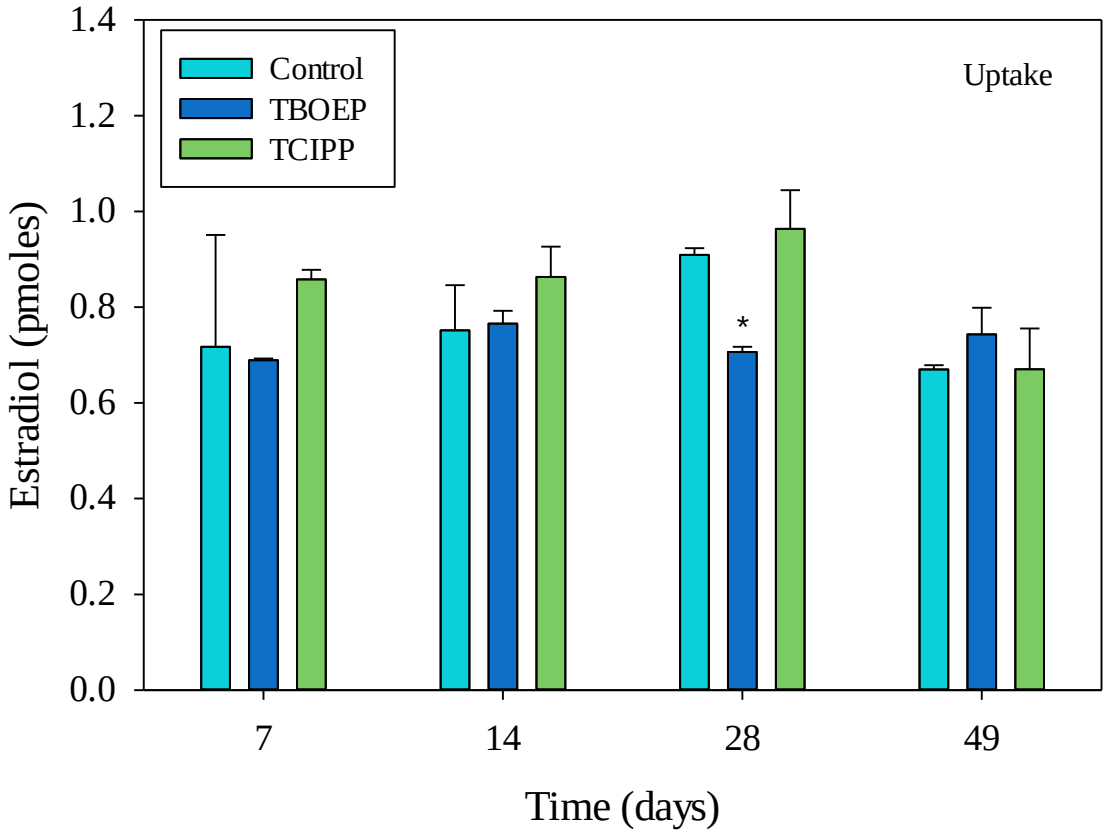


Figure 3.11: Plasma concentrations (pmoles) of E2 in fish fed reference diets or diets enriched with TBOEP or TCIPP for 49 days (uptake). Data are presented as arithmetic mean ± standard error. * indicates p-value < 0.05.

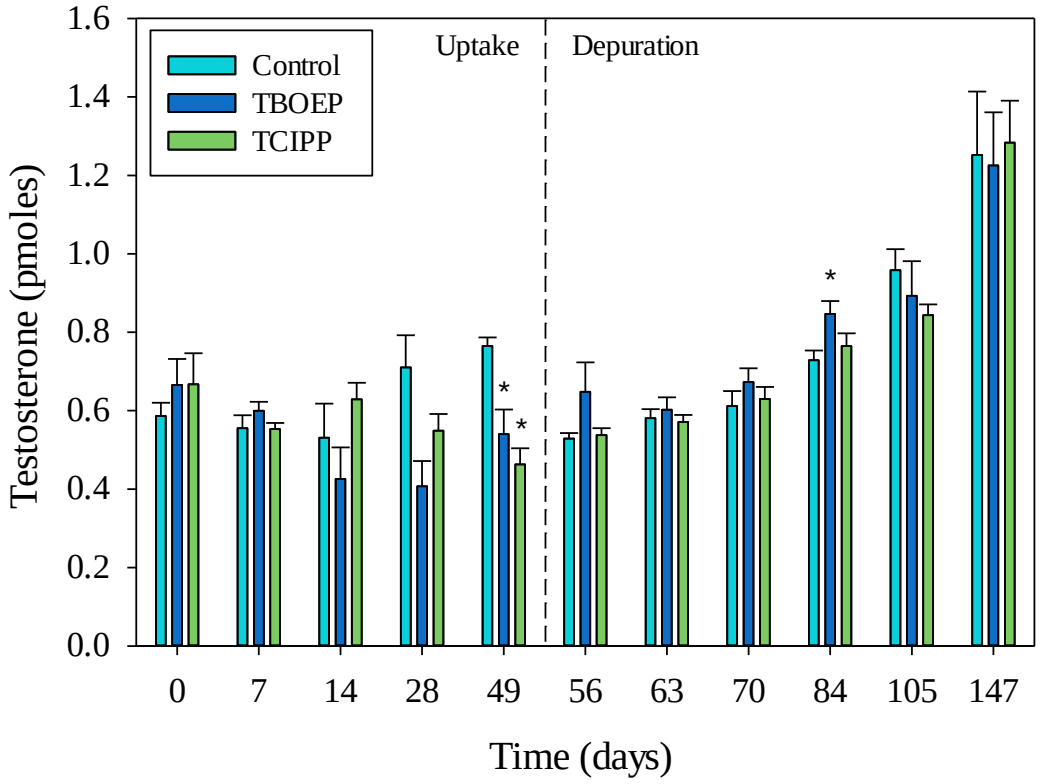


Figure 3.12: Plasma concentrations (pmoles) of T in fish fed reference diets or diets enriched with TBOEP or TCIPP for 49 days (uptake), followed by reference diet for 98 days (depuration). Data are presented as arithmetic mean ± standard error. * indicates p-value < 0.05.

4. Summary

The purpose of this study was to address the environmental fate and toxicity of two organophosphate flame retardants, tris (2-butoxyethyl) phosphate (TBOEP) and tris (1-chloro-2-propyl) phosphate (TCIPP) and determine if these compounds fulfill the persistent, bioaccumulative, and inherent toxicity criteria as defined by the SC. Juvenile rainbow trout were exposed via their diet to environmentally relevant concentrations, as determined by the Lake Ontario lake trout, of either TBOEP, TCIPP, or no OPFR (as a reference). Bioaccumulation was assessed by feeding fish with known amounts of each OPFR for a prescribed duration. Biological persistence was assessed by comparing the experimental determined half-life ($t_{1/2}$) to those of other known P compounds (i.e., several PBDE congeners). The T criterion was determined by measuring circulating plasma concentrations of sex, stress, and thyroid hormones at various time points in the exposed fish and comparing them to those in reference fish. Both TBOEP and TCIPP were detectable in lake trout from Lake Ontario, at concentrations of 6.46 ± 2.03 and 0.60 ± 0.19 pmole/g ww, respectively. These were the target concentrations used to drive the exposure study. The results of the exposure study suggests that TBOEP did not bioaccumulate in rainbow trout at the environmentally relevant concentration that was tested. Possible reasons for this include rapid elimination, biotransformation, or a poor assimilation efficiency. That TBOEP was measureable in other fish species from Lake Ontario suggests that there might be interspecies differences in

bioaccumulation potential of this compound. TCIPP did bioaccumulate in the rainbow trout with a BMF value of 0.81 ± 0.39. A BMF value less than 1 suggests that a chemical has a low potential to biomagnify in aquatic food webs. The calculated $t_{1/2}$ (50.2 ± 19.3 days) was similar to those of other POPs that have now been regulated by SC (i.e., penta- and octa-BDEs). I was unable to measure either compound or their postulated metabolites in the liver samples at the peak of uptake (day 49U).

There were limited significant effects on the rainbow trout in terms of growth and biological endpoints. There were some differences in the amounts of measured E2, T, CT, T4, and T3 between fish in the treatment groups and the reference group. There was a significant decrease ($p < 0.05$) in the amounts of TT4 and T in TCIPP exposed fish at the peak of uptake relative to the reference fish. However, there were no consistent treatment-affected trends among any of the hormones. Based on the findings in this study on rainbow trout, it could be concluded that TCIPP, and not TBOEP, fulfill the two criteria of P&B as defined by the SC, but whether either compound is iT remains inconclusive, at the environmentally relevant concentrations tested.

4.1 Recommendations for Future Work

There are several considerations for future studies regarding OPFR compounds. Further work on method development would be necessary in order to improve

recoveries and consistency of the method. There is no standardized method for extraction and analysis of OPFRs in any type of media. The considerations on how to minimize blank contamination in the study by Brandsma *et al*. (2013) is vital to obtaining more accurate and reliable data; including pre-cleaning all glassware, equipment, and SPE columns and silica with solvent, covering samples and glassware with aluminum foil, and minimizing the surface contact of the sampling. The lack of inadequate mass-labelled standards at the time of extraction was problematic. The internal standard for TBOEP was not available until after we had finished extractions, and currently there is still no labelled standard for TCIPP. Instead we used mass-labelled TCEP, the most structurally similar compound available. Labelled standards have similar physicochemical properties to the analyte, with identical optimized LC-MS conditions and a similar elution pattern. They are used for quantitative analysis to ensure accuracy of the data. Internal standards are important in order to minimize the variations resulting from sample preparation and extractions, and mass detector fluctuations. There is also a lack of OPFR metabolite standards. The metabolized forms may be used as biomarkers for their parent triester OPFRs in biota samples. The biotransformation products of OPFRs require further examination because it may be a major factor determining the *in vivo* metabolism and toxicity of the compounds. Sometimes metabolites are more toxic than their parent compounds (e.g., some OH-BDEs).

This study was limited by using only a single tank for each treatment, and it could be argued that true replicates were not available. However, despite the limitations of the experimental design, the data obtained in this study is still scientifically valid and can be used as a stepping stone for future work. Also, the use of more mature fish would have been beneficial in determining the effects of our test compounds on sex hormone levels; in addition, more plasma would be available for further tests, including testing OPFR levels in plasma samples as well as in the liver and muscle tissue.

Looking at other endpoints of these compounds would also be beneficial to furthering this study, especially ones based on effects of OP pesticides, since there are some structural similarities between them and OPFRs. The primary target of OP pesticides is the inhibition of the acetylcholinesterase (AChE) enzyme. AChE sends chemical signals to terminate nerve impulses, but OP pesticides inhibit this process causing the nervous system to become overstimulated resulting in immediate neurological dysfunction (PAN, 2013). The disruption of AChE activity is also linked to interferences with the reproductive system, resulting in reduced T levels (Recio *et al*., 2005). Lower T levels were observed in our study at the peak of uptake and perhaps this could be explained by disruption of AChE because of exposure to TCIPP and TBOEP.

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Appendix

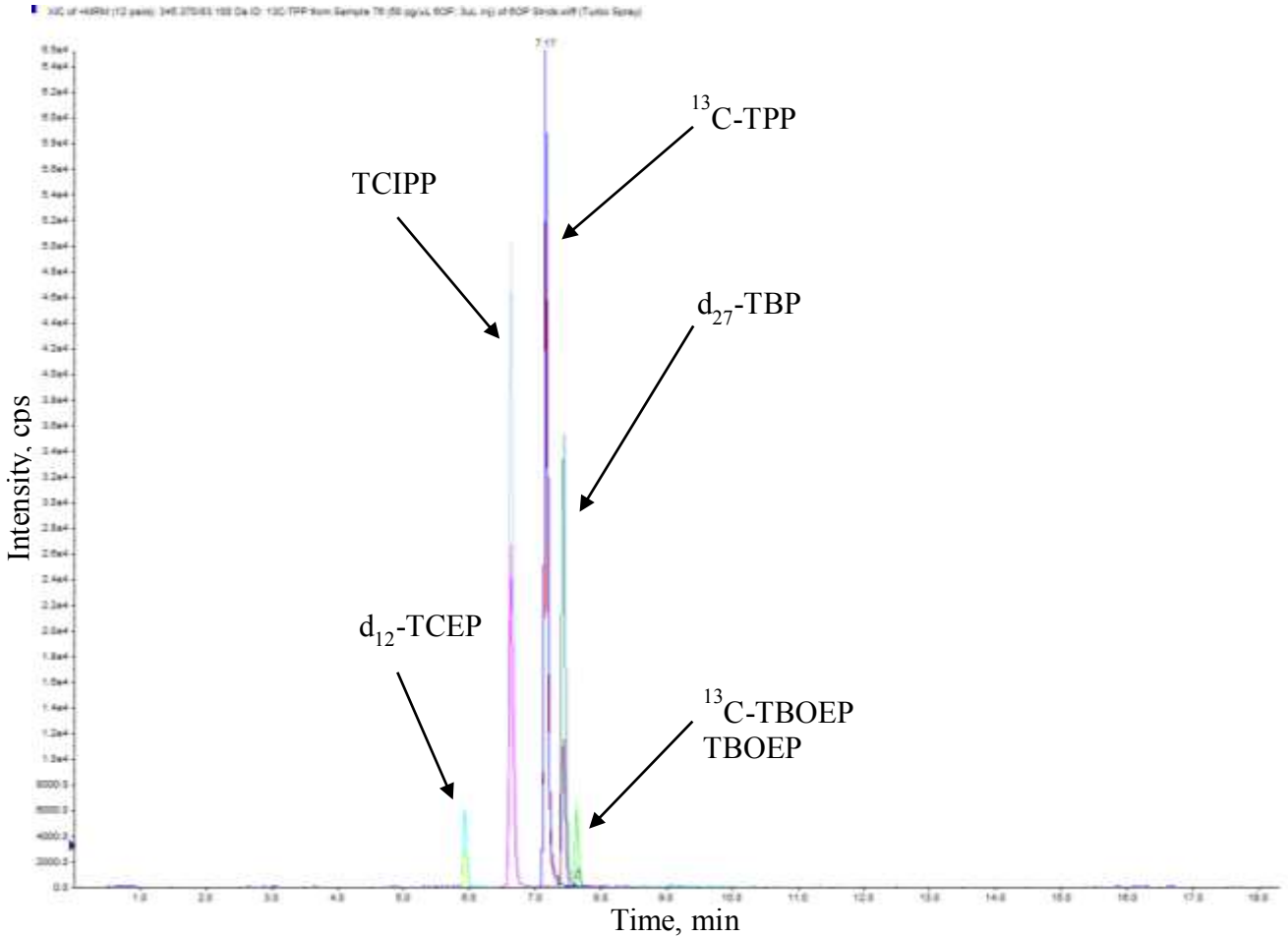
Appendix A

A.1: Parameters for negative ion mode of MS/MS.

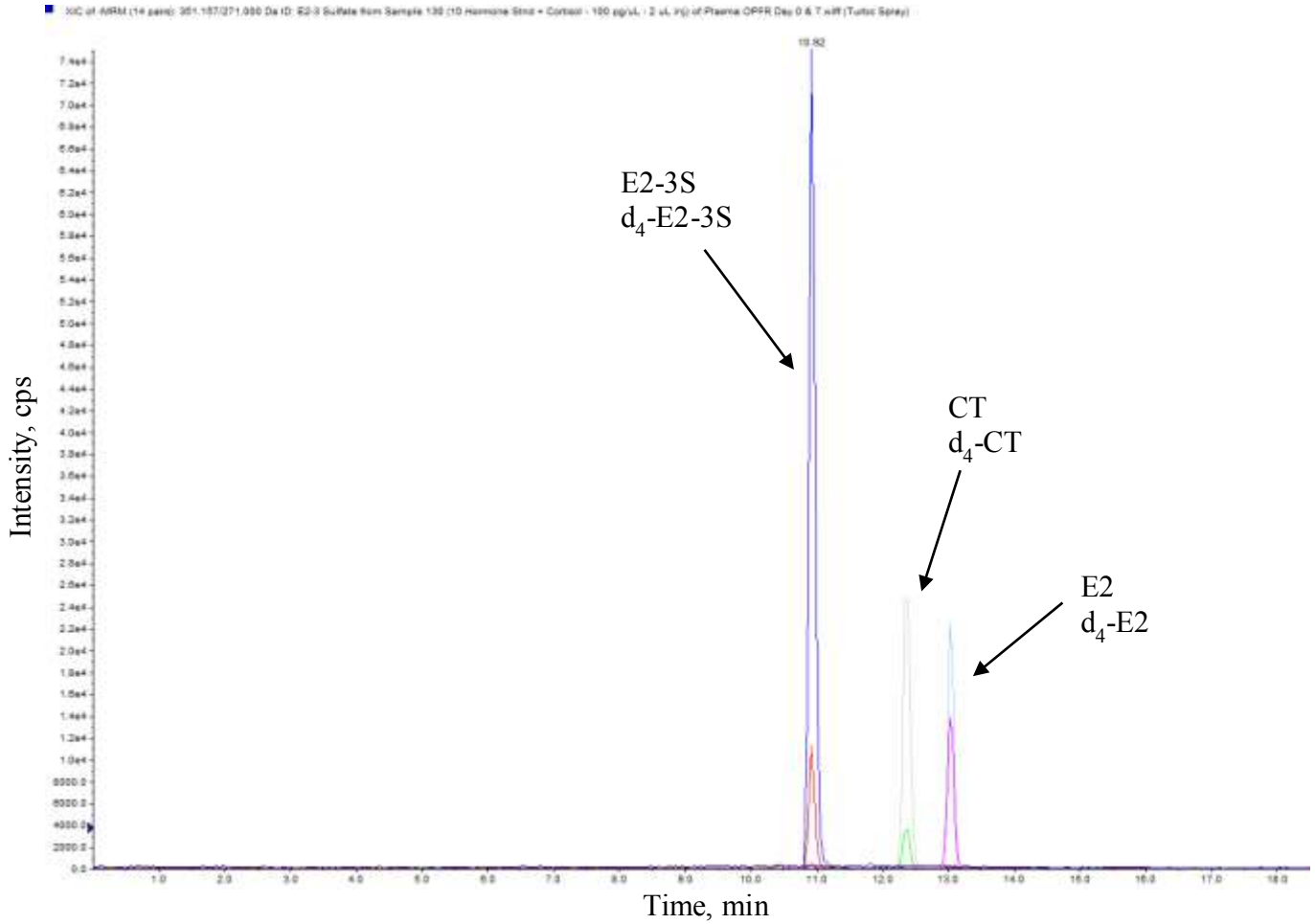
Parameter	**Abbreviation**	**E2**	**d_4-E2**	**E2-3S**	**d_4-E2-3S**	**d_4-E1**	**CT**	**d_4-CT**
Curtain gas	CUR	30	30	30	30	30	30	30
Sheath gas	GS1	20	20	20	20	20	20	20
Turbo gas	GS2	80	80	80	80	80	80	80
Ionspray voltage	IS (V)	-4400	-4400	-4400	-4400	-4400	-4400	-4400
Turbo-gas temperature	TEM (°C)	550	550	550	550	550	550	550
Declustering potential	DP (V)	-66	-86	-31	-31	-56	-35	-21
Focusing potential	FP (V)	-330	-230	-320	-350	-110	-330	-340
Entrance potential	EP (V)	-12	-10.5	-11	-11	-9	-10	-10
Collision gas	CAD	8	8	8	8	8	8	8
Collision cell entrance potential	CEP (V)	-16	-18	-18	-18	-16	-20	-20
Collision energy	CE (V)	-50/-52	-50/-52	-38/-46	-38/-52	-46/-74	-12/-28	-12/-28
Collision cell exit potential	CXP (V)	-10/-16	-12/-16	-18/-10	-20/-10	-12/-14	-18/-20	-18/-20

A.2: Parameters for positive ion mode of MS/MS.

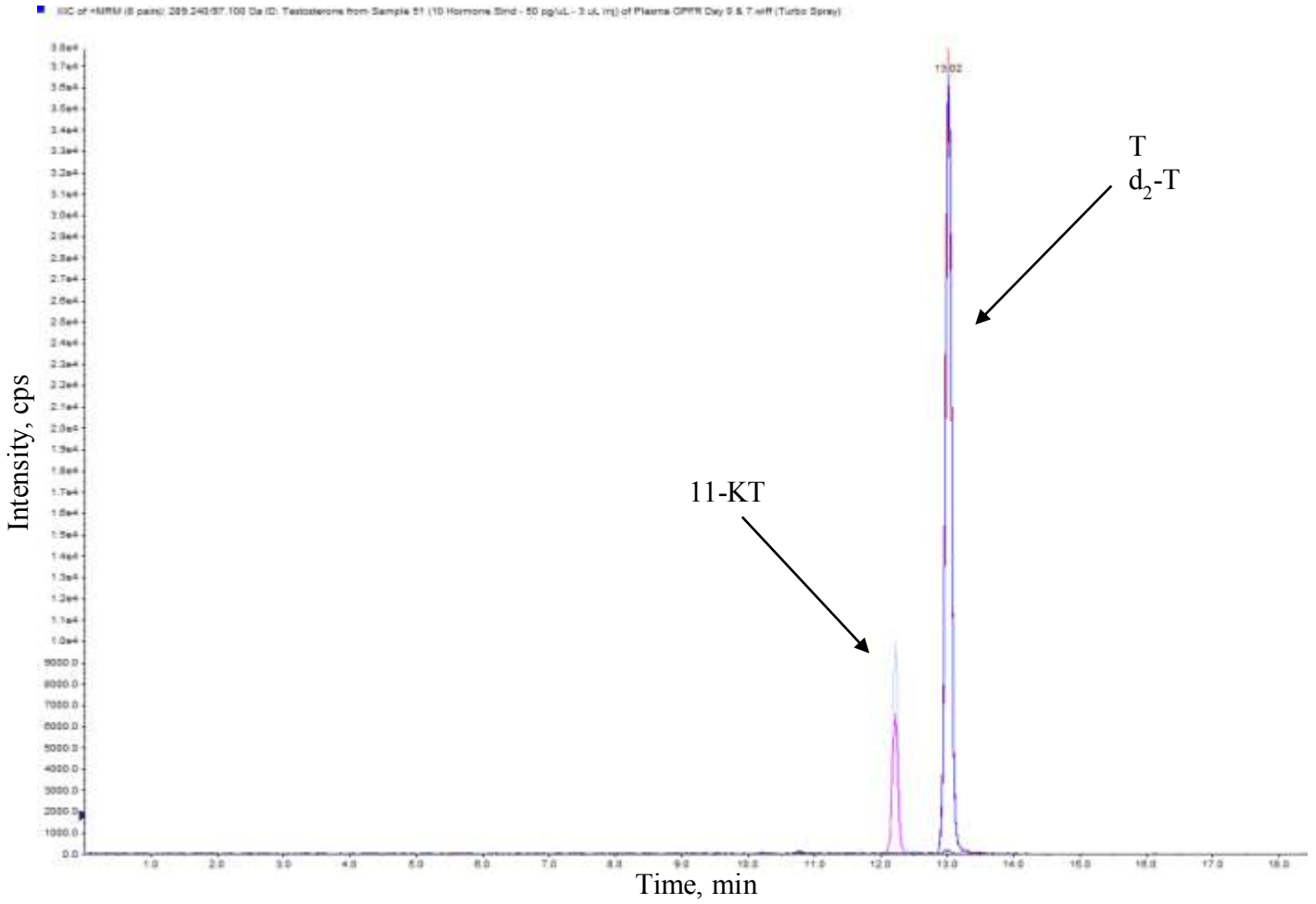
Parameter	**Abbreviation**	**T**	**11-KT**	**d_2-T**	**TCIPP**	**TBOEP**	**^{13}C-TBOEP**	**d_{12}-TCEP**	**d_{27}-TBP**	**^{13}C-TPhP**
Curtain gas	CUR	30	30	30	55	55	55	55	55	55
Sheath gas	GS1	30	30	30	50	50	50	50	50	50
Turbo gas	GS2	55	55	55	60	60	60	60	60	60
Ionspray voltage	IS (V)	5000	5000	5000	4500	4500	4500	4500	4500	4500
Turbo-gas temperature	TEM (°C)	550	550	550	500	500	500	500	500	500
Declustering potential	DP (V)	26	31	31	16	26	26	46	51	46
Focusing potential	FP (V)	340	370	370	360	370	350	370	330	360
Entrance potential	EP (V)	8.0	10.0	8.0	10.5	12.0	10.0	6.5	12.0	8.5
Collision gas	CAD	8	8	8	8	8	8	8	8	8
Collision cell entrance potential	CEP (V)	26	26	14	14	16	18	12	12	14
Collision energy	CE (V)	33	33/29	35	29/19	21/23	19/23	49/37	27/17	61/51
Collision cell exit potential	CXP (V)	10/8	8/16	8/12	6/14	18/12	14	6/10	6/8	6/14



A.3: Chromatogram of a 50 pg/µL OPFR standard analyzed by HPLC-MS-MS.



A.4: Chromatogram of a 100 pg/µL estrogen and cortisol standard analyzed by HPLC-MS-MS.



A.5: Chromatogram of a 100 pg/µL androgen standard analyzed by HPLC-MS-MS.

Appendix B

B.1: Average concentrations (pmoles/g ww, growth corrected) in fish prior to control/blank correction. Outliers have been removed using Q-test with a 95% confidence interval.

Sampling Day	**Control (TBOEP)**	**TBOEP**	**Control (TCIPP)**	**TCIPP**
0	16.42	10.54	2.60	1.55
7	1.97	2.99	1.48	1.57
14	2.52	3.24	2.31	3.99
28	6.15	7.01	2.93	3.64
49	3.04	3.59	2.50	5.92
56	3.34	4.37	2.17	3.08
63	4.67	2.64	1.85	1.99
70	9.53	8.79	2.35	3.96
84	9.34	7.90	2.10	2.14
105	11.65	10.68	4.19	4.47
147	10.09	8.45	4.25	2.47

Appendix C

C.1: Results of the zero- and first-order reaction kinetic plots for TCIPP.

	Zero-Order	**First-Order**
r^2	0.1676	0.1857
p-value	0.0200	0.0138
Slope	-0.0191	-0.0138
$t_{1/2}$	94.0 days	50.2 days

Appendix D

D.1: Significant values (p-value, Student's t-test) for the comparison of the amount of testosterone, estradiol, and cortisol between treatment groups and reference fish at each sampling time.

Day	**Testosterone**		**Estradiol**		**Cortisol**	
	TBOEP	**TCIPP**	**TBOEP**	**TCIPP**	**TBOEP**	**TCIPP**
7	0.283 (5,5)	0.944 (5,5)	0.915 (4,2)	0.482 (2,3)	0.171 (5,4)	0.224 (5,4)
14	0.348 (4,3)	0.210 (4,5)	0.876 (2,2)	0.352 (3,4)	0.611 (4,5)	0.942 (4,4)
28	0.104 (5,1)	0.156 (5,5)	**0.008 (3,4)**	0.824 (2,3)	**0.049 (5,5)**	0.945 (5,5)
49	**0.010 (5,4)**	**0.001 (5,5)**	0.430 (2,4)	0.997 (2,3)	0.107 (5,4)	**0.004 (5,4)**
56	0.175 (5,4)	0.707 (5,5)	n/a	n/a	0.236 (5,5)	0.056 (5,5)
63	0.562 (5,5)	0.742 (5,5)	n/a	n/a	0.217 (5,5)	**0.015 (5,4)**
70	0.278 (5,5)	0.746 (5,5)	n/a	n/a	**0.041 (5,5)**	**0.017 (5,5)**
84	**0.035 (5,5)**	0.783 (5,5)	n/a	n/a	0.985 (5,5)	0.662 (5,5)
105	0.569 (5,4)	0.103 (5,5)	n/a	n/a	**0.029 (5,4)**	**0.004 (5,5)**
147	0.913 (5,5)	0.886 (5,5)	n/a	n/a	0.240 (4,5)	0.887 (4,5)

Values in parenthesis are number of fish in each treatment group that are being compared (control, treated). Values in bold are statistically significant ($p < 0.05$).

D.2: Significant values (p-value, Student's t-test) for the comparison of the amount of thyroid hormones between treatment groups and reference fish at each sampling time.

Day	**TT3**		**FT3**		**TT4**		**FT4**	
	TBOEP	**TCIPP**	**TBOEP**	**TCIPP**	**TBOEP**	**TCIPP**	**TBOEP**	**TCIPP**
14	0.780 (5,5)	0.875 (5,5)	0.342 (3,3)	0.249 (3,5)	0.477 (5,5)	0.447 (5,5)	0.997 (5,5)	0.264 (5,5)
28	0.336 (5,5)	0.797 (5,5)	n/a	n/a	0.609 (5,5)	0.945 (5,5)	0.572 (5,5)	**0.008 (5,5)**
49	0.434 (5,5)	0.289 (5,5)	0.676 (5,3)	0.426 (5,4)	0.725 (5,5)	**0.050 (5,5)**	0.086 (5,4)	0.322 (5,5)
56	0.950 (5,5)	0.983 (5,5)	0.983 (5,4)	0.572 (5,5)	0.224 (5,5)	0.259 (5,5)	0.222 (5,5)	0.192 (5,5)
63	0.546 (5,5)	0.215 (5,5)	0.723 (5,5)	0.284 (5,5)	0.183 (5,5)	0.837 (5,5)	0.865 (5,5)	0.744 (5,5)
70	0.321 (5,5)	0.703 (5,5)	0.638 (5,5)	0.795 (5,5)	0.139 (5,5)	0.626 (5,5)	0.108 (5,5)	0.173 (5,5)

Values in parenthesis are number of fish in each treatment group that are being compared (control, treated). Values in bold are statistically significant ($p < 0.05$).