Fate of organophosphate esters (OPEs) in East Greenland polar bears and their ringed seal prey using *in vitro* lab- and field-based studies to investigate metabolism

by

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ABSTRACT

Arctic wildlife can be exposed to environmental chemical contamination due to long-range transport and subsequent deposition. Organophosphate esters (OPEs) are emerging chemicals of concern, found at high concentration in Arctic abiota but low levels in biota, including in polar bear and ringed seal adipose tissue currently under study. OPE metabolism was investigated in East Greenland polar bears and ringed seals using enzymatically-active liver microsomes. Organophosphate triester metabolism rate and extent were found to be greater in polar bears than ringed seals. Chemical structure and physical properties of OPEs affected metabolism; notably, bulkier, alkyl-substituted triphenyl phosphates had decreased metabolism in both species. The degradation of OPEs with aryl and alkyl substituents occurred at a greater rates in polar bears and ringed seals respectively. The structure-dependent metabolism of eleven OPEs have important implications for regulatory and risk assessments, as some compounds have little to no toxicokinetics data available in the current literature.

LIST OF WORKS ASSOCIATED WITH THE THESIS

Journal Publications

Strobel, A., Willmore, W.G., Sonnne, C., Dietz, R., Letcher, R.J., 2018. Organophosphate esters in East Greenland polar bears and ringed seals: adipose tissue concentrations and *in vitro* depletion and metabolite formation. Chemosphere 196, 240–250. doi:10.1016/j.chemosphere.2017.12.181

Strobel, A., Willmore, W.G., Sonnne, C., Dietz, R., Letcher, R.J., 2018 Structure-dependent *in vitro* metabolism of alkyl-substituted analogues of the organophosphate ester, triphenyl phosphate, in East Greenland polar bears and ringed seals. Environ. Sci. Technol. Letters, in preparation.

Conference Abstracts

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"Trust in the Lord with all your heart, and do not lean on your own understanding. In all your ways acknowledge him, and he will make straight your paths." ~Proverbs 3:5-6

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LIST OF ABBREVIATIONS

ADME	absorption, digestion, metabolism and excretion
APCI	atmospheric pressure chemical ionization
ANOVA	analysis of variance
BBOEP	bis (2-butoxyethyl) phosphate
BCIPP	bis(1-chloro-2-propyl) phosphate
BDE	brominated diphenyl ethers
BDCIPP	bis(1,3-dichloro-2-propyl) phosphate
BFR	brominated flame retardant
BMF	biomagnification factor
BSA	bovine serum albumin
CMP	chemical management plan
CYP	cytochrome P450
DCM	dichloromethane
CREATE	collaborative research and training experience program
DEHP	di (2-ethylhexyl) phosphate
DNBP	di (n-butyl) phosphate
DPHP	diphenyl phosphate
EHDPP	2-ethylhexyl diphenyl phosphate
HBCDD	hexabromocyclododecane
FR	flame retardant
GST	glutathione s transferase
HLM	human liver microsome
HPLC	high performance liquid chromatography

IDDPP	isodecyl diphenyl phosphate
L	litres
LOD	limit of detection
LRTP	long-range transport potential
MFO	mixed function oxidases
mL	milliliter
MLOD	method limit of detection
MLOQ	method limit of quantification
MS	mass-spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
ng	nanograms
OPEs	organophosphate esters
OCRL	organic contaminants research laboratory
PB	polar bear
PBDE	polybrominated diphenyl ether
PBT	persistence, bioaccumulation and toxicity
POPs	persistent organic pollutants
P_{OV}	overall persistence
Q-TOF	quadrupole-time-of-flight
REACT	research in environmental and analytical chemistry and toxicology
RS	ringed seal
t	tonnes
T2IPPP	tris (2-isoproylphenyl) phosphate
T4IPPP	tris (4-isopropylphenyl) phosphate
TBOEP	tris (2-butoxyethyl) phosphate
TCEP	tris(2-chloroethyl) phosphate
TCIPP	tris(1-chloro-2-propyl) phosphate
TCP	trimethylphenyl phosphate
TBPDPP	p-(tert-butylphenyl) diphenyl phosphate
TDCIPP	tris (1,3-dichloro-2-propyl) phosphate
TEHP	tris (2-ethylhexyl) phosphate

TEP	triethyl phosphate
TNBP	tri (n-butyl) phosphate
TPHP	triphenyl phosphate
TNBP-$(OH)_2$	dihydroxylated tri(n-butyl) phosphate
TTBPP	tris(p-tert-butylphenyl) phenyl phosphate
UPLC	ultra-high performance liquid chromatography
V_{MAX}	rate assuming the enzyme system is saturated

1 Chapter: General Introduction

1.1 Fate of OPEs: The Arctic Environment and Wildlife Exposure

The Arctic is a sink for anthropogenic contaminants at the global scale leading to regional model systems such as Eastern Greenland which is subject to ongoing extensive environmental contamination (Dietz et al., 2016). Substances enter the Arctic ecosystem via long-range oceanic and atmospheric transport and persist due to cold-trapping in biota. Thus, Arctic wildlife and Inuit subsistence hunters could be exposed to novel contaminants for which little toxicological data currently exists, as has previously been shown for legacy contaminants (Letcher et al., 2010; Rigét et al., 2016).

Evidence for long-range transport potential of OPEs has been demonstrated by detection and quantification of OPEs in airborne particles - from East Asia to the high Arctic (230 to 2900 pg/m^3), Svalbard (33 to 1450 pg/m^3), and Resolute Bay/Alert (nd-2340 pg/m^3) (Castro-Jimenez et al., 2016; Mcgoldrick et al., 2014; Möller et al., 2012). These compounds may subsequently persist in the environment depending on their physical-chemical properties (Möller et al., 2012; Wang et al., 2015; Wei et al., 2015). To further investigate transport and persistence potential, modeling has determined atmospheric lifetimes for OPEs. For tris (2-ethylhexyl) phosphate (TEHP), triphenyl phosphate (TPHP), and tris (1,3-dichloro-2-propyl) phosphate (TDCIPP) the atmospheric lifetimes were estimated at 4.3, 5.6, and 13 days respectively (Liu et al., 2014). Thus, providing further evidence for atmospheric transport of OPEs to off-source areas.

Various methods were used to ascertain the concentration of OPEs in the Arctic ocean: samples were taken in tributaries, Arctic seawater from a ship was sampled, and

"hotspots" were estimated based on air concentrations. In Europe, tributaries to the North Sea were sampled including the River Elbe where some major non-halogenated OPE contributors were found in the dissolved phase, notably TEP (10-180 ng L^{-1}) and TBOEP (<LOD-80 ng L^{-1}) (Bollmann et al., 2012). Additionally, Bollman *et al.* found seasonal variability suggesting biodegradation and photolysis of non-halogenated OPEs during summer months (Bollmann et al., 2012). Secondly, during 2007 samples were acquired in Canadian Archipelago water from Coast Guard ships (Louis S. St. Laurent and Amundsen) (Jantunen et al., 2007). Prominent OPEs were found to be tris (1-chloro-2-propyl) phosphate (TCIPP; 6-13 ng/L), tris (2-chloroethyl) phosphate (TCEP; 4.4-4.5 ng/L) and TNBP (4.2-8 ng/L). These concentrations were much higher than legacy compounds such as polybrominated diphenyl ethers (PBDEs) but comparable to North Sea levels (Bollmann et al., 2012; Jantunen et al., 2007; Su et al., 2007). Finally, contamination in the Canadian Arctic water systems have been inferred based on air concentrations of OPEs using the Organisation for Economic Co-operation and Development (OECD) overall persistence (P_{OV}) long-range transport potential (LRTP) Screening Tool (Sühring et al., 2016). At river mouths the salt concentration increases causing the solubility of OPEs to decrease and thus be evaporated into the atmosphere. Thus, air concentrations above these river mouths can give "hotspot" indications of discharges into the North Atlantic and Arctic Ocean. Non-chlorinated OPEs were detected at high concentrations (TPHP, tri-n-butyl phosphate (TNBP), trimethylphenyl phosphate (TCP), 2-ethylhexyl diphenyl phosphate (EHDPP)) around river mouths, even though they were predicted to have similar or greater persistence in water compared to air. This led to the authors suggesting that both air and water processes contributed to the

transport of OPEs into the Arctic environment (Sühring et al., 2016). Moreover, deposition of OPEs to surface sediment has been measured (sum of OPEs ranging from 159 – 4658 pg/g dry weight) in the North Pacific to Arctic Ocean (Ma et al., 2017). These investigations provide a basis to suggest that legacy and emerging substances, along with other triggers such as climate change, may affect Arctic biota. Thus, the fate of OPEs in Arctic biota is worthy of further research.

The environmental matrix detection and quantification of OPEs has motivated research measuring OPEs in biota residues, including some marine species (See **Table 1-1**). In summary, total OPE concentrations have been reported as high as 15 000 ng/g in global fish surveys (Hou et al., 2016; Sundkvist et al., 2010). In the Arctic Circle, OPEs have been reported in lake trout (*Salvelinus namaycush*), walleye (*Sander vitreus*), white-tailed sea eagle nestlings (*Haliaeetus albicilla*), peregrine falcon nestlings (*Falco peregrinus*), ringed seal (*Pusa hispida*; RS), arctic fox (*Vulpes lagopus*), and polar bear (*Ursus maritimus;* PB) (Eulaers et al., 2014; Fernie et al., 2017; Hallanger et al., 2015; Mcgoldrick et al., 2014). Additionally, low concentrations of OPEs have been detected in Western and Southern Hudson Bay polar bear liver and adipose tissue (Letcher et al., 2017b). These low concentrations comparative to the high environmental exposure data suggest rapid OP triester metabolism. Thus, there is environmental exposure concern relevant to the further study of toxicokinetics in Arctic marine biota. The present study will provide much needed data on the toxicology and fate (e.g. metabolism and biotransformation) of a subset of OPEs in polar bear and ringed seal collected from the East Greenland area of Scoresby Sound.

Table 1-1: OPEs measured in various Arctic biota, selected as the highest OPE levels in the studies identified below. A more comprehensive list can be found in the Supplementary Tables by Hou *et al*. (Hou et al., 2016)

Study	Collection Year	Sample Location	OPE	Species, samples	Range (ng/g)
Letcher *et al*., 2018 (Letcher et al., 2018)	2013-2014	South Hudson Bay, Canada	TEHP	Polar bear	0.308 ng/g lw
		West Hudson Bay, Canada	TEHP	Polar bear	0.163 ng/g lw
Fernie *et al.,* 2017 (Fernie et al., 2017)	2007	Ungava Bay, Canada	TBOEP	Peregrine falcon nestlings plasma	0-7.5 ng/g ww
Hallanger *et al.,* 2015 (Hallanger et al., 2015)	2010	Storfjorden, Svalbard	TCEP	Polar bear $(1/10)^{+}$	52.5 ng/g lw
	2010	Storfjorden, Svalbard	TDCIPP	Polar bear $(4/10)^{+}$	2.72± 1.24 ng/g lw (LOD: 6.89)
	2008	Storfjorden, Svalbard	TPHP	Polar bear $(1/10)^{+}$*	5.36 ng/g lw
Eulaers *et al*, 2014 (Eulaers et al., 2014)	2011	Trøndelag, Norway	Total	White-tailed sea eagle nestlings feathers	0.95 – 3000 ng/g
Evenset et al., 2009 (Evenset et al., 2009)	2008	Moffen, Greenland	TDCIPP	Polar cod	5.1 ng/g ww
	2008	Moffen, Greenland	TCEP	Polar cod	2.38 ng/g ww

+number of samples with detectable quantities of the total number of samples
*in 2008, TPHP was measured in 1/10 samples but in 2010, TPHP was measured in 0/10 samples.
lw, lipid weight; LOD, limit of detection; Total, sum of all OPEs measured in the corresponding study

1.2 OPEs as Global Emerging Chemicals of Concern

Today's world is global in many senses, from economy to interconnected communication, but no factor is as universal as the environment. Thus, concern regarding environmental contamination has led to strict regulation instituted at an international level through the Stockholm Convention. Recently, amendments to the Stockholm Convention Annex A list of Persistent Organic Pollutants (POPs) has resulted in the restriction of some brominated flame retardants (BFRs). Additions include penta- and octa-brominated diphenyl ethers (BDEs) in 2009, hexabromocyclododecane (HBCDD) in 2013, and very recently deca-BDEs in April 2017 (UNEP 2011, 2013, 2017). Restrictions have been mandated due to their potential to bioaccumulate, persist in the environment, and have hazardous health outcomes (Crump et al., 2012). These regulations lead to lack of commercial products available to serve specific purposes such as flame retardants.

There are many novel compounds produced as replacements to those which have been restricted or banned. Organophosphate esters (OPEs) are one such class of replacement flame retardants (FRs) that also function as plasticizers or performance additives. They can be found in industrial and consumer products such as polymers, resins, electronics, building materials, furniture, foams, plastics, engine oil and the list continues. Most recently, the European Flame Retardants Association published that global sales of FR were greater than 2 million tonnes (t) in 2013 (European Flame Retardants Association, 2015) and production estimates were 620,000 t/yr in 2013 (Sühring et al., 2016). Meanwhile, in 2011, OPE FR consumption reached 290,000 t globally (Du et al., 2015). An estimation of the growth in this market sector is an increase of 90,000 t over a 7-year span (Du et al., 2015). More specifically, in 2012, TDCIPP,

TPHP, and TCIPP, in the United States contributed approximately 38,000 metric t (Schreder et al., 2016) (See **Figure 1-2**, **Figure 2-3**, and **Figure 2-4** for full chemical names). Due to this increase in production of novel FRs, toxicity data is required to evaluate potential health and environmental hazards. This project will provide much needed data on the toxicokinetics of this subset of major, current-use FRs, OPEs.

OPEs functioning as additives to industrial and consumer products are not chemically bound (Hou et al., 2016). This results in potential for release into the environment during production, use, or disposal of products containing OPEs. Thus, OPEs have been found in many matrices such as air, wastewater effluent, household dust, sediment, and biota (Cristale et al., 2016; Greaves et al., 2016a; Kim et al., 2013; Möller et al., 2012; Su et al., 2015a). This potential exposure hazard is a strong motivator for further investigation into the fate of OPEs.

1.3 Adverse Effects of OPEs

Research regarding adverse health effects of OPEs in biota through *in silico, in vitro,* and *in vivo* studies have been conducted; as this class of compounds is numerous, findings have been diverse and structure-dependent. Wang et al., demonstrated this clearly when aryl and chlorinated OPEs inhibit lysine decarboylase enzymes *in vitro* and alkyl-substituted OPEs did not. This was further explored *in silico*, showing that OPE binding sites differed based on their inhibitory effects (Wang et al., 2014). This is just one example of structure-dependent inhibition of a key enzyme in a process affecting cell proliferation, hypertrophy and tissue growth.

OPEs demonstrate many other negative health effects. These range from cellular cytotoxicity (Porter et al., 2014; Su et al., 2014), cardiotoxicity in the zebra fish model species (Du et al., 2015; Isales et al., 2015), neurotoxicity (Pei et al., 2015; Yuan et al., 2016), endocrine disruption (Chen et al., 2015a; Kojima et al., 2016, 2013; Ma et al., 2015; Schang et al., 2016; Suzuki et al., 2013), oxidative stress (Chen et al., 2015a, 2015b; Schang et al., 2016), thyroid concerns (Fernie et al., 2017; Hill et al., 2018a), and carcinogenicity (Hoffman et al., 2017). While this is a very brief list of general toxicity and health related research, there is substantial evidence that negative effects of OPEs require further investigation into the toxicokinetic parameters, especially in terms of groups of structurally similar OPEs.

OPE metabolite toxicity is much less studied; however, there is indication that metabolites have structural dependent effects on toxicity end points. A cell based transcriptional assay showed that hydroxylated TPHP-metabolites had increased human estrogen receptor activity than the parent and diester metabolites (Kojima et al., 2016). This indicates that percent conversion to specific classes of metabolites may be critical for determining effect levels and OPE fate.

1.4 OPE Metabolism

OPEs have been shown to be degraded through abiotic processes such as pH-dependent hydrolysis as well as biotic processes such as enzymatic metabolism (Greaves et al., 2016b; Su et al., 2016b). Abiotic degradation should lead to consideration of effective controls in experiments to determine the contribution of enzymatic metabolism. Additionally, these processes should bring about awareness that wildlife may be exposed

to a mixture of parent and breakdown products. Thus, identifying environmentally relevant parent or active metabolite compounds is crucial for biomonitoring. In order to further this progress, the review by Hou *et al*., suggested study of the proposed diesters and monoesters of OPEs in wildlife (Hou et al., 2016). This thesis project aims to begin to fill this knowledge gap by studying the metabolism of a strategic subset of parent OPEs and their corresponding diester metabolites in polar bear and ringed seal.

Enzymatic biotransformation at a subcellular level can occur at various locations, where cytochrome P450 (CYP) enzymes are typically found in the endoplasmic reticulum (microsomal fraction) and many enzymes can be found in the cytosol (S9 fraction). Some CYP enzymes are known to metabolize xenobiotic compounds and thus, the microsomal fraction is the focus of the current project. Many of these CYP enzymes require nicotinamide adenine dinucleotide phosphate (NADPH) as a reducing agent and coenzyme, especially in the hydroxylation of aromatic compounds. Some metabolomics research has been conducted for OPEs, identifying some putative phase I and II pathways for OPEs (Van den Eede et al., 2013). Experiments were conducted utilizing human liver microsomes and high performance liquid chromatography, quadrupole-time-of-flight, mass-spectrometry (HPLC, Q-TOF-MS). The main pathways described were oxidative-dealkylation, hydroxylation, and oxidative-dearylation (see **Figure 1-1** for a more detailed overview of pathways). Further research to determine metabolite identities, rates of formation, and the mass balance are essential for designing biomonitoring studies to better assess internal exposure.

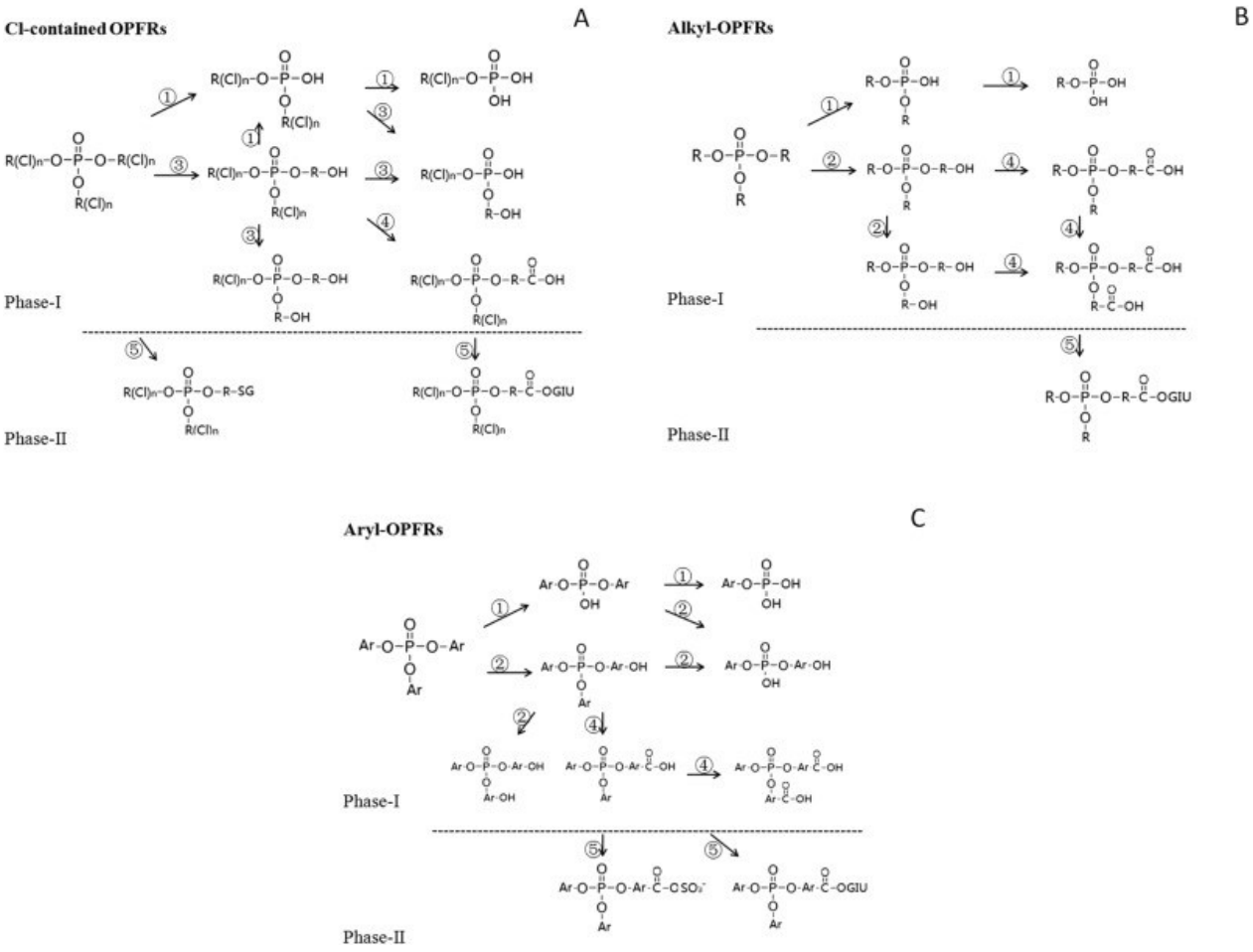


Figure 1-1: Review by Hou *et al*. summarizing *in vivo* and *in vitro* studies of Phase I and Phase II metabolic pathways for structurally categorized OPEs (A: chlorinated OP triesters; B: alkyl-substituted OP triesters; C: aryl-substituted OP triesters). Metabolic reactions are labeled as follows: ①: Oxidative dealkylation;②: Hydroxylation;③: Oxidative dechlorination;④: Oxidation; ⑤: Conjugation (Hou et al., 2016).

OPEs are metabolized in a structure dependent manner that has been shown through comparison of rates between OPEs, the quantification of products, and the analysis of pathways. Thus far, the number of OPEs tested is limited with representative halogenated, alky and aryl substituted triesters chosen for given studies. These few studies have produced key *in vitro* data to frame the concept of structural-dependent internal fate of OPEs as they explore potential metabolites.

Pioneering this concept, Sasaki *et al.* 1984, found that the order of metabolic depletion in Wistar rat liver microsomes from most to least rapid to be TNBP > TPHP > TDCIPP (Sasaki et al., 1984). More recently, Greaves *et al*. 2016 found that herring gull microsomes depleted OPE parent structures in the order of TNBP > TBOEP > TCIPP > TPHP > TDCIPP (Greaves et al., 2016b). Research progressed to reviewing specific rates. For example, in the case of TCIPP metabolized in human liver microsomes, the Michaelis-Menten model was used to determine the V_{MAX} (rate assuming the enzyme system is saturated) of 1470 ± 110 pmol/min/mg protein (Van den Eede et al., 2016). For herring gull liver microsomes, the V_{MAX} rates varied from 5.0 ± 0.4 to 29 ± 18 pmol/min/mg protein for TPHP and TBOEP respectively (Greaves et al., 2016b). The next layer of investigation reviewed the mass balance to determine the degree of parent depletion. For example, Van den Eede *et al.*, 2013 used human liver microsomes to show that 81 % of the initial quantity of TBOEP was depleted after the 60 minute assay, along with 46 % of TDCIPP, 41 % of TPHP, 33 % of TCIPP, and 7 % of TCEP (Van den Eede et al., 2013). Next, the metabolic products were analyzed and discussed considering putative biochemical pathways. For example, TNBP was metabolized to the intermediate metabolite TNBP-OH and further processed to TNBP-$(OH)_2$ and DNBP (Sasaki et al., 1984). A follow-up experiment showed that Wistar rat microsomes dosed with DNBP did not degrade this metabolite, providing an indication of internal fate of TNBP through a two-step process (Sasaki et al., 1984). The last piece of evidence that the literature reveals is regarding the conditions of OPE degradation. Study design controls have included presence and absence of assay additives such as cofactors (NADPH, GST etc.) and enzyme inhibitors (e.g. SKF-525A which inhibits mixed function oxidases or MFO).

In the absence of NADPH, two studies determined TPHP could be degraded and its diester metabolite, DPHP would form in human and Wistar rat liver microsomes (Sasaki et al., 1984; Van den Eede et al., 2013). This indicates that other enzymes beyond NADPH-dependent CYP enzymes may play a role in the metabolism of OPEs. One method of investigating further was through the use of enzyme inhibitors, demonstrating NADPH-dependent mixed function oxidases (MFO; i.e. CYP enzymes) played a key role in the metabolism of OPEs in Wistar rat liver microsomes (Sasaki et al., 1984). NADPH-independent enzymes were suggested to be paraoxonases and aryl esterases (Testa and Kramer, 2010; Van den Eede et al., 2013). During these broader processes to understand biochemical pathways, research has presented diverse results within classes of OPEs as is true for the chlorinated OP triester compounds. It has been shown that TCIPP forms the major metabolite of bis(1-chloro-2-propyl) phosphate (BCIPP) in human liver microsomes and serum (Van den Eede et al., 2016). Meanwhile, another study found TCEP was not metabolized (Sasaki et al., 1984). This indicates that broader research is required for this class of compounds compared to simply using representative OPEs. The current project aims to better understand structurally-dependent metabolic trends and rates by including a greater number of OPE parent compounds.

1.5 Thesis Research Questions, Objectives, and Hypothesises

The overall objective of this thesis is to better understand the fate of eleven OPEs in terms of their diverse structures and compare predator-prey species – polar bear and ringed seal. This goal was approached through two lines of evidence: (1) determining the depletion of OP triesters and formation of relevant OP diesters in the context of species

and structural comparisons; (2) determining the current status of OP triester residues in polar bear and ringed seal adipose tissue. It was hypothesized that metabolism would be fundamental in the fate of OP triesters, where there would be rapid rates of metabolism corresponding with low tissue residue levels. However, it was predicted that there would be species-specific trends as polar bear are known to be highly enzymatically active while there was minimal xenobiotic metabolism data available for ringed seal. In comparing tissue residue levels in the predator-prey species pair, bioaccumulation could be considered if applicable. Additionally, structural features were projected to have an effect on metabolism where more bulky OP triesters were postulated to be metabolised less rapidly and less completely than smaller analogues.

The objectives and hypothesizes of the project were described in major research questions as follows.

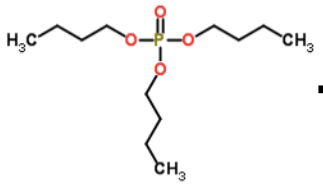
1. Do the quantities of OPEs found in storage tissues correspond to metabolic rates measured?
2. Are there any trends correlating OPE structure to metabolic rate or percent depletion?

1.6 Study Rationale

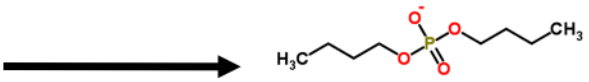
Due to long-range transport of contaminants, Arctic wildlife and Inuit subsistence hunters could be exposed to novel contaminants. Legacy compounds are regulated through the international Stockholm Convention list on POPs due to PBT concerns. Thus, novel replacement compounds are produced with little toxicological data available,

including OPEs. Data could support risk assessments used to evaluate these same PBT parameters in an environment comprised of a cocktail of anthropogenic chemicals.

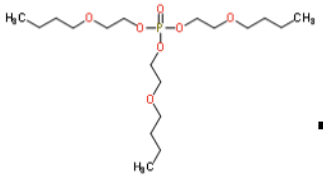
Therefore, this thesis project aims to provide degradation, respective metabolite formation, and internal fate data in the representative Arctic wildlife predator-prey relationship between polar bear and ringed seal. OPE metabolic rate was determined utilizing the established ultra-high performance liquid chromatography-electrospray ionization (+) triple quadrupole mass spectrometry (UPLC-ESI(+)-QQQ-MS/MS) method (Chu et al., 2011) along with some modifications (Greaves et al., 2016b). Substances chosen for metabolic and structural-activity relationship analysis are environmentally relevant and have priority under the mandates of the Chemical Management Plan and Arctic Monitoring and Assessment Program. More specifically, the structure-activity analyses of OPEs will be compared using toxicokinetic data.



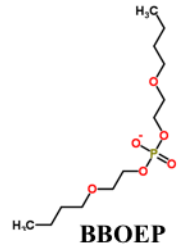
TNBP
tri (n-butyl) phosphate; 126-73-8
266.3 g/mol; log K_{OW} 4[a]



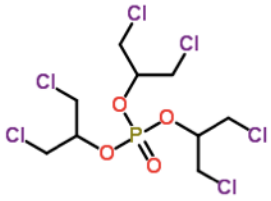
DNBP
di (n-butyl) phosphate; 107-66-4
209.2 g/mol; logP 2.20



TBOEP
tris (2-butoxyethyl) phosphate; 78-51-3
398.5 g/mol; log K_{OW} 3.65[a]

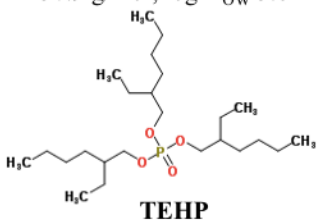


BBOEP
bis (2-butoxyethyl) phosphate;
297.3 g/mol; logP 2.22

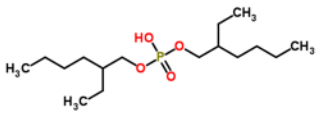


TDCIPP
tris (1,3-dichloro-2-propyl) phosphate; 13674-87-8
430.9 g/mol; log K_{OW} 3.8[a]

BDCIPP
bis (1,3-dichloropropyl) phosphate;
318.9 g/mol; logP 1.66



TEHP
tris (2-ethylhexyl) phosphate; 78-42-2
434.6 g/mol; log K_{OW} 9.49[a]



DEHP
di (2-ethylhexyl) phosphate;
322.4 g/mol; logP 6.09

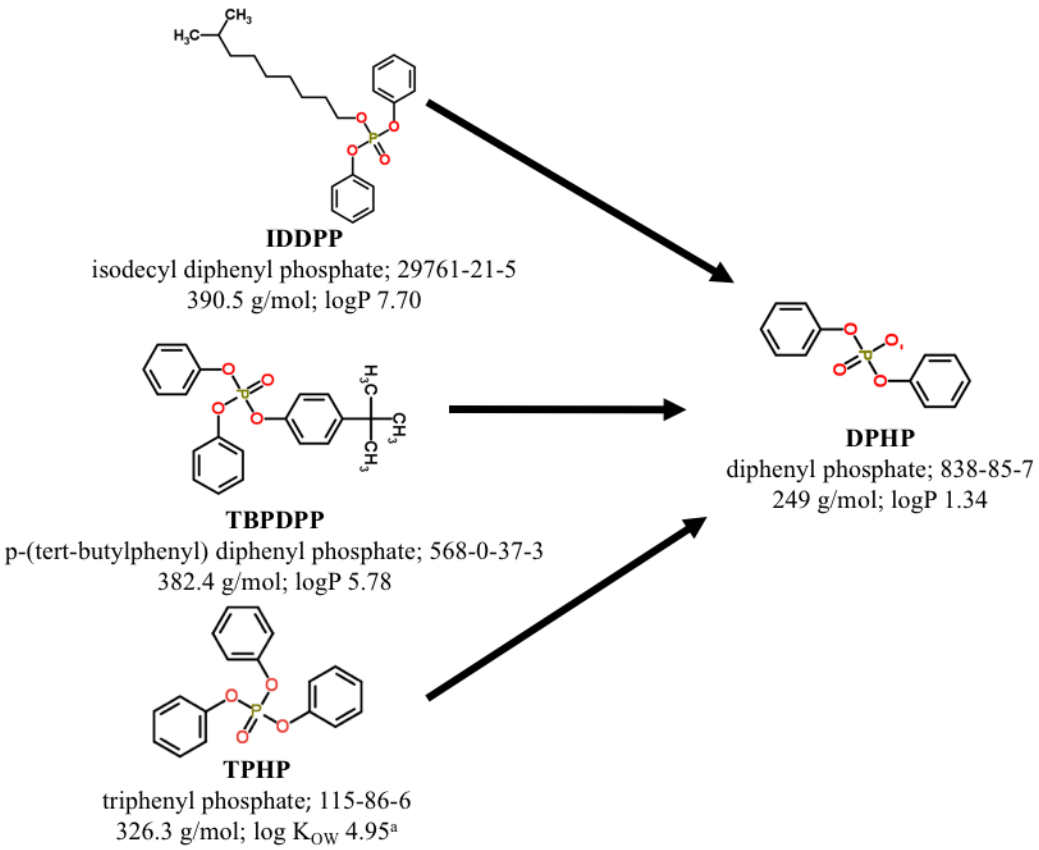


Figure 1-2: Organophosphate ester (OPE) ketcher chemical structures where the organophosphate (OP) triester is presented on the left and corresponding OP diester is on the right. Additional information includes: CAS identification numbers, molecular mass, along with either log K_{ow} from Wei et al., 2015 or predicted logP from Chemspider.

2 Chapter: Experimental Materials and Methods

2.1 Chemicals and reagents

The standards for the OP triesters and OP diesters under study were obtained as follows. All purchased standards, unless specified, had a chemical purity ranging between 94 and 99.8 %. All purchased standards were prepared to concentration in methanol. The OP triesters TDCIPP, TPHP, TNBP, TBOEP, TEP, TEHP and TTBPP (purity unknown) and the OP diesters DNBP, DPHP, and DEHP were purchased from Sigma-Aldrich (Oakville, ON, Canada). IDDPP (Santicizer-148 Tech Mix including TPHP) and TBPDPP (Tech Mix) were purchased from Chromatographic Specialties Inc. (AccuStandard; Brockville, ON, Canada). The specific purity of IDDPP and TBPDPP technical mixtures were not available from the suppliers at the time of the present study. T4IPPP (> 98 % purity) and T2IPPP (96 % purity) was purchased from Santa Cruz Biotechnology Inc. BDCIPP and BBOEP along with the mass-labeled internal standards d_{15}-TDCIPP, d_{15}-TPHP, d_{10}-BDCIPP, d_4-BBOEP, and d_{10}-DPHP were purchased from Dr. Belov at the Max Planck Institute for Biophysical Chemistry (Germany). d_{27}-TNBP and d_{15}-TEP were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Mass-labeled internal standards d_{33}-T4IPPP (> 97 % purity) and d_{21}-TCrP (> 99 % purity) were purchased from Caledon (Georgetown, ON, Canada). Wistar-Han rat liver microsomes (20 mg microsomal protein/mL), NADPH regenerating system solution A and NADPH regenerating system solution B were purchased from Corning (Corning, NY, USA). Potassium phosphate buffer, pH 7.4, was purchased from Biotage (Charlotte, NC, USA).

2.2 Sampling details and location

All polar bear and ringed seal sampling was conducted in co-operation with local indigenous subsistence hunters, within the yearly quota of 35 polar bears. Samples were acquired from Scoresby Sound/Ittoqqortoormiit, Eastern Greenland (70°-71°N, 20°-21°W). All fat/blubber samples that were collected were immediately and completely wrapped in aluminum foil and then put into a Whirlpak bags and immediately frozen. The aluminum foil was chemically rinsed with acetone and then hexanes and thus the aluminum foil surface was stripped of any possible OPE cross-contamination. Once at the National Wildlife Research Center (NWRC) in Ottawa, the bear and seal fat samples were sub-sampled and stored in glass jars that had been chemically rinsed and heated at 450 °C for 8 hours according to NWRC protocols.

Fat tissue samples were immediately frozen after harvesting from six polar bears in February – March 2011 and 2012. Five polar bears were subadults at 2-3 years old and one polar bear was an adult which altogether composed of three males and three females (**Table 2-1**). Blubber tissue were immediately frozen after harvesting from seven ringed seals in February-March 2012 where samples were comprised of two subadults and five adults altogether five males and two females (**Table 2-1**). Liver tissue samples for the *in vitro* metabolism studies were flash frozen in liquid nitrogen within 1 hour of harvesting and stored at -80 °C in aluminum foil.

Table 2-1: Liver and fat/blubber samples and sampling details for each polar bear (n=6) and ringed seal (n=7) individual collected from East Greenland.

Polar Bear (PB) ID	Age (Years)	Sex	Location	Date of Collection	Volume of microsome contribution to pool (mL)
PB 43101	5-6	M	South of Kap Tobin	2011-02-25	20.45
PB 43104	2-3	M	1 km south of Scoresby Sound	2011-03-02	9.74
PB 43105	2-3	F	South of Kap Tobin	2011-03-10	5.98
PB 43106	2-3	F	South of Kap Tobin	2011-03-10	1.91
PB 43107	2	F	South of Kap Tobin	2011-03-11	4.88
PB 43171	2	M	Kap Toblin	2012-03-10	10.54
Ringed Seal (RS) ID					
RS 43183	adult	M	Scoresby Sound	2012-02-26	3.86
RS 43185	adult	F	Scoresby Sound	2012-02-27	4.5
RS 43187	adult	M	Scoresby Sound	2012-02-27	2.47
RS 43188	subadult	M	Scoresby Sound	2012-02-27	2.9
RS 43189	adult	M	Scoresby Sound	2012-02-29	6.98
RS 43190	subadult	M	Scoresby Sound	2012-02-29	3.89
RS 43192	adult	F	Scoresby Sound	2012-03-01	6.04

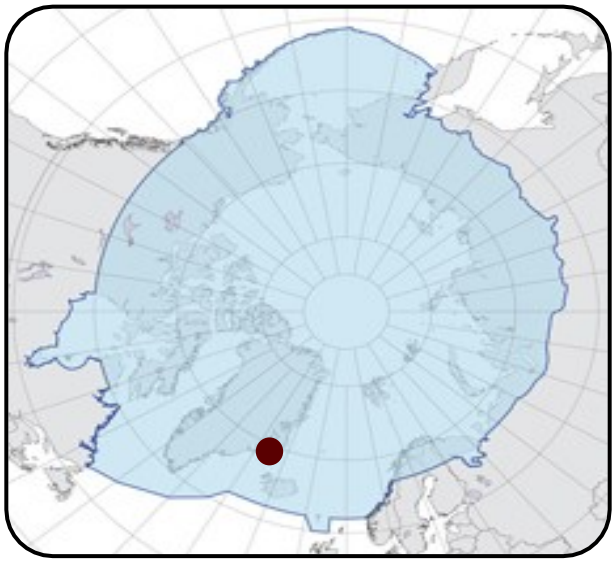


Figure 2-1: Sampling location in Scoresby Sound/Ittoqqortoormiit, Eastern Greenland (70°-71°N, 20°-21°) indicated by dark circle (Letcher et al., 2017a).

2.3 Liver microsome preparation and pooling

Polar bear and ringed seal liver tissue was processed into microsomal suspensions following procedures published previously (Greaves et al., 2016b; **Figure 2-2**). Briefly, liver tissue was thawed on ice, and the microsomes were prepared for each individual by homogenization and centrifugation using previously cooled microcentrifuge tubes and a Beckman TLA 100.4 rotor. First, approximately 500 mg of hepatic tissue was washed with phosphate buffer solution (0.1 M, pH 7) and diced using buffer-cleaned scissors. The tissue was homogenized with 800 µL of cold phosphate buffer using an IKA Ultra Turrax homogenizer (Wilmington, NC, USA). The tissue was transferred and centrifuged using a Beckman-Coulter Optima TLX Ultracentrifuge (Mississauga, ON, Canada) set at 17,000 RPM (15 670 X *g*) at 4 °C for 15 minutes. The supernatant was re-centrifuged at 56,000 RPM (170 043 *g*) at 4 °C for 60 minutes. The supernatant was stored as the S9 fraction, and the pellet was gently resuspended with 125 µL of buffer as the microsomal fraction. These components were snap-frozen in liquid nitrogen and stored in cryovials. Microsomes were combined into species-specific pools (polar bear: 53.50 mL and ringed seal: 30.65 mL) so that there would be sufficient volumes of microsomal suspension to accomplish the project objectives.

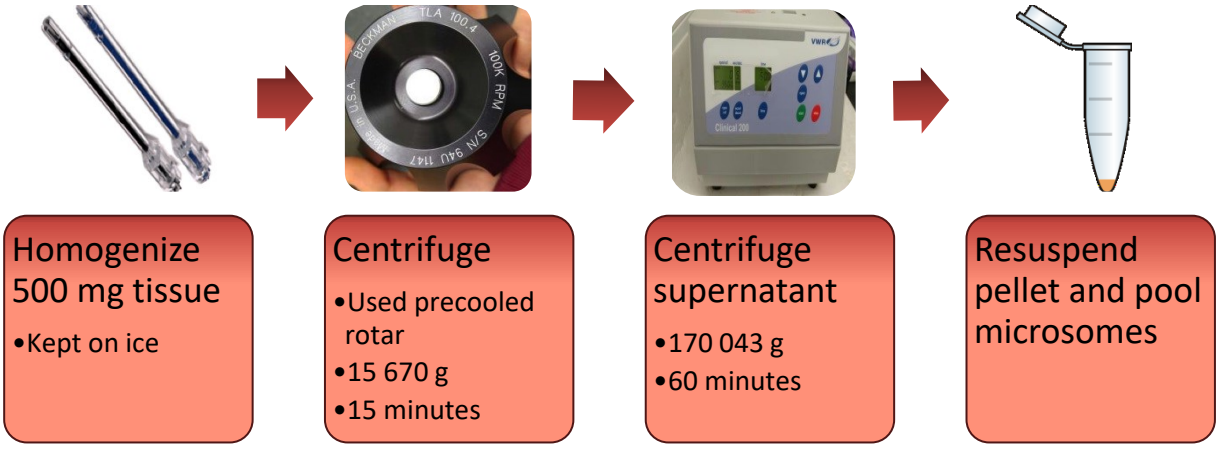


Figure 2-2: Summary of microsomal preparation with critical details described. Notably, this method requires samples to be kept on ice.

2.4 Quantification of microsomal protein content and enzyme activity

Total protein content and CYP1A-associated enzyme activity in each microsomal pool were determined using the ethoxyresorufin-O-deethylase (EROD) assay following the methods in Kennedy and Jones (1994). The EROD assay provides a measurement of the general enzyme activity and thus the enzymatic viability and relative activity level of the microsomes. EROD activity, which catalyzes the reaction of 7-ethoxyresorufin to resorufin in the presence of NADPH, was quantified against a concentration-dependent standard curve for resorufin. A 48-well plate was prepared with sodium phosphate buffer (0.05 M, pH 8.0) and 7-ethoxyresorufin (1.02 µM measured using a UV 6300PC Double Beam spectrophotometer). The plate containing blanks, standard curve solutions, and microsomal solutions was incubated at 37 °C for 5 minutes. NADPH regenerating system (composed of NADPH A and NADPH B) was added to start the reaction and incubation continued for two minutes. The reaction was terminated by adding 100 µL of 2.16 mM

fluorescamine in acetonitrile. Fluorescence was measured using a Beckman DTX880 multimode detector, multiwell microplate reader. Excitation and emission filters were used to measure resorufin (530 nm and 590 nm) and proteins (400 nm and 460 nm) respectively. Then, measurements were compared to a resorufin standard curve which was highly linear ($r^2 > 0.99$). Total protein content was quantified against a concentration-dependent and 5-point standard curve based on bovine serum albumin (BSA) with $r^2 > 0.99$. Once the total protein concentration had been determined, the microsomes were diluted to 20 mg protein/mL, and were frozen at −80 °C until further use.

2.5 *In vitro* metabolism assay for OP triester depletion and OP diester formation

The *in vitro* metabolism biotransformation assay for OP triesters was conducted according to previously published methods for rat and herring gull liver microsomes (Chu et al., 2011; Chu and Letcher, 2015; Greaves et al., 2016b). Briefly, liver microsomes and NADPH regenerating solutions were thawed slowly on ice and the dosing OP triester solution (see **Figure 2-3** and **Figure 2-4** for individual OPEs) was used at room temperature. The biotransformation solution contained potassium phosphate buffer (0.5 M, pH 7.4), NADPH regenerating solution (ratio of solutions 5A:1B), and OP triester dosing solution (final concentration 2 μM). This 2 μM dose was chosen following Greaves et al. (2016b) which determined zero-order enzyme kinetics for OPEs that 2 μM was $\gg 2 \times K_M$. This meant that there was adequate OPE to saturate enzymes such that there was no substrate concentration dependency. An assay solution containing one OP triester solution was incubated for 2 minutes at 37 °C with the shaker set to move horizontally 2.4 cm for 176 times per minute (80 RPM) prior to adding 50 μL of

microsomes (containing 1 mg of protein). The reaction was vortexed well and 100 µL aliquots were taken at designated time intervals (0, 1, 2, 5, 10, 40, 70 and 100 minutes). These aliquots were placed in a methanol quenching solution containing internal standard mixture (25 ppb). Solutions were capped to prevent evaporation. The solution was then microcentrifugated using an Eppendorf Mini Spin Plus with a F-45-12-11 rotor in prewashed tubes at 10,000 RPM (6 708 *g*) for 5 minutes. The solutions were then kept in UPLC-ready vials for analysis at 4 °C.

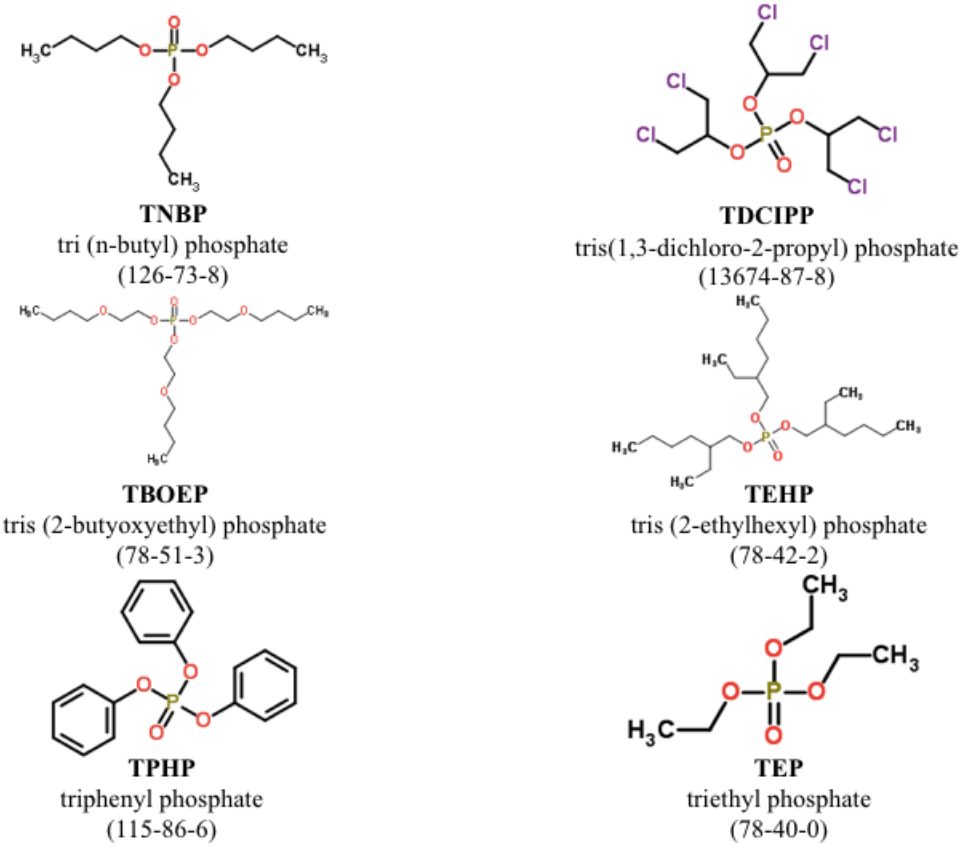


Figure 2-3: Ketcher chemical structures of the organophosphate (OP) triesters and OP diesters under study in Chapter 3 and including their full chemical names, abbreviations, and CAS numbers. Hydrogen atoms are omitted for clarity.

Prior to UPLC-vial preparation, a minor modification to the approach was made for Chapter 4 analysis, where the solutions were diluted in order to remain within the linear

analytical response range. Specifically, there was an additional 1:1 ratio dilution for TTBPP (i.e. 100 µL sample and 100 µL of MeOH) and there was a 1:6 ratio dilution for both T4IPPP and T2IPPP (i.e. 100 µL of sample and 500 µL of MeOH).

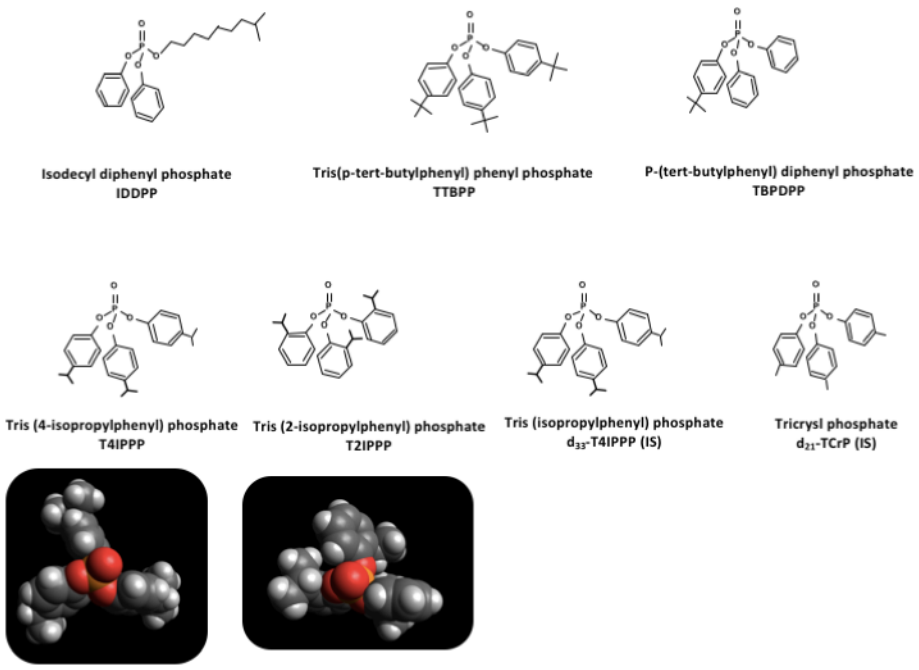


Figure 2-4: Ketcher chemical structures of the organophosphate (OP) triesters under study in Chapter 4 – including their full chemical names and abbreviations. Hydrogen atoms are omitted for clarity. Additionally, tris (isopropylphenyl) phosphate isomers (T4IPPP and T2IPPP) are shown as space-filled diagrams developed in Avogadro to aid in visualizing molecular structure (Hanwell et al., 2012).

2.6 Quality control / quality assurance for the *in vitro* assay

For every batch of two triplicates, a methanol blank and a negative control using deactivated Wistar-Han liver microsome was included as a negative control. This assay was spiked with 3 µL of the same 2 µM dose of the same OPE triester studied but the rat

microsomes were denatured at 100 °C for 5 min. Thus, this control was the same as a polar bear or ringed seal technical triplicate with the exception that the null hypothesis was that no OPE triester would be degraded. This showed that the OP triesters were not degraded in the absence of enzymatically viable microsomes. Likewise, a positive control (n=3) was conducted which used Wistar-Han rat liver microsomes dosed with TDCIPP. This demonstrated adequate replicability of TDCIPP depletion and BDCIPP formation compared with Chu et al., 2011. Additionally, a five to seven-point linear calibration curve was run with each batch (r^2 was consistently well above 0.90). Known quantities of internal standards were used to accurately compare unknown OP triester and diester concentrations.

2.7 OP triester and diester determination from the *in vitro* biotransformation assay for Chapter 3 OPEs

Quantification of OP triesters and their diester metabolites was conducted utilizing a Waters Acquity ultra-high pressure liquid chromatography (UPLC) coupled to a Waters Xevo TQ-MS operated in the electron spray ionization (ESI)+ mode (Chu et al., 2011; Greaves and Letcher, 2014). ESI+ mode conditions were as follows: capillary voltage 0.5 kV; desolvation temperature at 600 °C; desolvation gas flow rate of 800 L/hr; and cone gas flow rate at 150 L/hr. Analytes were separated using Waters Aquity UPLC BEH C_{18} column (50 mm L x 2.1 mm i.d., 1.7 μm particle size). The column temperature was set to 40 °C and the samples were maintained at 20 °C. Each measurement utilized 5 μL of injected sample. Mobile phases included Milli Q water with ammonium acetate (2 mM) and methanol with ammonium acetate (2 mM). The processing for each sample ran for 10

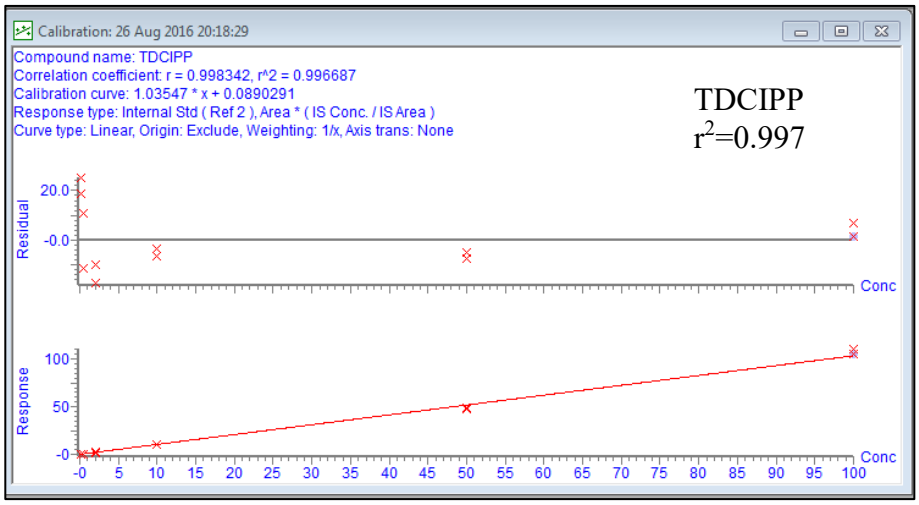
minutes following a gradient where the mobile phases started with a flow rate of 0.5 mL/min. Then, the sample was treated with a series of 5 % methanol with ammonium acetate (2 mM), where the methanol solution was increased over five minutes to 95 % where it was held for one minute, next it was dropped to 5 % and held for 4 minutes. A dicationic reagent was mixed with the aqueous mobile phase at a constant flow of 10 μL/min (decamethonium hydroxide, 0.1 mM).

The high sensitivity of TNBP standards resulted in responses above the upper detection limit; thus, the gain was set at 0.3 for all TNBP data collected. BDCIPP sensitivity may also have been extensive given the 2.0 μM triester dose and complete percent conversion (**Figure 3-2**; **Table 3-3**).

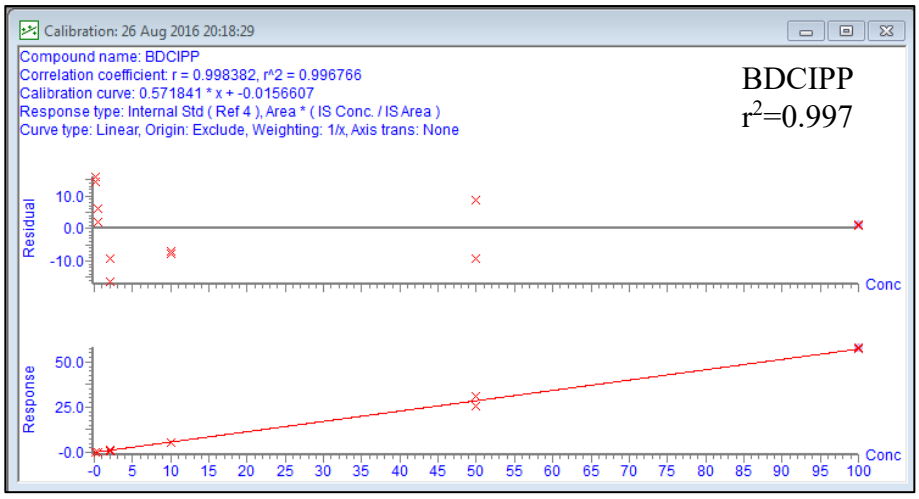
All compounds were identified by UPLC-ESI(+)-MS/MS based on their MRM ion channels (**Table 2-2**) and their UPLC retention times compared to authentic standards, and their characteristic mass transitions observed by multiple reaction monitoring. A seven-point calibration curve was performed daily for each OP triester under study to ensure ESI(+)-MS/MS linearity of response and to allow for quantification. Quantification was accomplished using MassLynx 4.1 software (**Figure 2-5**).

Table 2-2: Operating parameters of MS/MS for *in vitro* assay analysis including the multiple reaction monitoring (MRM) transitions. OPE triesters studied include triethyl phosphate (TEP), tris (1,3-dichloro-2-propyl) phosphate (TDCIPP), tris (2-ethylhexyl) phosphate (TEHP), triphenyl phosphate (TPHP), tri (n-butyl) phosphate (TNBP), and tris (2-butoxyethyl) phosphate (TBOEP) and their corresponding OP diesters: bis(1,3-dichloropropyl) phosphate (BDCIPP), di (2-ethylhexyl) phosphate (DEHP), diphenyl phosphate (DPHP), di (n-butyl) phosphate (DNBP), and bis (2-butoxyethyl) phosphate (BBOEP). Mass-labeled internal standards are also identified.

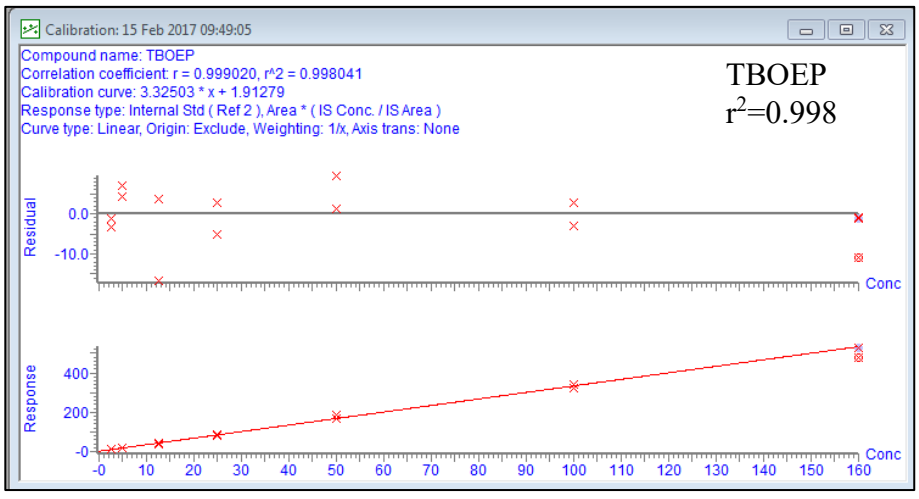
Compound	MRM Transition	Dwell (s)	Cone (V)	Collision (eV)	Internal standard
TEP	183.00>98.70	0.009	35	17	d_{15}-TEP
d_{15}-TEP	198.20>101.70	0.009	35	17	N/A
DNBP	467.40>243.30	0.009	0	26	d_{10}-BDCIPP
DPHP	507.30>243.30	0.009	42	28	d_{10}-DPHP
d_{10}-DPHP	517.40>243.30	0.009	42	28	N/A
BDCIPP	577.20>243.30	0.009	53	30	d_{10}-BDCIPP
d_{10}-BDCIPP	587.30>243.30	0.009	53	30	N/A
TNBP	267.10>99.00	0.009	35	20	d_{27}-TNBP
d_{27}-TNBP	294.30>102.00	0.009	35	20	N/A
TPHP	327.10>77.10	0.009	100	40	d_{15}-TPHP
d_{15}-TPHP	342.20>82.00	0.009	100	40	N/A
TBOEP	399.00>199.00	0.009	35	15	d_{15}-TDCIPP
TDCIPP	430.90>99.00	0.009	42	26	d_{15}-TDCIPP
d_{15}-TDCIPP	446.00>102.00	0.009	42	26	N/A
BBOEP	555.50>243.30	0.009	43	25	d_4-BBOEP
d_4-BBOEP	559.50>243.30	0.009	43	25	N/A
TEHP	435.40>99.30	0.164	24	12	d_{15}-TDCIPP
DEHP	579.50>243.30	0.053	40	28	d_4-BBOEP



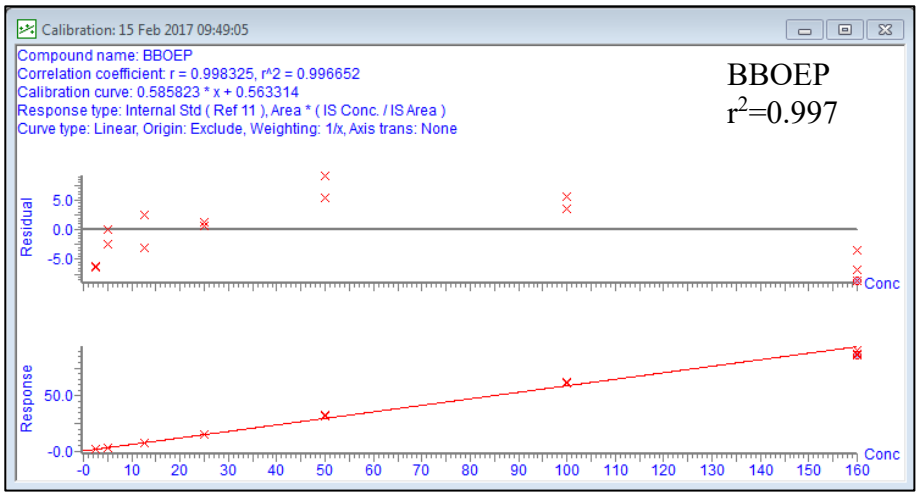
TDCIPP
r^2=0.997



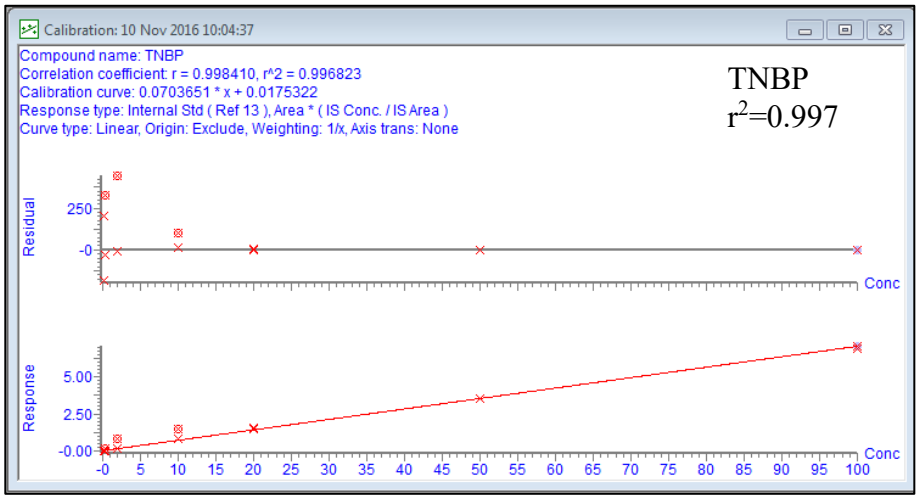
BDCIPP
r^2=0.997



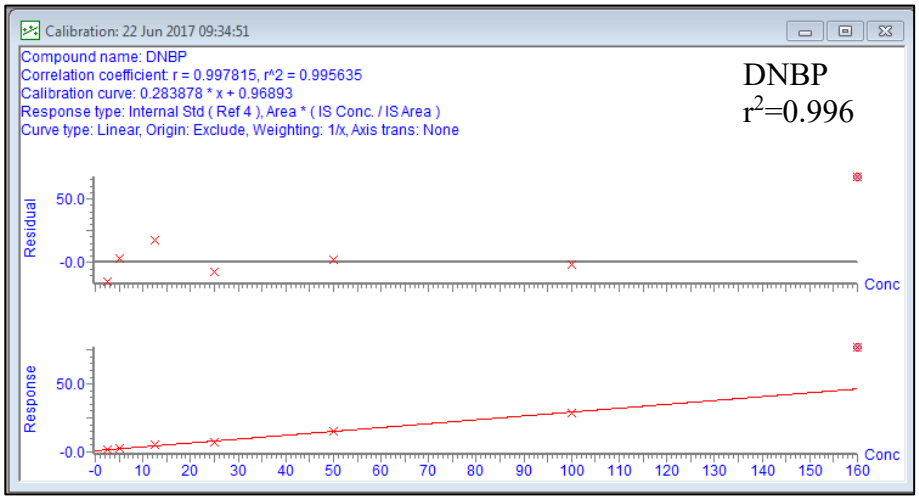
TBOEP
r^2=0.998



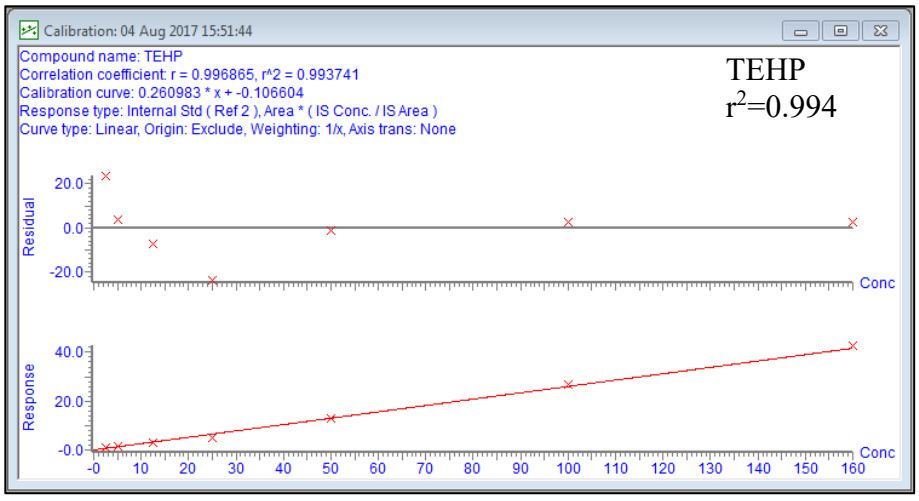
BBOEP
r^2=0.997



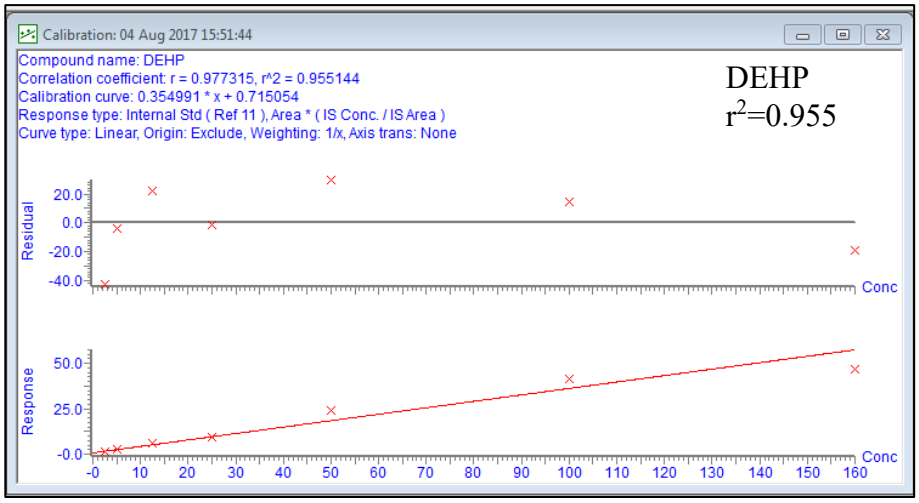
TNBP
r^2=0.997



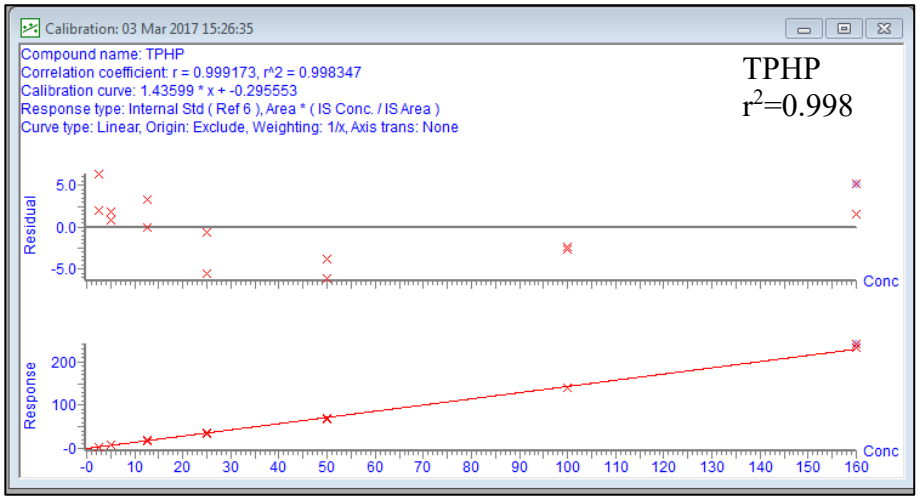
DNBP
r^2=0.996



TEHP
r^2=0.994



DEHP
r^2=0.955



TPHP
r^2=0.998

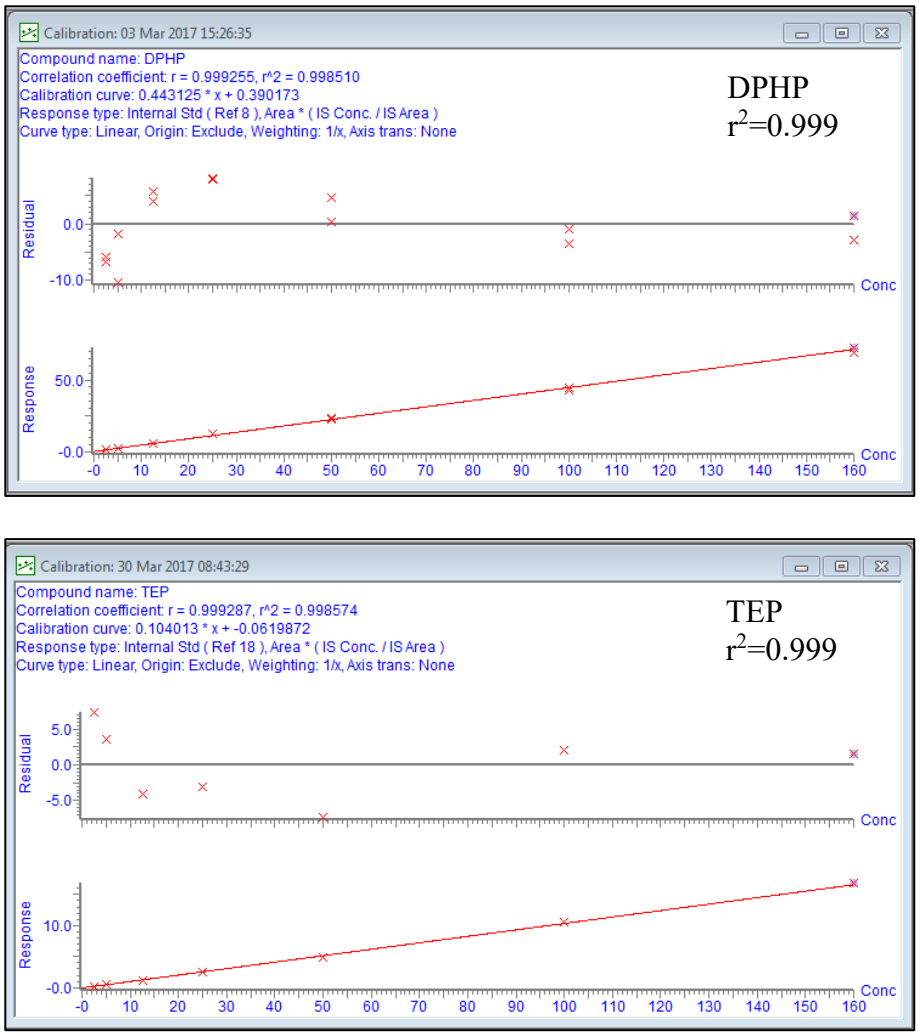


Figure 2-5: Representative linear regressions for OPE triester and corresponding diester calibration curves by UPLC-ESI(+)-MS/MS analysis.

2.8 OP triester and diester determination from the *in vitro* biotransformation assay for Chapter 4 OPEs

Quantification of OP triesters and their diester metabolites was conducted by utilizing a Waters Acquity ultra-high pressure liquid chromatography (UPLC) coupled to a Waters Xevo TQ-MS system operated in the electron spray ionization (ESI)+ mode. The approach was based on OPE analysis conducted previously (Chu et al., 2011; Greaves and Letcher, 2014; Strobel et al., 2018). Briefly, the injection volume was 10 µL. The

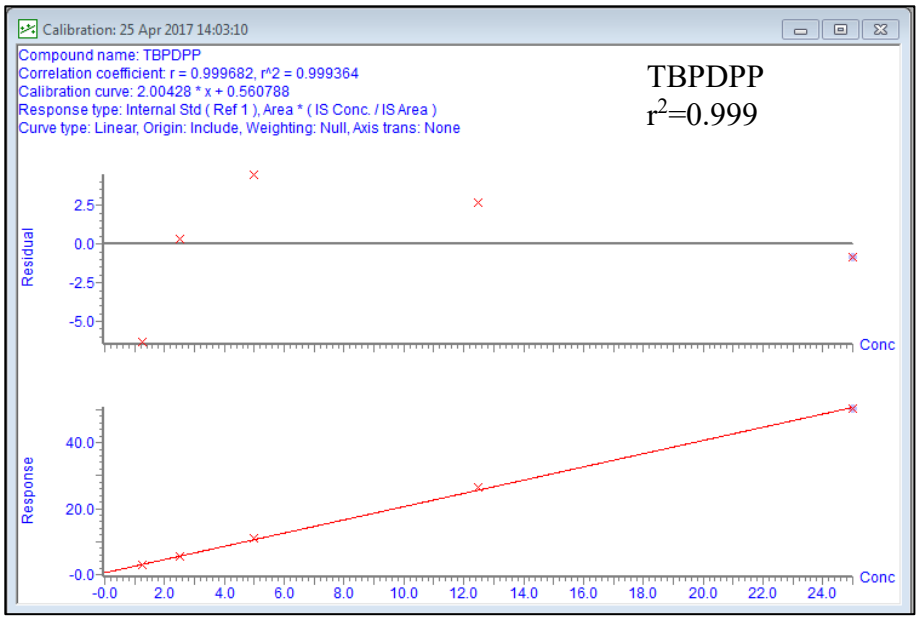
ESI(+) mode conditions were as follows: 1.0 kV capillary voltage, 150 °C and 600 °C were source and desolvation temperature, respectively; 800 L/h and 150 L/h were the desolvation gas and cone gas flow rates respectively. Analytes were separated using Waters Aquity UPLC BEH C_{18} column (50 mm L x 2.1 mm i.d., 1.7 μm particle size). The column temperature was set to 40 °C and the samples were maintained at 20 °C. The two mobile phases used were methanol and water, each with 2 mM ammonium acetate. The process for each sample with a total elution time of 12 minutes was following a gradient where the mobile phases started with a flow rate of 0.5 mL/min. The following procedure was set for the gradient: initially 5 % methanol with 2 mM ammonium acetate increasing to 95 % within 5 minutes where it was held for 2 minutes. Initial conditions of the 5 % methanol with 2 μM ammonium acetate were then maintained for an additional 5 minutes.

All compounds were identified by UPLC-ESI(+)-MS/MS based on their multiple reaction monitoring (MRM) ion channels (**Table 2-3**) and their UPLC retention times compared to authentic standards or in technical mixtures, and their characteristic mass transitions observed by MRM. A five-point calibration curve was performed daily for each OP triester under study to ensure ESI(+)-MS/MS linearity of response and to allow for quantification. Quantification was done using MassLynx 4.1 software (**Figure 2-6**).

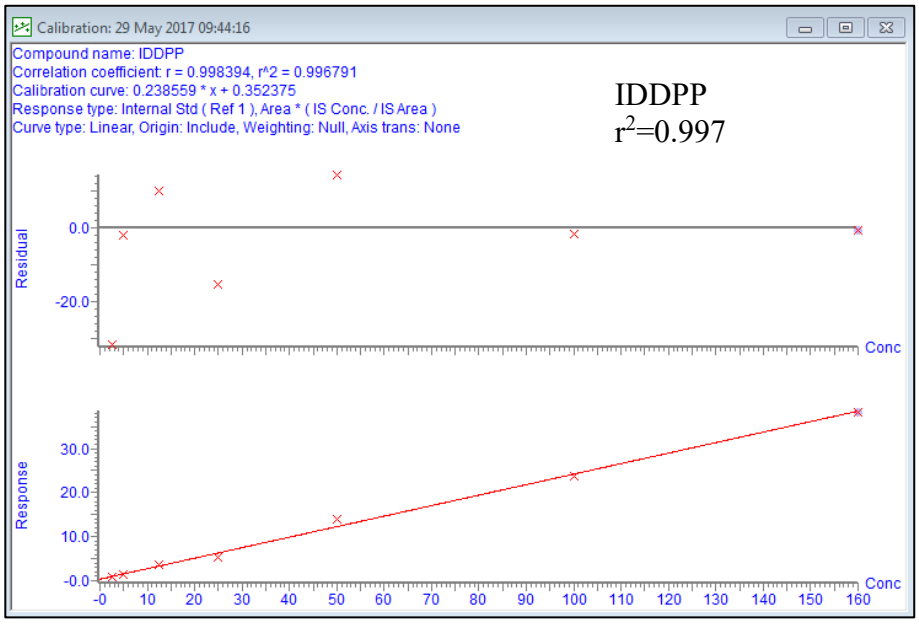
The metabolite DPHP was analyzed by UPLC-MS/MS in the ESI(-) mode for samples dosed with IDDPP and compared to the results from the ESI(+) mode. There was little difference in results and thus the ESI(-) based quantification results were used in the present study. However, TBPDPP dosed samples were solely measured using ESI(+) as the additional analysis under ESI(-) mode was deemed unnecessary.

Table 2-3: Operating parameters of the tandem quadrupole MS/MS for the organophosphate ester (OPE) analysis of the fractions from the *in vitro* assays, which includes the multiple reaction monitoring (MRM) transitions. The OP triesters studied were isodecyl diphenyl phosphate (IDDPP), p-(tert-butylphenyl) diphenyl phosphate (TBPDPP), tris(p-tert-butylphenyl) phenyl phosphate (TTBPP), and two tris (isopropylphenyl) phosphate isomers (TIPPPs) and where possible, the corresponding OP diester, diphenyl phosphate (DPHP). Mass-labeled internal standard parameters are also listed.

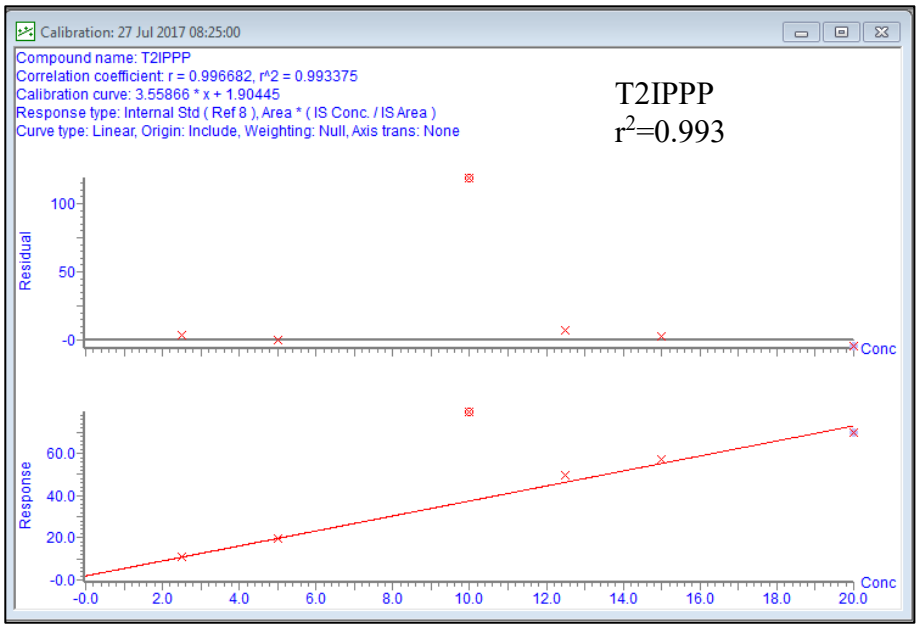
Compound	**MRM Transition**	**Dwell (s)**	**Cone (V)**	**Collision (eV)**	**Internal standard**
DPHP	507.3>243.3	0.009	42	28	d_{10}-DPHP
TBPDPP	383.1>327.3	0.029	10	18	d_{21}-TCrP
d_{21}-TCrP	390.3>98.3	0.036	30	37	N/A
IDDPP	391.2>251.3	0.029	16	12	d_{21}-TCrP
TIPPP	453.2>327.3	0.029	8	30	d_{33}-T4IPPP
d_{33}-T4IPPP	486.4>342.2	0.029	8	30	N/A
TTBPP	395.3>439.4	0.029	19	22	d_{33}-T4IPPP
DPHP in ESI(+)	507.3>243.3	0.197	42	28	d_{10}-DPHP
d_{10}-DPHP in ESI(+)	517.4>243.3	0.197	42	28	N/A
DPHP in ESI(-)	248.90>92.80	0.197	76	23	d_{10}-DPHP
d_{10}-DPHP in ESI(-)	259.00>97.80	0.197	76	23	N/A



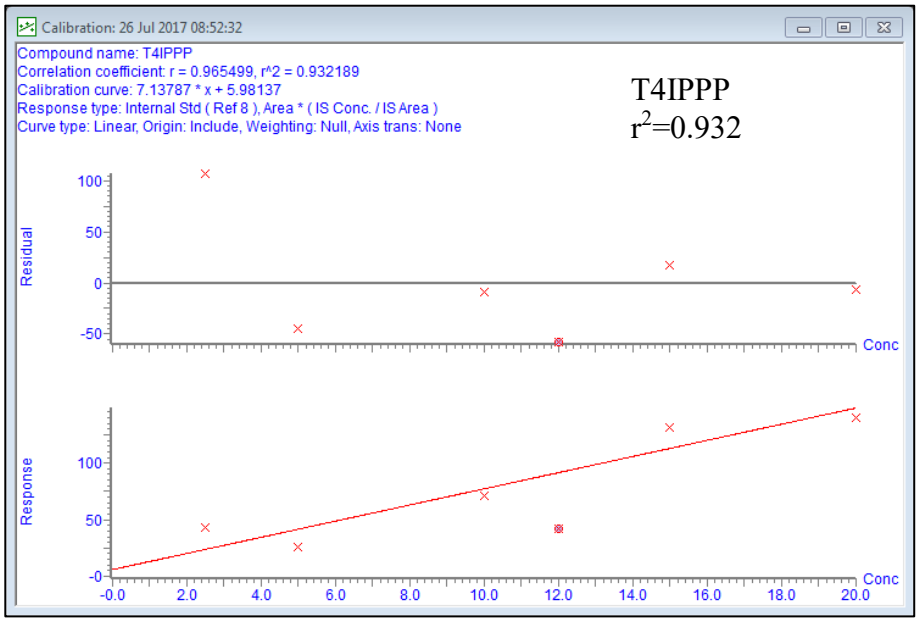
TBPDPP
r^2=0.999



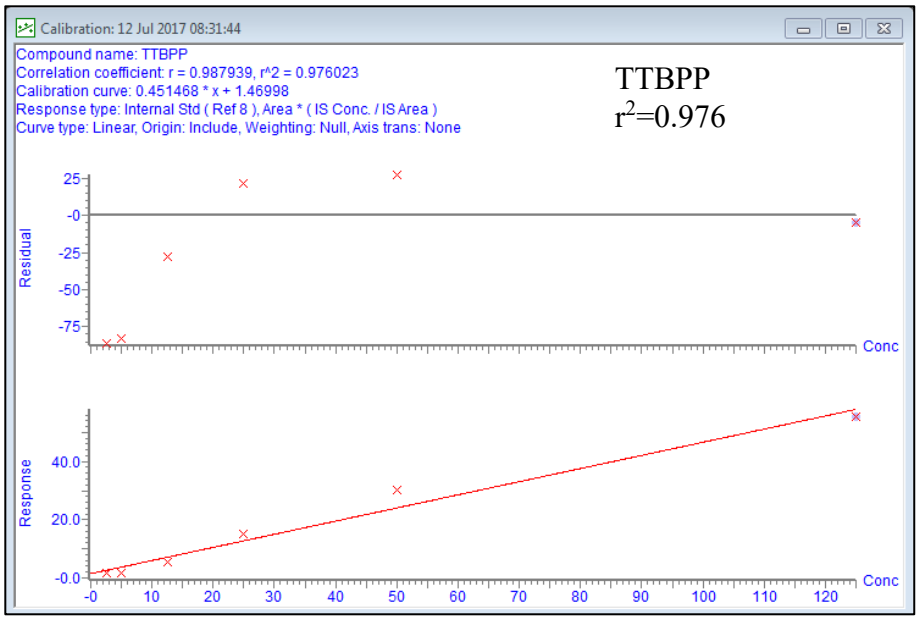
IDDPP
r^2=0.997



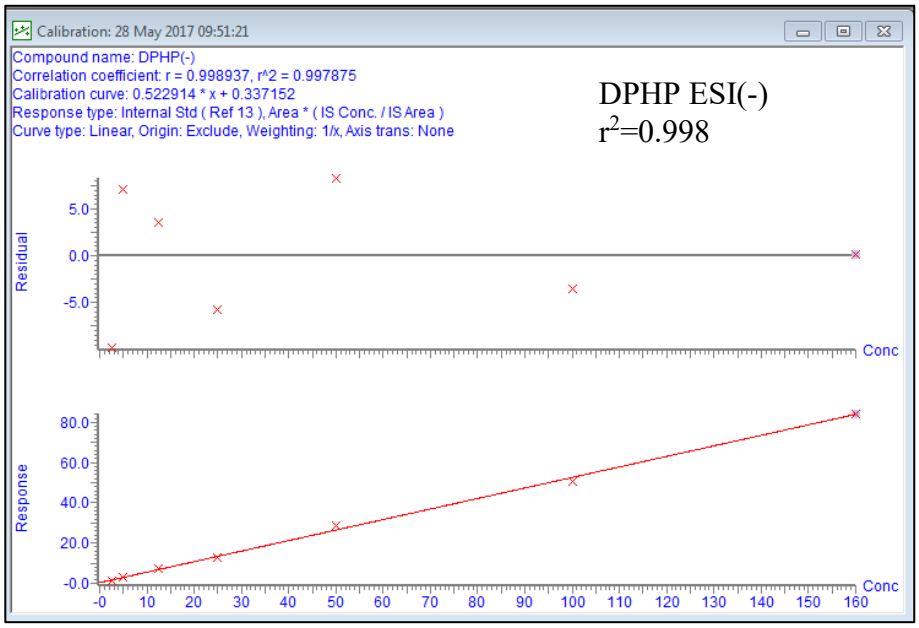
T2IPPP
r^2=0.993



T4IPPP
r^2=0.932



TTBPP
r^2=0.976



DPHP ESI(-)
r^2=0.998

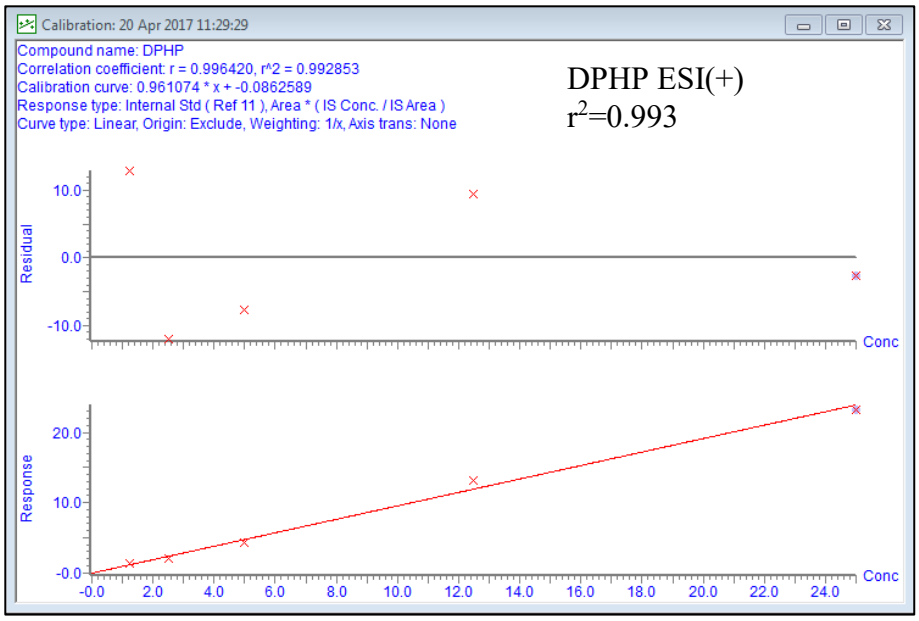


Figure 2-6: Representative linear regressions for OPE triester and corresponding diester calibration curves by UPLC-ESI(+)-MS/MS analysis.

2.9 OPE extraction and clean-up of field fat/blubber tissue samples

Methods for the determination of OP triesters in wildlife tissues, including adipose tissue, have been developed and are described in detail elsewhere (Chu and Letcher, 2015; Letcher et al., 2018; Su et al., 2017). Briefly, approximately 1 g of fat or blubber sample was extracted using 1:1 dichloromethane (DCM): hexane. Internal standard mixture (final concentration 1 ppb) was added to sample, blank, and positive controls. Samples were capped, vortexed, and left for approximately 45 minutes. After 0.2 g of sodium chloride and 1.2 g magnesium sulphate were added, the samples were vortexed, sonicated at room temperature and centrifuged for 5 min. at 4,000 RPM (3,309 *g*). This extraction process was repeated two additional times and the three extracts were combined. After taking the solution to dryness with a gentle stream of nitrogen gas, 1 mL of methanol was added, then vortexed for 1 minute, sonicated for 10 minutes, and centrifuged for 5 minutes at 3,500 RPM (2,534 *g*). This was followed by the addition of 0.3 g PSA bonded silica with vortexing and centrifugation at 3,500 RPM (2,534 *g*). The final supernatant was transferred to glass vials with extreme caution to remove the polar fraction without disrupting the lipid phase. Vials were capped using cleaned aluminium foil. Samples were maintained at -20 °C and brought to room temperature prior to chemical analysis.

2.10 OP triester determination in adipose fractions by UPLC-MS/MS

Quantification of OP triesters in the final fractions from section 2.9 was conducted using a Waters Acquity UPLC coupled to a Waters Xevo triple quadrupole mass spectrometer (TQ-S) operated in the atmospheric pressure chemical ionization (APCI (+))

mode (Chu and Letcher, 2015). The APCI(+) mode conditions were as follows: corona voltage 3.0 kV; APCI(+) probe temperature 400 °C; desolvation gas flow of 100 L/hr; and cone gas flow rate at 150 L/h. Analytes were separated using Waters Aquity UPLC BEH C_{18} column (50 mm L x 2.1 mm i.d., 1.7 μm particle size). The column temperature was set to 60 °C and the samples were maintained in 25 °C. Each measurement used 10 μL of injected sample. The two mobile phases used were Milli Q water and methanol. The processing for each sample ran for 12 minutes following a gradient where the mobile phases started with a flow rate of 0.5 mL/min. The following procedure was set for the gradient: initially 5 % methanol increasing to 100 % within 3 minutes where it was held for 8 minutes. Initial conditions of 5 % methanol were then maintained for 4 minutes.

All compounds were identified by UPLC-APCI(+)-MS/MS based on their MRM ion channels (**Table 2-4**). Other MS/MS parameters was the same as described in Section 2.7.

Table 2-4: Operating parameters of MS/MS for adipose analysis including the multiple reaction monitoring (MRM) transitions. OPE triesters studied include triethyl phosphate (TEP), tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), tris (2-ethylhexyl) phosphate (TEHP), triphenyl phosphate (TPHP), tri (n-butyl) phosphate (TNBP), and tris (2-butoxyethyl) phosphate (TBOEP). Mass-labeled internal standards are also identified.

Compound	MRM Transition	Dwell (s)	Cone (V)	Collision (eV)	Internal standard	% IS[a] Recovery PB | RS
TEP	183.00>98.98	0.024	14	16	d_{15}-TEP	74.6 | 77.2
d_{15}-TEP	198.17>102.00	0.024	14	16	N/A	
TNBP	267.17>98.98	0.010	32	18	d_{27}-TNBP	137 | 75.1
d_{27}-TNBP	294.34>102.00	0.010	32	18	N/A	
TPHP	327.08>152.06	0.010	48	32	d_{15}-TPHP	70.9 | 75.1
d_{15}-TPHP	342.17>160.11	0.010	48	36	N/A	
TBOEP	399.25>299.16	0.017	26	12	d_{15}-TDCIPP	137 | 166
TDCIPP	430.89>98.98	0.010	34	26	d_{15}-TDCIPP	59.5 | 59.6
d_{15}-TDCIPP	445.98>102.00	0.010	34	26	N/A	
TEHP	435.36>98.98	0.017	16	12	d_{15}-TDCIPP	59.5 | 59.6

[a] % IS Recovery – average percent internal standard recovery for PB fat and RS blubber OPE extraction analysis

2.11 Quality control / quality assurance for OPE determination in fat samples

For each batch of eight polar bear or eight ringed seal samples, a blank and an OP triester-spiked pork liver sample was included to assess replicate analysis precision and accuracy. To the pork liver samples was added a standard mixture of OP triesters including TEP, TEHP, TNBP, TPHP, TBOEP, and TDCIPP (final concentration 1 ppb each), as well as a standard mixture of the corresponding mass-labeled OP triesters (final

concentration 1 ppb each). Additionally, one ringed seal or polar bear sample per batch of eight samples was analyzed in duplicate.

The OP triester concentrations in all fat and blubber samples were determined on a wet weight basis. To determine the method limit of quantification (MLOQ) and detection (MLOD), eight replicate pork liver homogenate samples were spiked with standards, extracted, and analyzed as one single batch. The MLODs of the OP triesters were calculated as three times the average signal-to-noise ratio of the MRM responses. The MLOQs were determined by using Student t-test, where a 99% confidence interval and 7 degrees of freedom were used for the t statistic. The Student t-test compared the variance in standard deviation of the n = 8 spiked pork samples. MLOD range was found to be 0.07 – 0.33 ng/g ww and MLOQ to be 0.14 – 2.2 ng/g ww for the current OPEs under study (**Table 2-5**).

Table 2-5: Method limit of quantification (MLOQ) and method limit of detection (MLOD) for tissue residue measurements (**Table 3-4**) based on n = 8 pork liver samples.

	TEP	TDCIPP	TEHP	TPHP	TNBP	TBOEP
MLOQ(ng/g)	0.14	0.22	0.32	0.40	0.78	2.2
MLOD (ng/g)	0.25	0.17	0.07	0.14	0.33	0.27

2.12 Data analysis and statistics

Data analysis was determined from standard curves run for each assay or tissue residue measurement. Integration of peaks detected on the UPLC was automatic and adjusted based on the internal standard concentration. Analyte concentrations are presented in ppb and converted to nmol/L while accounting for prior dilutions.

For metabolic *in vitro* assay data, time-course nonlinear regressions were plotted using Prism Version 7.0c GraphPad (GraphPad Software, San Diego, CA, USA; http://www.graphpad.com). Regressions for Chapter 3 OPEs used the one phase decay or one phase association and least squares fit functions. R Studio Version 1.0.143 was used to determine the normality of the data (RStudio Team, 2015). Shapiro Wilk's and Levene's tests were used to determine whether parameteric (ANOVA) or non-parameteric tests (Friedman) would be utilized to determine significance of OP triester depletion. The appropriate test in GraphPad was conducted to determine the level of significance at each time point compared to the initial concentration measured at 1 minute. ANOVA was paired with Dunnett's multiple comparisons test and Friedman non-parametric test was paired with Dunn's multiple comparisons test. The level of significance that was acceptable was $p < 0.015$.

For Chapter 4 OPEs, regressions used the one phase decay (polar bear degradation of IDDPP), one phase association (DPHP) or linear regressions (all other OP triesters presented) and least squares fit functions. The non-parametric Friedman test paired with Dunn's multiple comparisons test were conducted to determine the level of significance ($p < 0.01$) at each time point compared to the initial concentration measured at 1 minute.

3 Chapter: Organophosphate Esters in East Greenland Polar Bears and Ringed Seals: Adipose Tissue Concentrations and *In Vitro* Depletion and Metabolite Formation[1]

Abstract

East Greenland is a contamination "hot spot" for long-range transported anthropogenic chemicals, including organophosphate esters (OPEs). High concentrations of OPEs have been reported in Arctic air while very little is known for wildlife where OPE tissue residues levels appear to be strongly influenced by biotransformation. In the present study, the hepatic *in vitro* metabolism of six environmentally relevant organophosphate (OP) triesters and corresponding OP diester formation were investigated in East Greenland polar bears and ringed seals. The *in vitro* metabolism assay results were compared to adipose levels in field samples from the same individuals. OP triester metabolism was generally rapid and structure-dependent, where polar bears metabolized OPEs more rapidly than ringed seals. Exceptions were the lack of triethyl phosphate metabolism and slow metabolism of tris (2-ethylhexyl) phosphate in both species. OP diester metabolites were also formed with the exception of diethyl phosphate due to lack of standard available. Tris(1,3-dichloro-2-propyl) phosphate was completely converted to its corresponding diester. However, the mass balances showed that OP diester formation corresponding to tris (2-ethylhexyl) phosphate, tri (n-butyl) phosphate, and tris (2-

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[1]**Adapted from** Strobel, A., Willmore, W.G., Sonnne, C., Dietz, R., Letcher, R.J., 2017. Organophosphate esters in East Greenland polar bears and ringed seals: adipose tissue concentrations and *in vitro* depletion and metabolite formation. Chemosphere submitted.

butoxyethyl) phosphate did not account for 100 % of the OP triester depletion, which indicated alternate pathways of OP triester metabolism. Triphenyl phosphate was completely converted to its OP diester metabolite in polar bears but not in ringed seals suggesting species-specific differences. The results demonstrated that OP triester bioaccumulation and fate in polar bears versus their ringed seal prey is substantially influenced by biotransformation.

Keywords**:** Organophosphate esters (OPEs); *In vitro* metabolism; Liver and Adipose; Polar bear; Ringed seal; East Greenland

3.1 Introduction

The Arctic includes regional anthropogenic chemical contaminant "hot spots", such as Eastern Greenland (Scoresby Sound) and Hudson Bay, which are sinks for persistent organic pollutants (POPs) (Dietz et al. 2016; Letcher et al. 2010). POPs enter the Arctic ecosystem via long-range oceanic and/or atmospheric transport and can bioaccumulate in biota and especially via the marine food web. There are a growing number of POPs of high relevance to the Arctic, listed in Annex A of the Stockholm Convention on POPs (United Nations Environment Programme (UNEP)). Additions to Annex A include the flame retardants (FRs) penta- and octa-brominated diphenyl ethers (PBDEs) in 2009, hexabromocyclododecane (HBCDD) in 2013, and deca-BDE in 2017 (UNEP, 2017, http://chm.pops.int). However, there are a growing number of new FRs being produced as replacements for those that have been banned and regulated.

Organophosphate esters (OPEs) are a large class of replacement FRs, which are also used as plasticizers or performance additives in consumer products (van der Veen and de Boer 2012; Hou et al 2016). Most recently, the European Flame Retardants Association published that global sales of FR were greater than 2 million tonnes (t) in 2013 (European Flame Retardants Association, 2015) while in 2011, OPE FR consumption reached 290,000 t globally (Du et al., 2015). An estimation of the growth in this market sector is an increase of 90,000 t over a 7-year span (Du et al., 2015). More specifically, in 2012, tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), triphenyl phosphate (TPHP), and tris(1-chloro-2-propyl) phosphate (TCIPP), in the United States contributed approximately 38,000 metric t (Schreder et al., 2016).

OPEs have been reported in numerous environmental samples such as in air,

wastewater effluent, household dust, sediment, and biota (Cristale et al., 2016; Fernie et al., 2017; Greaves et al., 2016a; Kim et al., 2013; Möller et al., 2012; Su et al., 2015a). There are limited reports for various Arctic media. However, atmospheric particle concentrations of OPEs in air have been reported to be greater than for PBDEs, from monitoring in the Canadian Arctic (Sühring et al., 2016), in Svalbard at Longyearbyen (Salamova et al., 2014) and the Chukchi Sea (Arctic Ocean) (Möller et al., 2012). In contrast, reports on OPEs in arctic biota and wildlife are extremely limited. Among the limited numbers of reports in fish, low OPE levels were reported in whole body lake trout (*Salvelinus namaycush*) and walleye (*Sander vitreus*) from Lake Athabasca, Canada (2009-2010) (Mcgoldrick et al., 2014). Generally low to moderate ng/g wet weight levels of nine OPEs were reported in whole body caplin (*Mallotus villosus*) collected from 2007 to 2010 in the Svalbard Archipelago, Norway. In the same Norwegian study, low to moderate ng/g wet weight levels of these OPEs were reported in the liver of arctic fox (*Vulpes lagopus*) and kittiwakes (*Rissa tridactyla*), blood of polar bear (*Ursus maritimus*) and harbour seal (*Phoca vitulina*), blubber of ringed seal (*Pusa hispida*), and eggs of Brünnich's guillemot (*Uria lomvia*) and glaucous gull (*Larus hyperboreus*). Other limited studies in arctic resident birds include mean concentrations of 4-110 ng/g of six OPEs in the feathers and sub-ng/g wet weight levels in the plasma of white-tailed sea eagle nestlings (*Haliaeetus albicilla*) from the Trøndelag region, Norway (2011) (Eulaers et al., 2014). Fernie et al. (2017) recently reported non-detectable to low ng/g wet weight concentrations of four OPEs in the plasma of peregrine falcon nestlings (*Falco peregrinus*) from Ungava Bay, Canada (2007) (Fernie et al., 2017).

The polar bear is the top marine-feeding predator in the circumpolar Arctic, which feed primarily on seals, and in particular on ringed seal. To our knowledge, the only known reports on OPEs in the tissues of these species were by Hallanger et al. (2015) from Svalbard, and very recently in the adipose tissue of polar bears from Western and Southern Hudson Bay subpopulations (Letcher et al., 2018). In these limited reports, OPEs were largely not detectable or at very low concentrations. Given the relatively high OPE concentrations reported in Canadian Arctic air particle phase samples (Sühring et al. 2016; Salamova et al. 2014), the low concentrations of OPEs in polar bears and ringed seals suggest that in these species that biological persistence and food web accumulation is low due to poor assimilation from the diet and/or rapid metabolism.

Toxicokinetic studies which are limited to a few avian and mammalian model systems have demonstrated the rapid metabolism *in vitro* of OP triesters. Greaves et al. (2016b) used a 100-minute microsomal biotransformation assay based on the liver of herring gulls (*Larus argentatus*) from the Great Lakes and revealed that OP triester depletion was rapid and structure-dependent and corresponding OP diester metabolite formation was observed. Using a human liver microsomes (HLM), van den Eede et al. (2013) suggested putative Phase I and II biotransformation pathways for OPEs such as oxidative-dealkylation, hydroxylation, and oxidative-dearylation. In mammals, two categories of implicated enzymes catalyzing these pathways are NADPH-dependent cytochrome P450 enzymes (CYP450) (Sasaki et al., 1984; Van den Eede et al., 2013) and NADPH-independent paraoxonases (e.g. PON1) and aryl esterases (Sasaki et al., 1984; Testa and Kramer, 2010; van den Eede et al., 2013). Thus, there is little information on

the metabolic depletion and pathways for OPEs in wildlife, especially in the Arctic context, which is fundamental to understanding fate and exposure.

The objective of the present study was to determine the comparative metabolism and metabolite formation *in vitro* of six environmentally relevant OP triesters using a liver microsomal assays for polar bears and ringed seals from East Greenland. *In vitro* metabolism results were then compared to OPE levels in fat or blubber samples from the same animals.

3.2 Results and Discussion

3.2.1 Total protein and enzyme catalytic activity for liver microsomes

Enzymatic activity based on CYP1A-mediated activity using the EROD assay has been conducted previously for both polar bears and ringed seals (Chabot-Giguère et al., 2013; Greaves et al., 2016b; Letcher et al., 2014; McKinney et al., 2006). Each individual ringed seal and polar bear liver microsomal suspension had EROD measured to determine the relative variability of enzyme activity among individuals. Enzymatic activity percent coefficient of variation (% CV) were low for each individual among the ringed seal (7.93 %) and polar bear (23.3 %) species. Since the inter-individual variation was low, species-specific pools were created to allow for sufficient quantities of microsomes to conduct *in vitro* assays (**Table 3-2**). As listed in **Table 3-1**, replicate EROD analysis of the ringed seal and polar bear pools at eight times dilution had average activities of 392.4 ± 22.84 and 1,784 ± 82.11 pmol*min^{-1}*mg^{-1} protein, respectively, meaning that polar bear microsomes were approximately four times more EROD active compared to ringed seal microsomes. This clearly indicated that the polar bear and ringed

seal microsomes were enzymatically viable. The average replicate EROD activity and total protein levels for Wistar-Han rat control were consistent with the 210 pmol/min/mg protein activity and 20 mg/mL protein content provided by the supplier (Corning Inc.). Furthermore, the EROD activity and protein content of the polar bear and ringed seal microsomal pools were within the same order of magnitude as was published previously (Letcher et al., 2014). A total of 60.95 g of polar bear and 30.19 g of ringed seal liver tissue were processed.

Table 3-1: Pooled ringed seal and polar bear liver microsome protein content and enzymatic specific activity at dilution factors of 4 and 8 along with pooled microsomal standard error of the mean (SEM) for technical replicates (n=3). The positive control used was Wistar-Han rat microsomes.

Pooled Microsomes, Dilution Factor	Protein Content (mg/mL) (±SEM) (n=3 rep.)	Specific Activity pmol*min^{-1}*mg^{-1}) (±SEM) (n=3 rep.)
Wistar-Han rat, 4	21.04 (± 1.633)	163.7 (± 14.45)
Ringed seal, 4	18.32 (± 6.163)	423.0 (± 7.906)
Ringed seal, 8	19.77 (± 1.109)	392.4 (± 22.84)
Polar bear, 4	22.21 (±1.479)	1006 (± 65.07)
Polar bear, 8	23.13 (±0.7614)	1784 (± 82.11)

Table 3-2: Individual polar bear microsomal, ringed seal microsomal, and control rat microsomal protein concentration (mg protein / mL) and specific activity (pmol*min^{-1}*mg^{-1}). All polar bear and ringed seal samples are presented for dilution factors of 4 and 8 with n=3 technical replicates except for 43107 (n=2) for dilution factor 8 and 43192 which only has dilution factor of 8.

Sample ID	Dilution Factor	Average Protein Concentration (mg protein / mL)	SEM[a] Protein (mg / mL)	Average Specific Activity (pmol*min^{-1}*mg^{-1})	SEM[a] activity (pmol*min^{-1} *mg^{-1})
Polar Bear					
43101	4	51.2	7.7	125.6	23.6
43101	8	71.5	5.1	172.0	12.6

43104	4	34.9	1.7	160.5	8.9
43104	8	59.1	6.1	198.1	19.1
43105	4	23.8	3.4	246.9	42.6
43105	8	24.5	1.8	284.1	46.9
43106	4	42.1	2.5	138.0	16.0
43106	8	41.5	0.5	242.4	2.4
43107	4	69.9	2.4	82.6	8.0
43107* (n=2)	8	7.8	6.6	990.2	481.4
43171	4	37.9	6.6	171.6	33.9
43171	8	53.8	2.1	226.9	10.5
Ringed Seal					
43183	4	68.8	0.6	82.2	1.6
43183	8	104.5	1.1	53.7	0.0
43185	4	76.5	7.4	105.3	11.2
43185	8	97.4	2.0	89.6	1.3
43187	4	85.3	1.5	147.9	2.1
43187	8	134.5	2.6	170.2	0.9
43189	4	30.8	4.3	72.3	7.6
43189	8	53.7	13.2	112.9	29.3
43190	4	55.0	0.6	135.3	1.8
43190	8	83.8	2.5	103.4	2.2
43192	8	124.7	4.7	136.7	4.0
43188	4	53.2	1.2	118.6	0.3
43188	8	73.3	2.7	151.0	0.5
Control: Wistar Han Rat Microsomes					
	4	38.7	3.8	64.5	7.0
	4	23.6	1.4	44.7	1.8
	4	28.7	8.3	48.3	20.4
	4	23.0	1.9	50.6	4.0
	4	24.5	0.3	41.7	0.7
	4	46.1	0.1	107.2	0.6
	4	40.1	0.6	53.2	0.3
	4	10.2	0.5	45.3	4.3
Total PB (n=35)		44.5	3.08	232	39.2
Total RS (n=39)		80.1	4.75	114	5.69
Total Rat (n=24)		29.4	2.47	56.9	4.80

[a] SEM is the standard error mean = standard deviation / square root (n=3)

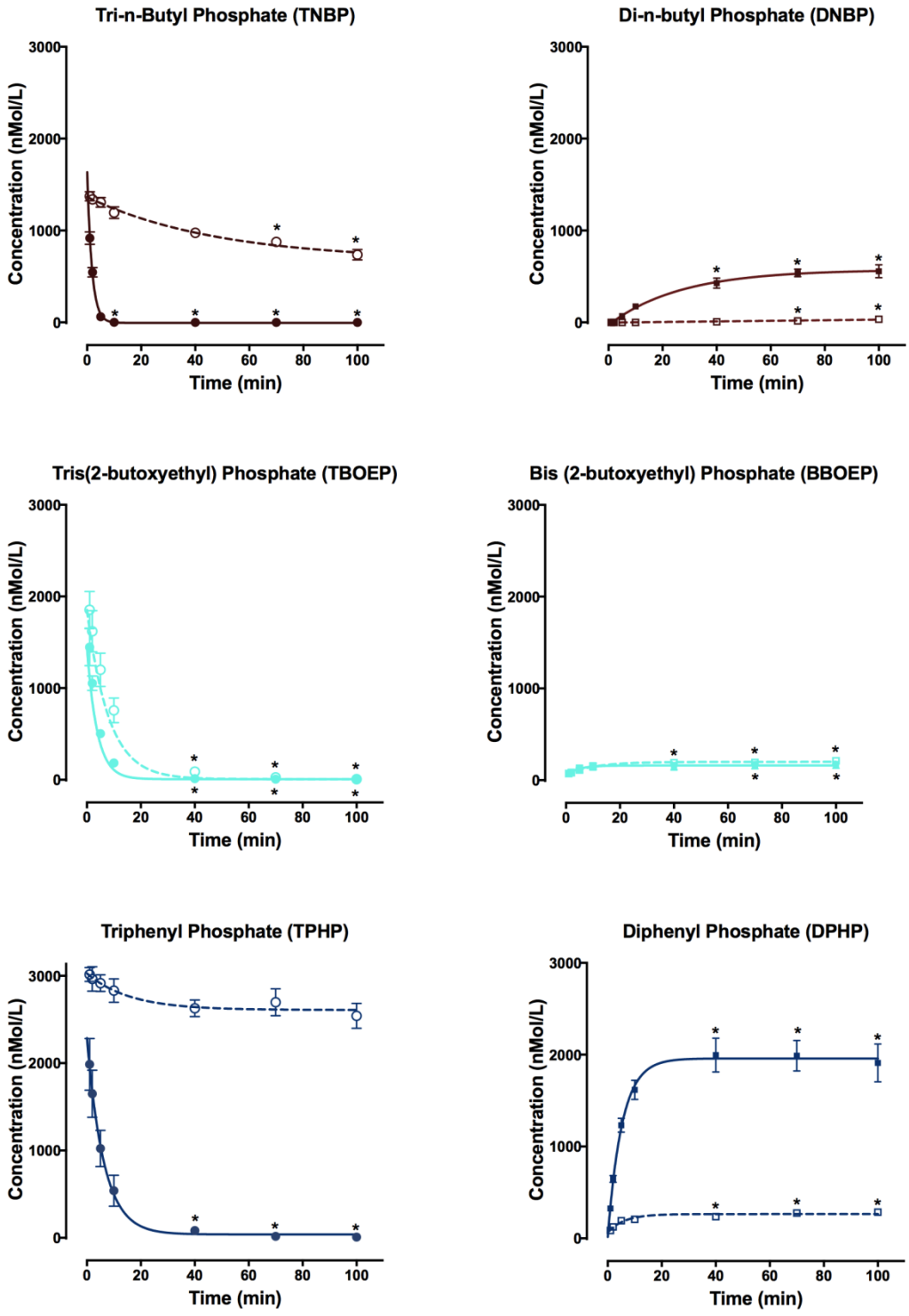
3.2.2 In vitro OP triester metabolism and OP diester formation

The six OPEs for the present *in vitro* studies were chosen based on a few relevant factors. First, these structures represent a diverse range of OPE structures, e.g. from short hydrocarbon chain and non-halogenated TEP to longer chain and halogenated TDCIPP to the aryl-substituted TPHP. Such a OPE structural range was logical in the present study to examine how OPE structure influenced *in vitro* metabolism. Second, there is demonstrated environmental relevancy to the study of these OPEs with a limited number of reports showing low levels of these six OPEs in Arctic mammalian wildlife (e.g. Hallanger et al., 2015). The *in vitro* metabolism of these same six OPEs were reported in a herring gull bird model system (Greaves et al., 2016b) and thus a comparison between the present Arctic mammals and that of a bird model system was possible. Finally, we have robust and validated analytical methods for the determination of these six OPE (triesters) as well as their OP diester metabolites (Chu and Letcher, 2015; Letcher et al., 2018).

During the 100 min incubation of the *in vitro* metabolism assay, the OP triester depletion and OP diester formation curves are shown in **Figure 3-1** and **Figure 3-2**. It is evident that OP triester depletion and OP diester formation occurred *in vitro* for both ringed seals and polar bears, and that the observed metabolism is dependent on the OP triester structure and species. It was ruled out that the observed OP triester depletion and OP diester formation was non-enzyme-mediated. Su et al. (2016) recently reported on the base-catalyzed hydrolysis (pH 7 to 13) of OP triesters and subsequent OP diester formation in aqueous solution (20 °C). At pH values of 7 or 9 and within 7 days, there was no depletion or corresponding diester metabolite formation of the OP triesters

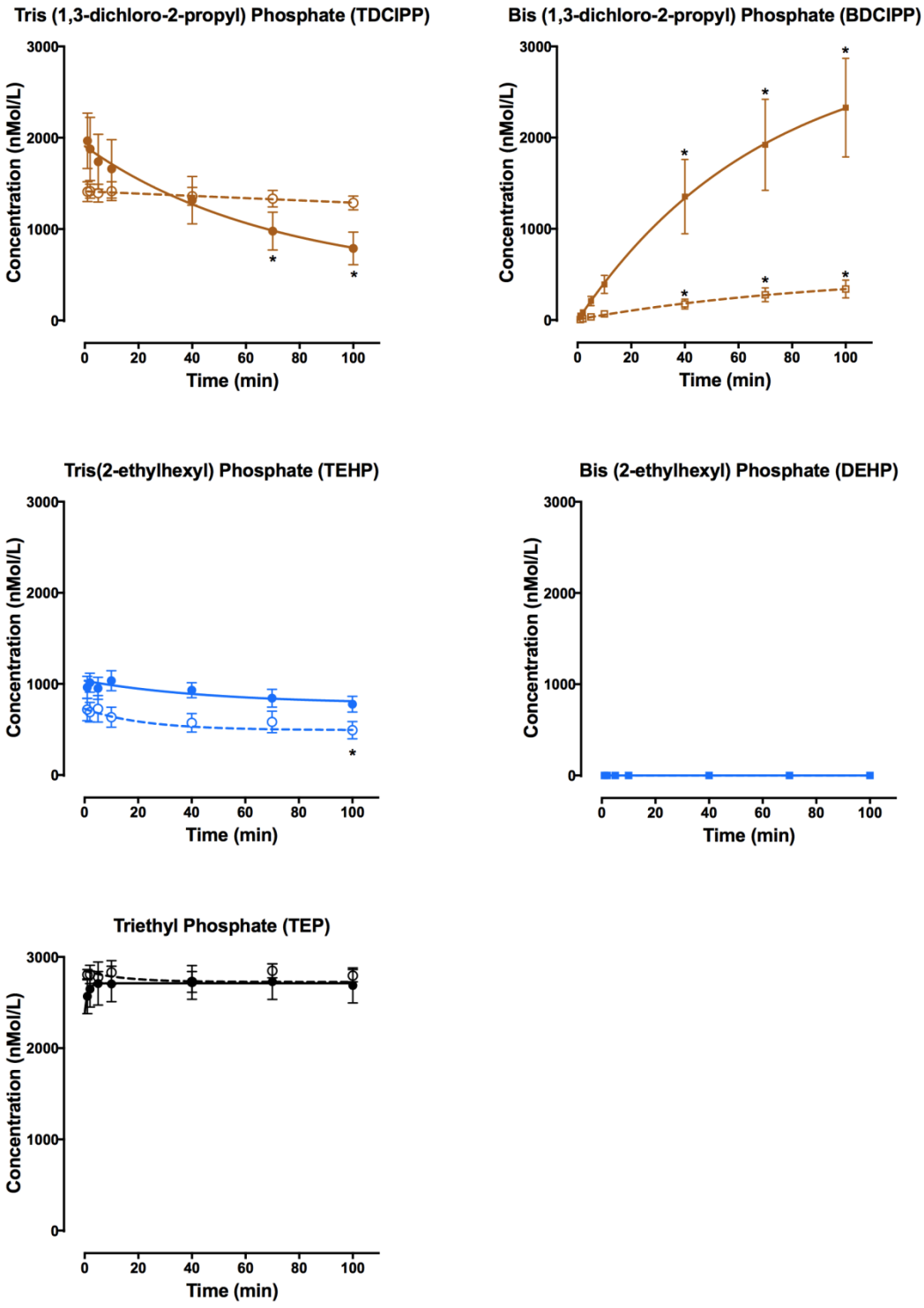
presently under study: TEP, TNBP, TDCIPP, TEHP, TBOEP and TPHP. The assay incubations were conducted at pH levels of 7.5 – 8 along with negative controls run for every assay. The negative controls demonstrated no aqueous depletion as there was consistently high levels of OP triesters with no production of corresponding OP diester metabolites.

As illustrated in **Figure 3-1** and **Figure 3-2**, any enzyme-mediated depletion of OP triesters was more efficient in polar bear microsomes relative to those of ringed seal. Notably, complete depletion of TBOEP was shown in both species, along with TNBP and TPHP by polar bears. However, in the case of ringed seal, TPHP and TNBP were metabolized slowly and incompletely after 100 minutes (**Figure 3-1**). There was partial depletion of TDCIPP by polar bears and marginal depletion by ringed seals. TEHP showed minimal depletion and TEP was not depleted at all by either polar bears or ringed seals over the 100 min. period (**Figure 3-2**). These species-specific metabolic differences were consistent with the greater general enzyme activity of the polar bear versus ringed seal microsomes (**Table 3-1**). These trends for polar bear were also consistent with previous studies, for example as reported in Greaves et al. (2016b), which used a comparable assay with herring gull liver microsomes. Ringed seals generally had less rapid metabolism of OPEs than herring gull microsomes. EROD activity for the present ringed seal microsomes was about four times greater than herring gull microsomes (Greaves et al., 2016b).



Legend:
solid symbols, solid lines - polar bear liver microsomal pool
white symbols, dashed lines - ringed seal liver microsomal pool

Figure 3-1: Time course incubation of ringed seal and polar bear microsomal assays with the non-halogenated OP triesters, tri-n-butyl phosphate (TNBP), tris(2-butoxyethyl) phosphate (TBOEP) and triphenyl phosphate (TPHP), showing the curves for OP triester depletion (left plots) and OP diester metabolite formation (right plots) for polar bears (dark symbols, solid lines) and ringed seals (open symbols, dotted lines). Each data point is the mean of n=3 replicate assays conducted on n=2 days (total of n=6 replicates) and the error bars represent the ± SEM. Asterisks indicate time points when OP triester depletion or OP diester formation differs significantly ($p < 0.015$) from the concentration at one minute.



Legend:
solid symbols, solid lines - polar bear liver microsomal pool
white symbols, dashed lines - ringed seal liver microsomal pool

Figure 3-2: Time course incubation of ringed seal and polar bear microsomal assays with the OP triesters, tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), tris(2-ethylhexyl) phosphate (TEHP) and triethyl phosphate (TEP), showing the curves for OP triester depletion (left plots) and OP diester metabolite formation (right plots) for polar bears (dark symbols, solid lines) and ringed seals (open symbols, dotted lines). Each data point is the mean of n=3 replicate assays conducted on n=2 days (total of n=6 replicates) and the error bars represent the ± SEM, except for TDCIPP (total of n=5 replicates). Asterisks indicate time points when OP triester depletion or OP diester formation differs significantly ($p < 0.015$) from the concentration at one minute.

OP triester depletion was structure-dependent especially for polar bears, where more rapid metabolism appeared to be the case for non-halogenated and shorter hydrocarbon chain OP triesters, except for TEP. This suggests that the bulkiness or polarity of OP triesters had an effect on the metabolic rate. Likewise, Greaves et al. (2016b) demonstrated that halogenated OPEs were slowly metabolized and TEP was not metabolized at all using an assay based on herring gull liver microsomes. More research is warranted to explore how OP triester structural elements (e.g. bulkiness, steric hindrance etc.) affects metabolism.

As shown in **Figure 3-1** and **Figure 3-2**, OP diester formation occurred as a result of OP triester metabolism, but with the exceptions of TBOEP, TEHP, and TEP. Not all of the depleted OP triester was converted to OP diester as shown by the mass balances in **Table 3-3**. This indicates that OP triester metabolism by polar bears and ringed seals can proceed via alternate metabolic pathways. The percent conversion gives an indication that OP diesters are the primary metabolite for TPHP and also in the case of polar bear, for TDCIPP. The other OPEs (TEHP, TBOEP and TNBP) all had less than 50 % conversion to the OP diester. Any additional diester produced (i.e. > 100 % conversion) may be due to endogenous liver OP triester or diester.

Table 3-3: The percent conversion of nmol of parent organophosphate (OP) triester to the corresponding OP diester metabolite. See **Figure 1-2** and **Figure 2-3** for names and abbreviations of the OP triesters and diesters.

Polar Bear				
OP triester or diester	Administered conc. (μM)	OP Triester Depletion (nmol) [a]	OP Diester Formation (nmol) [b]	% Conversion [c]
TDCIPP	2.0	1178		
BDCIPP			2328.2	198
TEHP	2.0	186		
DEHP			0	0
TPHP	2.0	1979		
DPHP			1910.5	97
TBOEP	2.0	1440		
BBOEP			165.2	11
TNBP	2.0	917		
DNBP			557	61
Ringed Seal				
OP triester or diester	Administered conc. (μM)	OP Triester Depletion (nmol) [a]	OP Diester Formation (nmol) [b]	% Conversion [c]
TDCIPP	2.0	123		
BDCIPP			339.4	276
TEHP	2.0	226		
DEHP			0	0
TPHP	2.0	474		
DPHP			285.1	60
TBOEP	2.0	1847		
BBOEP			211.2	11
TNBP	2.0	635		
DNBP			34.5	5

[a] OP triester depletion is the concentration measured at 1 minute less the remained at 100 minutes.

[b] OP diester formation is the concentration quantified at 100 minutes.

[c] Where % conversion = (OP diester formed) ÷ (OP triester depleted) x 100 %.

The *in vitro* conversion of TPHP to DPHP by polar bear of approximately 1:1 (**Table 3-3**) was much greater than was reported in similar assay studies with human liver

microsomes (HLM) (van den Eede et al., 2013). In the case of TPHP, the HLM clearance (41 %) was dissimilar to either polar bears or ringed seals. More similarly to ringed seals, for HLMs, DPHP was not the major metabolite, and a glucuronide metabolite and hydroxyl metabolite were found to contribute the most to the mass balance (van den Eede et al., 2013). Moreover, the HLM assay with TBOEP resulted in a high depletion (81 %) but as with the current study, BBOEP was not the major metabolite. Rather, a hydroxylated metabolite was found to be the major metabolite. Additionally, *in vitro* studies with herring gull microsomes demonstrated different percent conversion to diesters for TPHP (15 ± 3 %) and TNBP (14 ± 2 %) (Greaves et al., 2016b). In comparing gulls to the current study, the OP diesters constituted a major portion of the depleted TPHP in polar bear and ringed seal and TNBP in polar bear microsomes. TPHP percent conversion to DPHP was more similar to HLMs (van den Eede et al., 2013) than with the present polar bear microsomes.

TBOEP conversion to BBOEP was minimal at 11 % for both polar bear and ringed seal (**Table 3-3**). However, DNBP was a major metabolite formed at 61 % conversion for the polar bear microsomal assay, while for ringed seal there was only 5 % produced. Likewise, the difference was striking for TPHP between polar bear (97 %) and ringed seal (60 %). The species differences were further apparent when comparing OP triester depletion, where polar bear showed greater degradation for both TNBP and TPHP over the same time, compared to ringed seal. The difference in depletion and formation patterns for these OP triesters suggest that the key polar bear biochemical pathway leading to hydrolysis and diester formation is likely constitutively active. Specifically, polar bears are at the top of the marine food chain and would have a higher exposure of

contaminants through diet, and species-specific metabolism has been described as differences in constitutively versus induced enzyme activity (e.g. Boon et al., 1997; McKinney et al., 2006). In the present study, enzyme presence, active-site binding interaction, and induction may have influenced the observed species- and/or OPE-specific metabolism, where contaminant-mediated enzyme induction has been shown to be species-specific via aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR/SXR), and constitutive androstane receptor (CAR) mediated gene expression (e.g. Köhle and Bock, 2009; Lille-Langøy et al., 2015; Nyman et al., 2001; Wang et al., 2014; Wolkers et al., 1998).

Structure-dependent OP triester metabolism in the polar bears and ringed seals are likely dependent on different biochemical pathways and enzymes (**Figure 3-1** and **Figure 3-2**; **Table 3-3**). Previous studies have implicated Phase I enzymes capable of OP diester metabolite formation to be NADPH-dependent enzymes, notably CYP450 enzymes (Testa and Kramer, 2010; van den Eede et al., 2013). However, DPHP formation has repeatedly been found in NADPH negative controls (e.g. Sasaki et al., 1984; van den Eede et al., 2013) implicating NADPH-independent enzymes including paraoxonases and aryl esterases. In the present *in vitro* assay, an NADPH negative control was conducted where polar bear microsomes were dosed with TPHP and the NADPH component was replaced with buffer. Metabolic depletion of TPHP could occur via NADPH-independent enzymes (**Figure 3-3**). The results show that there is 23 % conversion of TPHP to DPHP due to NADPH-dependent enzymatic metabolism; thus, NADPH-independent enzymatic metabolism likely plays a greater role in TPHP metabolism in polar bear. This mass

balance concept is critical in discovering the potential biochemical pathways, metabolite products, and fate of OPEs in exposed wildlife.

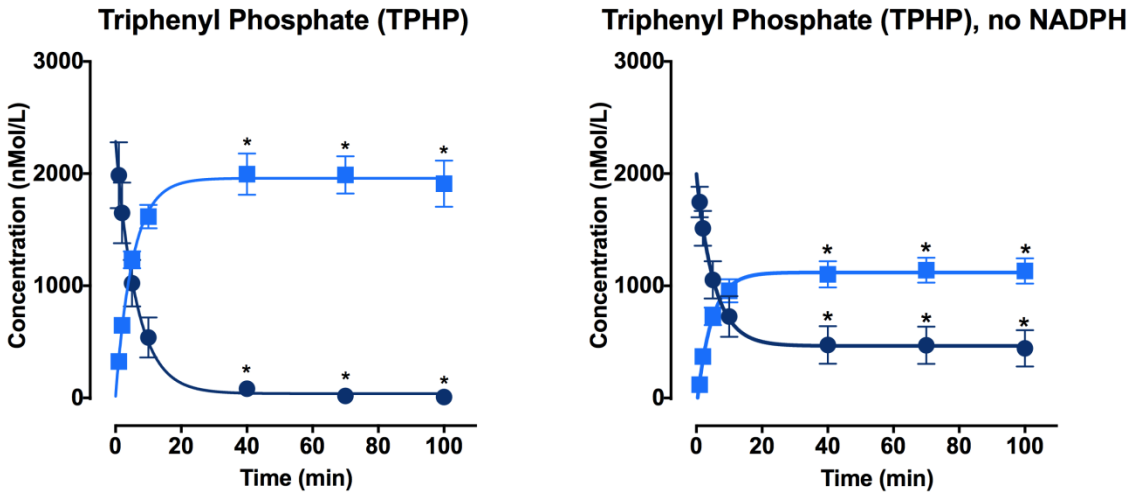


Figure 3-3: 100-minute time course incubation of polar bear microsomal assays with the OP triester triphenyl phosphate (TPHP) depletion (dark blue circles) and corresponding diphenyl phosphate (DPHP) metabolite formation (light blue squares). Left graph reproduced using data from **Figure 3-1** and right graph representing the assay without the addition of nicotinamide adenine dinucleotide phosphate (NADPH). Each data point is the mean of n=3 replicate assays conducted on n=2 days (total of n=6 replicates) and the error bars represent the ± SEM. Error bars are not shown where they are shorter than the height of the symbol. Asterisks indicate OP triester depletion or OP diester formation is significantly ($p < 0.01$) different compared to the concentration at one minute.

TDCIPP and TPHP metabolism for polar bears showed an essentially complete mass balance conversion to BDCIPP and DPHP, respectively, and thus indicated that no OP diester to OP monoester conversion occurred. It could be possible that if DPHP and BDCIPP are poorly depurated, there may be an exposure concern for these OP diesters. Moreover, HLM showed that conjugate metabolites were only seen to a limited extent, where TDCIPP and TBOEP had no to little conjugate formation (van den Eede et al., 2013). Previous research dosing chicken embryonic hepatocytes with DPHP showed greater number of genes were altered when compared to TPHP (Su et al., 2014). There is

a dearth of data on OPE metabolite toxicity and thus, the effects of nonconjugated metabolites should be investigated. The toxicokinetics, metabolite identity, and adverse effects of OPEs and their metabolites in exposed organisms requires further study including adsorption, distribution, metabolism and excretion (ADME).

3.2.3 Adipose tissue OPE concentrations

Fat and blubber samples of the same polar bears and ringed seals were all analyzed for the OP triesters under study (**Table 2-1**). Thus, it was possible to compare OP triester exposure with the predator to prey relationship, potential bioaccumulation, and the *in vitro* metabolism results. East Greenland polar bear diet has been estimated to have the greatest contribution at 47.5 ± 2.1 % from a diet of ringed seal and largely the blubber (McKinney et al. 2013).

Table 3-4 lists quantifiable concentrations of TDCIPP, TEP, TPHP, TBOEP, TEP and TEHP in polar bear adipose tissue and ringed seal blubber samples for the individuals listed in **Table 2-1**. For polar bear fat samples, the frequency of quantification was 100 % for TNBP, 67 % for TPHP, 50 % for TDCIPP, and 17 % for each TEP, TBOEP and TEHP. For ringed seal blubber samples, the frequency of quantification was 100 % for TNBP, 88 % for both TDCIPP and TPHP, 63 % for both TEP and TEHP, and 0 % for TBOEP. For the OP triesters that were quantifiable, all sample concentrations were very low (sub-ppb) levels. These results are consistent with OPE results for polar bear fat samples taken from Hudson Bay individuals (Letcher et al., 2018). Polar cod (*Boreogadus saida*) from Moffen, Greenland, 2008 was also reported to have low detection frequency and levels for all OPEs where the highest OPE level was TDCIPP

with a detection frequency of 9/25 fish analyzed and an average concentration of 5.1 ng/g ww (Evenset et al., 2009; Hou et al., 2016). In ringed seals from Svalbard, Norway, collected in 2010, TEHP was the only OPE detected, corresponding with the current study, that was above the MLOD with the low level of 1.96 ± 1.2 (detection frequency: 2/10 samples) (Hallanger et al., 2015). Most recently, peregrine falcon nestlings' plasma collected in 2007 from Ungava Bay, Canada showed TBOEP had the highest detection frequency of all OPEs studied (63 %) but the range of 0-7.5 ng/g ww showed low levels (Fernie et al., 2017). Thus, low levels of OPEs in this study and in other Arctic wildlife tissue is consistent with rapid metabolism and degradation of OP triesters, which suggests that the measurement of OP triesters in tissues is not an accurate biomarker for wildlife exposure.

Table 3-4: Polar bear fat and ringed seal blubber tissue concentrations of the organophosphate (OP) triesters, triethyl phosphate (TEP), tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), tris (2-ethylhexyl) phosphate (TEHP), triphenyl phosphate (TPHP), tri (n-butyl) phosphate (TNBP), and tris (2-butoxyethyl) phosphate (TBOEP). All concentrations are in ng/g wet weight. See **Table 1-1** for sample details.

Tissue Sample	TEP	TDCIPP	TEHP	TPHP	TNBP	TBOEP
Polar bear (PB)						
PB 43101	<0.25	<0.17	<0.32	0.44	0.38	<0.27
PB 43104	<0.25	0.39	<0.32	6.4	0.76	<0.27
PB 43105	<0.25	<0.17	<0.32	1.3	0.80	<0.27
PB 43106	<0.25	<0.17	0.48	<0.14	0.51	<0.27
PB 43107	<0.25	0.18	<0.32	<0.14	0.36	<2.2
PB 43171	0.57	54	<0.32	1.8	3.1	2.5
Mean Conc (±SEM) or Range	<0.25 - 0.57	<0.17 - 54	<0.32 - 0.48	<0.14 -6.5	0.98 (±0.54)	<0.27 - 2.5
Ringed seal (RS)						
RS 43183	<0.25	<0.17	0.51	0.88	1.5	<0.27
RS 43185	0.33	0.46	<0.32	0.52	1.7	<2.2
RS 43187	0.44	0.68	0.39	2.3	2.5	<2.2
RS 43188	0.32	0.54	0.33	5.1	1.2	<0.27
RS 43189a	0.28	0.69	0.38	2.4	0.99	<2.2
RS 43189b	<0.25	0.41	<0.32	1.1	0.82	<2.2
RS 43190	0.27	1.2	<0.32	7.2	1.5	<2.2
RS 43192	<0.25	0.27	0.65	<0.40	1.5	<0.27
Mean Conc. (±SEM) or Range	<0.25 – 0.44	<0.17 – 1.2	<0.32 – 0.65	<0.40 – 7.2	1.5 (±0.18)	<2.2

N/D non-detectable – below the MLOD.

N/Q non-quantifiable – above the MLOD but below the MLOQ.

Determining OP triester biomagnification factors (BMFs) was a challenge since in ringed seal blubber and polar bear fat most OP triester levels were below the MLOQ and/or had low detection frequencies, with the exception of TNBP. However, of the OPEs with 100 % detection frequency, BMF estimates were 1.1, 0.88, and 0.66 for TEHP, TPHP, and TNBP respectively. These estimates strongly suggest that OPEs do not

biomagnify from ringed seals to polar bears and that biotransformation of OPEs is the major confounding factor.

3.3 Conclusion

This study demonstrated that OP triester metabolism *in vitro* can be extensive in polar bears and (comparatively less so) for ringed seals, such that there are species-specific differences in terms of enzyme-mediated metabolic capabilities. The rapid rate of *in vitro* metabolism was consistent with the low levels found in the fat and blubber field samples of the same individuals, and thus further indicating that OP triester metabolism is extensive in these Arctic species, although in ringed seal the OP triester exposure from its marine food web is not known. Regardless, in ringed seal and certainly in polar bear these results emphasize that measuring OP triesters may be highly underestimating OP triester exposure in ringed seal via its marine food web. The observed structure-dependent metabolism of OP triesters in polar bears and ringed seals could suggest better prioritization for the study of POPs, especially where metabolites formed following biotic metabolism could require attention. Many OPE metabolites that could be formed remain unknown and require identification, and thus there is a lack of knowledge on the species-specific enzymes that mediate such metabolism. This will be required for a more complete understanding of any negative health effects of OPEs on biota, especially highly exposed Arctic wildlife.

4 Chapter: Structure-dependent *in vitro* metabolism of alkyl-substituted analogues of the organophosphate ester, triphenyl phosphate, in East Greenland polar bears and ringed seals[1]

Abstract

Organophosphate esters (OPEs) are used as plasticizers and flame retardants, and are increasingly being shown to be environmental contaminants. Some OPEs have been detected at high levels in the Arctic air and thus can undergo long range transport. Among the OPEs of emerging environmental interest, an increasing number are triphenyl phosphate (TPHP) structure-based, with an array of alkyl substitutions. TPHP has been shown to be metabolized rapidly by *in vitro* assays based on polar bear liver microsomes. The current study investigated the *in vitro* hepatic metabolism in polar bear and their ringed seal prey by comparing TPHP to isodecyl diphenyl phosphate (IDDPP), p-(tert-butylphenyl) diphenyl phosphate (TBPDPP), tris(p-tert-butylphenyl) phenyl phosphate (TTBPP), and tris (isopropylphenyl) phosphate (T2IPPP and T4IPPP). Polar bear metabolism of tert-butyl substituted OPEs, TBPDPP and TTBPP, had substantially lower rate and percent depletion compared to TPHP. Isodecyl and isopropyl-substituted OPEs, IDDPP, T2IPPP and T4IPPP were also more slowly depleted by polar bears relative to TPHP. Ringed seal microsomal depletion of TPHP was nonsignificant for TPHP along with TBPDPP, TTBPP and T4IPP. Meanwhile, IDDPP and T2IPPP showed a low level

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[1]**Adapted from** Strobel, A., Willmore, W.G., Sonnne, C., Dietz, R., Letcher, R.J., 2017 Structure-dependent *in vitro* metabolism of alkyl-substituted analogues of the organophosphate ester, triphenyl phosphate, in East Greenland polar bears and ringed seals. Environ. Sci. Technol. Letters In Preparation.

of depletion by ringed seal microsomes. Overall, the data showed that either *ortho*- and *para*-alkyl-substitution of TPHP can alter the rate of metabolism for both polar bear and ringed seal.

***Keywords*:** organophosphate esters (OPEs); triphenyl phosphate; alkyl-substitution; *in vitro* metabolism; liver; polar bear; ringed seal; East Greenland

4.1 Introduction

As organophosphate esters (OPEs) have entered the commercial market as additives and used as plasticizers, performance additives and flame retardants in various products; they are also entering the environment as novel environmental contaminants. In the Arctic, high concentrations of OPEs have been measured in atmospheric air particles and at concentrations greater than polybrominated diphenyl ethers (PBDEs) (Möller et al., 2012; Salamova et al., 2014; Sühring et al., 2016).

OPE exposure for Arctic biota is a concern and there is little toxicological data available. From the few monitoring studies that have been reported, top Arctic predators including polar bears (*Ursus maritimus*) and their main prey source, ringed seal (*Pusa hispida*), have consistently low concentrations of OPEs in various storage tissues (Eulaers et al., 2014; Fernie et al., 2017; Hallanger et al., 2015; Letcher et al., 2018; Muir et al., 2013; Strobel et al., 2018). This is proving to be largely due to rapid metabolism of OPEs as has been shown *in vitro* for rat, human, herring gull, polar bear, and ringed seal in assays using hepatic microsomes (Sasaki et al., 1984; Strobel et al., 2017; Su et al., 2016a; van den Eede et al., 2013). For example, shorter chain and less bulky OP triesters had more rapid metabolism when comparing tri (n-butyl) phosphate (TNBP) and tris(2-butoxyethyl) phosphate (TBOEP) or tris(2-ethylhexyl) phosphate (TEHP) in polar bear and herring gull liver microsomes (Greaves et al., 2016b; Strobel et al., 2018). However, none of these studies investigated alkyl-substituted OPEs, which could alter the rate and extent to which such OPEs are metabolized. These TPHP analogues are substituted with alkyl groups of varying bulkiness, including isodecyl diphenyl phosphate (IDDPP), p-(tert-butylphenyl) diphenyl phosphate (TBPDPP), tris(p-tert-butylphenyl) phenyl

phosphate (TTBPP), and tris(isopropylphenyl) phosphate isomers (e.g. T2IPPP and T4IPPP). An array of alkyl-substituted TPHPs are emerging environmental contaminants and appear to have been used commercially in Canada as early as 1991 and 1975 in the US (Monsanto Co, 1975; Schulz and Parker, 1991).

The increased chemical bulkiness of these TPHP-substituted OPEs raises questions regarding their environmental persistence and fate, particularly in the Arctic. Recently there has been an assessment of the long-range transport of OPEs using the Organisation for Economic Co-operation and Development (OECD) *Screening Tool*, which found that IDDPP and TTBPP had high estimates of environmental persistence (Zhang et al., 2016). Additionally, the soil half-life of TTBPP was reported to be 12 h based on estimates using the environmental model, CATALOGIC. There is concerns for the potential for these emerging alkyl-TPHP OPEs have similar properties of persistence to PBDEs, listed in Annex A of the Stockholm Convention of Persistent Organic Chemicals (UNEP, 2017). OPEs were once thought to distribute exclusively to the particle phase in air but recent modeling has demonstrated IDDPP has distribution in both the aerosol and gas phases (Sühring et al., 2016). Although TTBPP was not detected in any Canadian Arctic air samples as reported by Sühring et al. (2015), modelling of TTBPP and TIPPP isomers provided characteristic travel distances of 2,853 km and 2,739 km, and persistence time in air (P_{OV}) of 8,160 h and 2,724 h, respectively (Sühring et al., 2016). Thus, there is potential for transport and ultimately, exposure of more hydrocarbon-like alkyl-substituted TPHPs to Arctic wildlife.

Alkyl-substituted TPHPs have been investigated in a few biomonitoring or toxicology studies. Most recently TTBPP and T2IPPP have been reported in bald eagle

plasma samples from the Great Lakes (Simon, 2016). Results show that next to TPHP, T2IPPP was the second greatest contributor to the total sum OPE concentrations (contributing 20 %). However, concentrations were still low, ranging from below the method limit of detection (MLOD) to 28.60 ng/g ww, and with a geometric mean of 4.43 ng/g ww. Likewise, TTBPP was measured in the eagle plasma at around 0.013 ng/g ww but was only quantified in one sample (Simon, 2016). Toxicology studies showing effects of alkyl-TPHP OPEs (i.e. TBPDPP, IDDPP, T4IPPP, TTBPP) show zebrafish behavioural effects (Jarema et al., 2015; Noyes et al., 2015); upregulation of an *in vitro* avian genomics assay (Porter et al., 2014); and altered stereogensis in mouse leydig cells to a greater extent than the well-studied flame retardant, BDE-47 (Schang et al., 2016). Likewise, eleven different *in vitro* assays studying neurotoxicology and development have applied the weight-of-evidence approach, where TIPPP was active in ten assays, TBPDPPP was active in nine, and TPHP was only active in five assays (Behl et al., 2015). However, the US National Toxicology Program demonstrated that IDDPP did not have bacterial mutagenesis using the AMES test (Zeiger et al., 1987). It should be noted that, compound purity was a challenge in some cases, such as T4IPPP had 72.9 % purity (Jarema et al., 2015) and there was no purity information available for TBPDPP (Noyes et al., 2015).

The present study investigated the effect of differing molecular characteristics (e.g. alkyl-substituent bulkiness and position of substitution) on metabolism of several TPHP analogues. The microsomal-based assay used TPHP analogues with environmental importance and enzymatically viable liver tissue from polar bears and ringed seals from East Greenland. It was hypothesized that structure-dependent alkyl-TPHP OPE

metabolism would be decreased from the rapid and complete metabolism of TPHP in polar bear.

4.2 Results and Discussion

Strobel et al., (2017) previously studied the *in vitro* metabolism of TPHP in polar bear and ringed seal liver microsomal assays, and found that rapid and complete metabolism for polar bears had significant depletion from 40 minutes onward ($p < 0.01$). Meanwhile, there was no significant depletion of TPHP using ringed seal microsomes ($p = 0.1205$ at 100 min).

Of the *in vitro* assays with alkyl-substituted TPHP analogues, IDDPP was fairly rapidly metabolized in polar bears. However, compared to TPHP, the rate of IDDPP metabolism was decreased in polar bear as significant ($p < 0.01$) depletion only occurred after 70 minutes of incubation (**Figure 4-1**). For ringed seal, there was no significant depletion of IDDPP ($p = 0.0667$ at 100 min); however, there was 44 % IDDPP depletion compared to only 16 % TPHP depletion (**Table 4-1**). This indicates that ringed seals may have a slow but apparent capacity to metabolize IDDPP. In comparison to TPHP, the isodecyl alkyl chain on IDDPP may be more readily cleaved than a phenyl group by ringed seals, whereas for polar bears there is a decreased rate of isodecyl alkyl chain cleavage. The difference between ringed seals and polar bears suggests that there are species-specific biotransformation factors (such as enzymatic capacity, activity or induction) which may affect rate of OP triester metabolism in a structure-dependent manner.

Table 4-1: The percent depletion of organophosphate (OP) triesters following 100 min. of incubation in the *in vitro* assay using polar bear and ringed seal liver microsomes, for isodecyl diphenyl phosphate (IDDPP), p-(tert-butylphenyl) diphenyl phosphate (TBPDPP), tris(p-tert-butylphenyl) phenyl phosphate (TTBPP), and two tris (isopropylphenyl) phosphate isomers (T4IPPP and T2IPPP).

		Polar Bear	*Ringed Seal*
OP triester	Administered conc. (mM)	% Depletion [a]	% Depletion [a]
IDDPP	2	81	44
TTBPP	2	67	48
TBPDPP	2	58	25
T4IPPP	2	73	62
T2IPPP	2	83	86

a $\% \, Depletion = \frac{Administered\ concentration - OP\ triester\ concentration\ at\ 100\ minutes}{Administered\ concentration} \times 100$

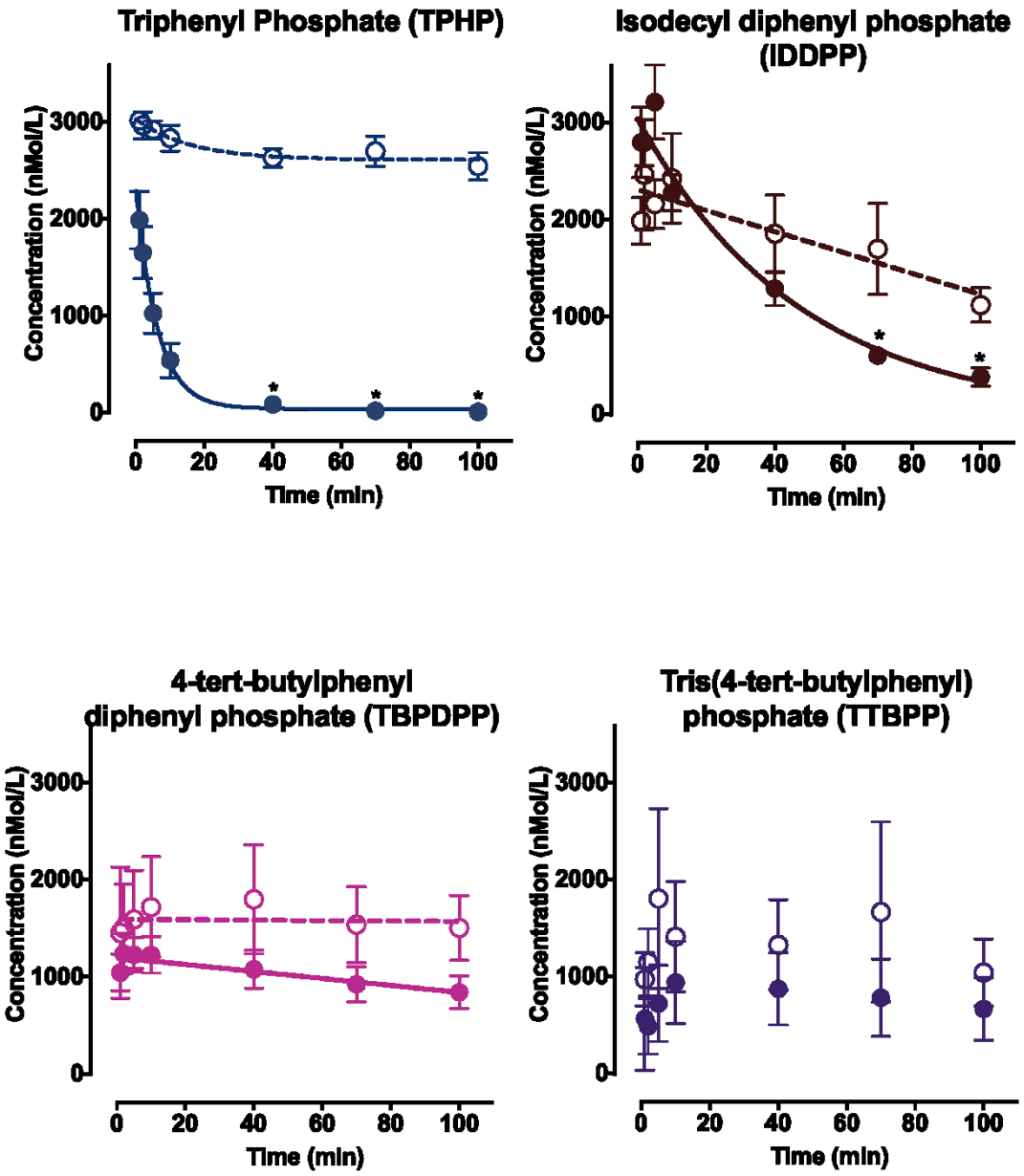

Figure 4-1: Time course *in vitro* assay incubation of organophosphate (OP) triesters in ringed seal and polar bear liver microsomal assays for isodecyl diphenyl phosphate (IDDPP), p-(tert-butylphenyl) diphenyl phosphate (TBPDPP), tris(p-tert-butylphenyl) phenyl phosphate (TTBPP). Triphenyl phosphate (TPHP) is reproduced from Strobel et al. (2017). Polar bears are indicated by solid symbols, solid lines and ringed seals clear symbols, dotted lines. Each data point is the mean of n = 3 replicate assays conducted on n = 2 different days (total of n = 6 replicates) and the error bars represent the ± SEM. Error bars are not shown where they are smaller than the height of the symbol. Asterisks indicate time points when OP triester depletion differs significantly ($p < 0.015$) from the concentration at one minute.

Further *in vitro* metabolism studies examined TPHP with the addition of a bulky tert-butyl group in the *para*-position of one phenyl ring in the case of TBPDPP, or at the *para*-positions of all three phenyl rings in the case of TTBPP, which saw a reduced depletion in polar bears (**Figure 4-1**). Specifically, there was no significant depletion for TBPDPP, and TTBPP did not follow any clear trend, indicating a lack of metabolism over the 100 min. incubation period. Likewise, the position (e.g. ortho or para) of alkyl groups affects *in vitro* metabolism (**Figure 4-2**). Specifically, depletion of T2IPPP (83 and 86 % loss for polar bear and ringed seal, respectively) is more rapid and complete than for T4IPPP (73 and 62 % loss for polar bear and ringed seal, respectively; **Table 4-2**). It was only after 100 min. of incubation that there was any significant ($p < 0.01$) depletion of T2IPPP for ringed seals (**Figure 4-2**), although at the same incubation time, depletion for polar bear was not significant ($p = 0.067$). Meanwhile, there was no significant depletion of T4IPPP in either species. This shows a clear structural-specific difference favouring metabolism of T2IPPP. The effect of substitution positions should be investigated further because there was no statistical significance when comparing each T4IPPP versus T2IPPP time point.

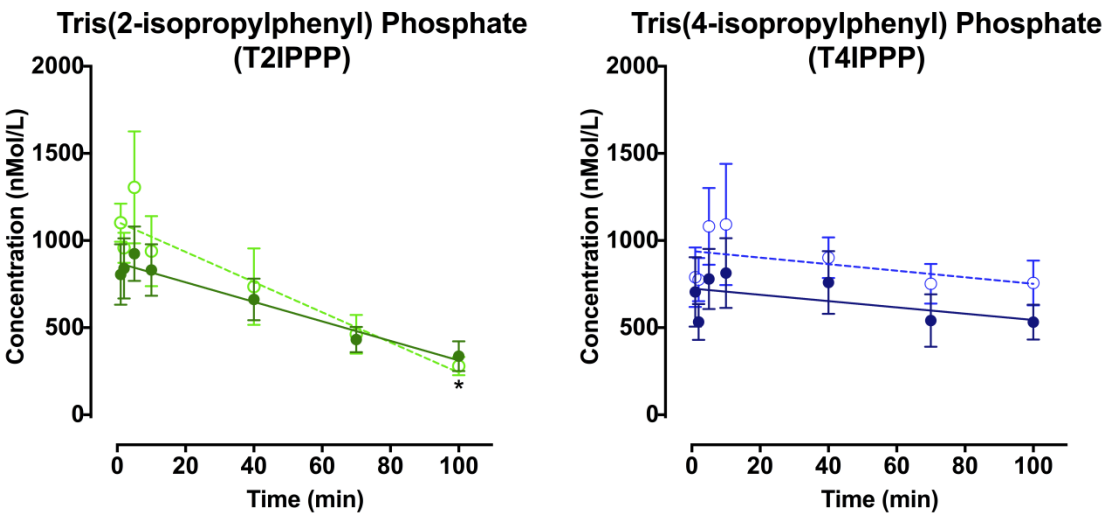

Figure 4-2: Time course *in vitro* assay incubation of organophosphate (OP) triesters in ringed seal and polar bear liver microsomal assays for *para*- and *ortho*- tris (isopropylphenyl) phosphates (T2IPPP and T4IPPP). Polar bears are indicated by solid symbols, solid lines and ringed seals clear symbols, dotted lines. Each data point is the mean of n = 3 replicate assays conducted on n = 2 different days (total of n = 6 replicates) and the error bars represent the ± SEM. Error bars are not shown where they are smaller than the height of the symbol. Asterisks indicate time points when OP triester depletion was significantly ($p < 0.01$) different from the concentration at one minute of incubation.

Table 4-2: After 100 min. of *in vitro* assay incubation, the percent conversion of nmol of parent organophosphate (OP) triester to the corresponding OP diester metabolite. The triesters presented are isodecyl diphenyl phosphate (IDDPP) and p-(tert-butylphenyl) diphenyl phosphate (TBPDPP), and their corresponding diester metabolite, diphenyl phosphate (DPHP).

Polar Bear

OP triester or diester	Administered conc. (mM)	OP Triester Depletion (nmol) [a]	OP Diester Formation (nmol) [b]	% Conversion [c]
IDDPP	2	1620		
DPHP			63	4
TBPDPP	2	1159		
DPHP			475	41

Ringed Seal

OP triester or diester	Administered conc. (mM)	OP Triester Depletion (nmol) [a]	OP Diester Formation (nmol) [b]	% Conversion [c]
IDDPP	2	879		
DPHP			60	7
TBPDPP	2	497		
DPHP			203	41

[a] OP triester depletion is the administered concentration less the measured concentration at 100 minutes

[b] OP diester formation is the concentration quantified at 100 minutes

[c] Where % conversion = (OP diester formed) ÷ (OP triester depleted) x 100 %

In agreement with a previous study by Strobel et al., diester formation is a fairly low contributor to the mass balance where DPHP was measured for IDDPP and TBPDPP (**Table 4-2**; **Figure 4-3**) (Strobel et al., 2018). DPHP contributed less than 10 % to the mass balance for IDDPP and 41 % for TBPDPP (**Table 4-2**). Notably, the percent conversion to diester were consistent between polar bears and ringed seals suggesting that enzymes capable of alkyl and substituted-aryl cleavage may be consistent between the two species. Moreover, it is clear other unknown metabolites are formed which require further investigation, especially as these pathways may play a greater role in the species-

specific rates. This is especially true as some OP metabolites have been identified as more potent than their parent compounds (e.g. Su et al., 2014).

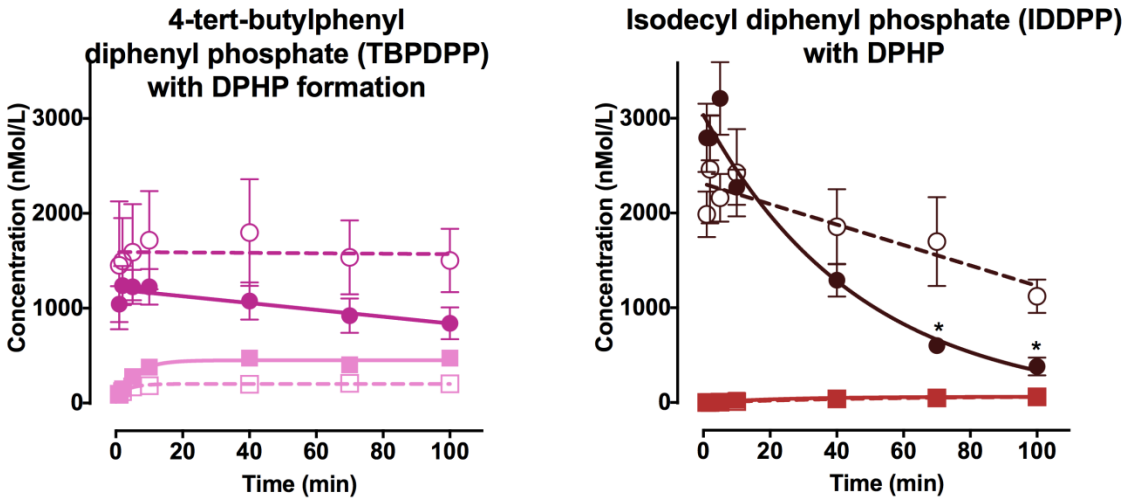

Figure 4-3: Time course *in vitro* assay incubation of organophosphate (OP) triesters in ringed seal and polar bear liver microsomal assays for IDDPP and TBDPP, which includes the diester metabolite formation, diphenyl phosphate (DPHP) over 100 minute time course. Polar bears are indicated by solid symbols, solid lines and ringed seals clear symbols, dotted lines. OP triester depletion is indicated by the darker colour and DPHP formation is shown with the lighter colour. Each data point is the mean of n = 3 replicate assays conducted on n = 2 different days (total of n = 6 replicates) and the error bars represent the ± SEM. Error bars are not shown where they are shorter than the height of the symbol. Asterisks indicate where the OP triester depletion or OP diester formation is significantly different ($p < 0.01$) from the concentration at one minute of incubation.

4.2.1 Structure Effects

Overall, the present study showed bulky, hydrophobic groups on TPHP phenyl rings results in changes (and generally decreases) the rate and percent depletion of OP triesters in both ringed seals and polar bears. Reduction in depletion rates for polar bear microsomes compared to TPHP may be due to increasing steric hindrance of bulky hydrocarbon substituents on the phenyl rings, potentially reducing the binding capacity of enzymes. The OPE-metabolizing enzymes in question may include cytochrome P450s,

paraoxonases, and/or aryl esterases (Testa and Kramer, 2009; Van den Eede et al., 2013). Another plausible explanation for the reduced metabolic depletion relative to TPHP, is the increased hydrophobicity with alkyl substituted TPHPs may have increased loss due to adsorption on glassware surfaces. Su et al. (2016b) postulated this concept when studying pH-dependent hydrolysis of OPEs.

It should be noted that the IDDPP standard used was the technical mixture Santicizer® 148, which also contains TPHP of unknown proportion. Likewise, TBPDPP was a technical mixture, however, it had an undetermined composition. Thus, the present IDDPP and TBPDPP results should be interpreted with caution since any species- and OPE structure-dependent metabolic depletion trends may be affected by purity. However, the available OPE technical mixtures can be viewed as being more environmentally relevant in terms of what could be released and with subsequent exposure to biota.

4.2.2 Arctic Species Context

There is little metabolism research available for OPEs, and specifically, we believe this is the first *in vitro* metabolism study of these five OP triesters. Thus, the mechanisms of OPE metabolism or lack thereof in biota, and the implications for potential bioaccumulation in the environment, requires further investigation. Previous studies on wildlife and humans have found rapid metabolism to be a key indicator of the fate and persistence of OPEs (e.g. Greaves et al., 2016; Strobel et al., 2017; van den Eede et al., 2013). Specifically, this rapid metabolism was paired with nondetectable to very low residue levels in adipose tissues of ringed seals and polar bears (Strobel et al., 2018). In comparison, urinary metabolites indicate human exposure to TIPPP mixtures and TTBPP,

where TTBPP metabolites were detected at low frequency suggesting reduced metabolism (Butt et al., 2014). This corresponds with the current study, where there was no clear trend of metabolism for TTBPP in either polar bear or ringed seal. Metabolism as a key driver of OPE fate may not hold for bulkier, alkyl-TPHP parent compounds, leading to alternative fates such as bioaccumulation.

As the polar bear is the top marine predator in the Arctic, it is well documented that they are highly exposed to POPs (e.g. Letcher et al., 2017; Muir et al., 2013); however, Arctic wildlife metabolism research is also limited. For example there are limited studies presenting POP metabolism in ringed seals, where small sample sizes made intra-species comparison challenging (Letcher et al., 2014; McKinney et al., 2011). Letcher et al., (2014) demonstrated species-specific depletion of *N*-ethyl-perfluorooctane sulfonamide (*N*-EtFOSA) where polar bears had 95 % depletion and ringed seals only had 65 % depletion. Meanwhile, McKinney et al., demonstrated structure-dependent depletion of various PBDEs in polar bear and ringed seal (2011). Likewise, EROD activity was the same order of magnitude to the pooled microsomes of the current study (Letcher et al., 2014; McKinney et al., 2011). The current study adds to the weight of evidence in the literature that metabolism is a key driver of internal fate of environmental contaminants. Thus, biomonitoring of various wildlife, could provide further evidence for the hypothesis of bioaccumulation potential of bulkier OPEs such as TBPDPP and TTBPP. Moreover, this should motivate further research in exposure hazard assessment to better quantify alkyl-TPHP produced, released and potentially stored /cold-trapped in various environmental matrices.

4.3 Conclusion

In the past, OPEs have shown to be rapidly metabolized and found in low concentrations in adipose, including in ringed seals and polar bears (Strobel et al., 2018). The current study provides examples of alkyl-substituted TPHP-like compounds which have decreased rates of metabolism and less depletion within the 100 minute *in vitro* microsomal assay compared with TPHP. This brings about questions of the potential for bulkier compounds to bioaccumulate in wildlife and persist in the environment. Further research should be investigated to better assess the exposure hazard and provide more mechanistic toxicokinetics data.

The history of legacy POPs (e.g. regulated by the Stockholm Convention) has often been responsive to extensive environmental contamination (e.g. the case of DDT); however, perhaps OPEs can have a unique story in that it may still be possible to apply preventative measures. If indeed there are high levels of alkyl-substituted TPHP produced and used in products, and if their fate is bioaccumulation in storage tissues of wildlife, the current research may provide direction for further study to curtail the current path of heavy chemical usage.

5 Chapter: Overall Conclusions and Future Directions

This project was designed to investigate the internal fate of eleven strategically chosen OP triesters through two lines of evidence. OP triester metabolism and where possible, corresponding OP diester metabolite formation, were measured using polar bear and ringed seal liver microsomal based *in vitro* assays. Additionally, data was collected for residue levels of six OP triesters in adipose tissue as a second line of evidence for OPE fate. OPEs were prioritized based on relevance to environmental risk and regulatory assessment (in Canada the federal Chemicals Management Plan (CMP)), and diverse but specific chemical structural differences.

A clear statement from this research and much other literature, is that OP triester fate is greatly affected by metabolism (e.g. Greaves et al., 2016; Strobel et al., 2017; Van den Eede et al., 2013). However, there is little research on wildlife metabolic capabilities especially considering biota in the Artic are often highly exposed. The current study found species-specific differences within a food chain, where polar bear generally metabolized OPEs more rapidly and completely than ringed seals (e.g. TPHP). The corresponding implication is that OPEs which are not rapidly metabolized or OP metabolites which are not eliminated efficiently in polar bear dietary source of ringed seal blubber have the potential to bioaccumulate. This principle of bioaccumulation potential also underlies the OP triester structures which had little metabolism in either species (e.g. alkyl-TPHP OPEs). This study provided a mechanism for better understanding high exposure (e.g. high atmospheric concentrations) with low tissue residue levels of certain OPEs in polar bear and ringed seal – metabolism.

Thus, this study determined two major factors driving rate and extent of metabolism – species capacity and OP triester structure. Structure-activity relationship (SAR) analysis may have the appeal to prioritize these factors, asking questions of whether structure or species plays a greater role in the internal fate of OPEs. However, such questions are much too simple, as certain OPEs indicated great species differences (e.g. TPHP) and others, structure played a greater role than species (e.g. T4IPPP). To better ascertain internal fate and bioaccumulation potential of OPEs, SAR analysis requires further, broader investigation.

There appears to be little bioaccumulation of "mainstream" OPEs while alkyl-TPHP OPEs should be investigated further in food chain contexts as they had little metabolism in ringed seals and polar bears. Thus, biomonitoring targets must be carefully chosen. For example, this is the first study investigating the metabolism of TTBPP, which did not appear to be degraded by polar bear or ringed seal. Thus, TTBPP may be an appropriate biomarker for biota exposure, however, many other OPEs (e.g. TNBP) would be more appropriately monitored by metabolites.

The current study analyzed *in vitro* metabolism data in terms of percent depletion by microsomes and percent conversion from parent OP triester to metabolite OP diester, demonstrating that OP degradation is multi-faceted. Small diester contributions (e.g. TBOEP → BBOEP), enzymatic degradation without NADPH requirements (i.e. TPHP can be partially degraded by polar bears without NADPH), and species-specific differences (e.g. TPHP) suggest various enzymes and biochemical pathway mechanisms underlie OPE metabolism. The mechanism of action was not investigated in the current study but there is strong evidence provided for such diversity. This evidence should be

applied to current project interpretations and future project directions. Briefly, while TPHP was metabolized rapidly in polar bear, most bulky alkyl-TPHP OPEs were poorly metabolized by polar bear and ringed seal. Meanwhile alkyl OPEs (e.x. TEP, TEHP, TNBP) had great diversity in metabolism for polar bear and ringed seal. Thus, the mechanisms of metabolic action, enzyme capacity, and weight of various factors (e.g. species vs. structure differences) should be investigated given the great physical characteristic diversity of OPEs.

5.1.1 Future Directions and Further Research

1. There is a lack of information regarding the possible metabolites that contribute to OP triester metabolism, as the percent conversion to diester clearly demonstrated that other metabolites are produced.
    a. Research should investigate the toxicity of metabolites as it is possible that they may be more readily bioaccumulative or toxic. For example, OPEs and relevant metabolites have demonstrated influence on thyroid protein interactions (Hill et al., 2018a, 2018b).
    b. Further, other components of the ADME paradigm (such as excretion and adsorption) could be investigated in partnership with metabolite formation to better assess the toxicokinetics of priority OPEs.
    c. Wildlife are exposed to a greater mixture of environmental contaminants which may affect toxicokinetics, such as competitive metabolism, which should be investigated further with OPEs.

d. Metabolites appropriate for biomonitoring should be identified. For example, DPHP was measured as a potential metabolite for three OP triesters in the current study. Thus, this metabolite may not be good for biomonitoring purposes as the OP source could not be identified. Furthermore, phase II processes such as conjugation, need to be addressed as they were not considered in this study. There is some research on urine metabolites (e.g. Su et al., 2015b); however, there is lack of metabolite information for alkyl-TPHP OPEs.

2. There should be investigation of metabolism in other cellular compartments (i.e. S9 cytoplasmic fraction) and tissue types (e.g. plasma, kidney, thyroid) to better understand the rapid metabolism of OPEs. This may explain the lack of microsomal depletion of TEP and the correspondingly low levels in biota. For example, if an *in vitro* assay using polar bear liver S9 fraction showed rapid metabolism of TEP, then a mechanism for understanding low fat residue levels could be clarified. However, if this same experiment did not demonstrate rapid metabolism, other metabolically active tissues (e.g. kidney) or target tissues of concern (e.g. thyroid) or tissues mobilizing xenobiotics (e.g. plasma) should be tested for their metabolic activity.
3. The enzymes responsible for OPE degradation should be identified explicitly. This project identified the presence of CYP1A enzymes in microsomes and identified that NADPH-independent enzymes were also necessary for OPE metabolism; however, this should be investigated further.

a. Binding assays can provide toxicokinetics data that can lead to more precise structure activity relationship analysis. Additionally, binding sites and binding fit may be identified.

b. This type of information could be used to add to the weight of evidence of OPE metabolism (n=11) which could be used for future predictive toxicology work. The extensive use of flame retardants, the novelty of some OPEs, and the diverse chemical structure of this class of compounds make them ideal candidates to apply in research to predictive toxicology models (such as a quantitative Adverse Outcome Pathway).

4. There are still literature gaps in understanding the metabolism and fate of OPEs in wildlife. This project addressed two species from the same class (Mammals) and previously, research was conducted on the metabolism of OPEs in herring gulls (same phylum: Chordata) and humans (same class: Mammals) (Greaves et al., 2016b; Van den Eede et al., 2013). Additionally, the "hot spots" of environmental contamination studied thus far are East Greenland (current study) and the Great Lakes (Greaves et al., 2016b). Diversification of species can strengthen a mechanistic understanding of metabolism and drivers of internal fate.

    a. Species from different locations (e.g. other hot spots of environmental contamination such as pelagic microplastic conglomerate) or different ecosystems (e.g. terrestrial or fresh water) could also provide weight of evidence for OPE toxicology.

b. Species at different trophic levels of the Arctic marine food chain should be investigated further (e.g. Arctic Cod, *Boreogadus saida*). This would allow better assessment of the degree of dietary assimilation in the fate of OPEs.

5. The current data could be further applied and synthesized to benefit stakeholders – wildlife and humans.
    a. Polar bears are top marine predators, leading them to be good indicators of toxicological exposure. Regulatory limits are often based on high-risk populations, such as highly exposed populations. Thus, the species-differences and potential bioaccumulation considerations mentioned throughout the thesis may be applicable to the hazard assessment process. Likewise, humans are a top predator species and while direct comparison is unwise, metabolism of OPEs in humans could be an avenue of consideration when considering OPE regulation in human environments.
    b. As polar bears are species of national and global cultural importance, and as the Arctic is an environment affecting world affairs, the food chain under current study can have regulatory implications. Moreover, ringed seals are a food source for northern communities. Thus, for economic, human well-being, and health reasons, the current study can be applied to the regulatory decision making process.
    c. This data set can aid other researchers in prioritizing OPEs studied further (e.g. taking into consideration substitution positions or prioritizing alkyl-TPHP OPEs that are not readily metabolised). Similarly, certain OP triesters may be of a higher priority based on this project, such as TTBPP. Due to the

lack of metabolism in the current study and dearth of data in the literature, this compound is an ideal candidate for further research regarding the fate of OPEs. Additionally, other structures may be identified as good comparative compounds (notably, TPHP). This process of prioritizing compounds that have the potential to biomagnify and are data poor, should be paired with any toxicity predictions and consumption data available.

d. The study design can also be applied elsewhere as an example of optimal use of resources for quality output. This type of application was already necessary for the current project, where a previous range study (Greaves et al., 2016b) provided a base for the zero-order kinetics dose used in this study. Likewise, study design components such as the structure-activity approach, pairing two lines of evidence in answering one question (adipose residue levels and metabolism), or pooling of microsomes could be applied elsewhere. It should be noted that these study design factors should be considered in detail, such as was the case in measuring individual EROD activity prior to pooling of microsomes to evaluate the consistency within the samples collected.

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