ORGANOPHOSPHATE ESTER *IN VITRO* METABOLISM, STRUCTURE-ACTIVITY RELATIONSHIPS, FATE AND BIOACCUMULATION POTENTIAL IN POLAR BEARS (*URSUS MARITIMUS*) AND THEIR RINGED SEAL (*PUSA HISPIDA*) PREY

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Introduction:
Organophosphate esters (OPEs) are used as alternative FRs to regulated use BFRs such as PBDEs.[1] Production of some brominated flame retardants (BFRs), e.g. PBDEs and hexabromocyclododecane, have been regulated due to their environmental persistence and bioaccumulation.[1] The Arctic is a sink for anthropogenic substances via long-range oceanic and atmospheric transport. OPEs have been detected and quantified in airborne particles, from East Asia to the high Arctic (230 to 2900 pg/m^3), Svalbard (33 to 1450 pg/m^3, Resolute Bay/Alert (nd-2340 pg/m^3) [2–4]. These compounds may subsequently persist in the environment depending on their physical-chemical properties.[2,5,6]

Thus, Arctic wildlife and other biota are exposed to both legacy and novel persistent organic pollutant (POP) contaminants. Several OPEs that are triesters have recently been detected in Arctic biota. Total OPE concentrations have been reported as high as 15,000 ng/g in global fish surveys[7,8]. In the Arctic Circle, OPEs have been reported in lake trout (*Salvelinus namaycush*), walleye (*Sander vitreus*), white-tailed sea eagle nestlings (*Haliaeetus albicilla*), ringed seal (*Pusa hispida*), arctic fox (*Vulpes lagopus*), and polar bear (*Ursus maritimus*)[3,9–11]. Additionally, low concentrations of OPEs have been detected in western and southern Hudson Bay polar bear liver and adipose tissue[12]. These low concentrations compared to the high environmental exposure data suggest rapid OP triester metabolism. Thus, there is environmental exposure concern relevant to the further study of toxicokinetics and -dynamics in Arctic marine biota[13]. 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 bears and ringed seals collected from the East Greenland area of Scoresby Sound.

Previously, OPEs were detected in herring gull eggs and the compound structure-activity relationships (SAR) were analyzed[14]. Greaves *et al.* demonstrated varying metabolic rate (TNBP > TBOEP > TCIPP > TPHP > TDCIPP) using *in vitro* herring gull microsomal assays, which showed structure-dependent depletion rates[14]. Putative Phase I and II metabolic pathways have been suggested for OPEs such as oxidative dealkylation, oxidative dearylation, oxidative dehalogenation, and hydroxylation[15]. In addition to OP triester depletion, this study will also focus on OP diester metabolite formation using available standards for quantification.

Methods:
Reagents: OP triesters studied are of environmental relevance and identified under the mandate of Environment and Climate Change Canada's, Chemicals Management Plan including TDCIPP, TCIPP, TNBP, TPHP, TBOEP, TEP. Standards of these OP triesters along with DNBP and DPHP were purchased from Sigma-Aldrich (Oakville, ON, Canada). BDCIPP, BBOEP, and BCIPP, along with the 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).

Microsome preparation: Hepatic microsomes were prepared from polar bears (n=6) and ringed seals (n=7) in Scoresby Sound, Eastern Greenland during 2011-2012. Briefly, tissue was homogenized with phosphate buffer solution (0.1M, pH 7) and centrifuged to separate subcellular fractions using previously cooled equipment. The Beckman-Coulter Optima TLX Ultracentrifuge (Mississauga, ON, Canada) was set to 17,000 RPM at 4° C for 15 minutes, and the supernatant was further centrifuged at 56,000 RPM at 4° C for 60 minutes. The final pellet was resuspended and diluted to concentrations of 20 mg protein/mL for use in the *in vitro* biotransformation assay.

Ethoxyresorufin-O-deethylase (EROD) Assay: The catalytic activity of CYP1A enzyme isoforms was used as a relative indicator for the overall CYP450 monooxygenase activity of the prepared microsomes. The substrate, 7-

ethoxyresorufin was catalyzed by CYP1A/ethoxyresorufin-*O*-deethylase (EROD) to resorufin. The reaction was carried out at 37° C in a 48 well plate containing solutions of sodium phosphate buffer (0.05 M, pH 8.0) and 7-ethoxyresorufin (1.02 μM). After five minutes, NADPH regenerating solution was added. Incubation was haltered after 10 minutes by addition of 100 μL of acetonitrile containing florescamine (2.16 mM). A Beckman DTX880 multimode detector, microplate reader measured resorufin (530 nm and 590 nm) and proteins (400 nm and 460 nm) using excitation and emission filters respectively. Resorufin and bovine serum albumin (BSA) standard curves were used to quantify activity and protein concentration respectively.

Metabolic Assay: The *in vitro* metabolic biotransformation assay solution was incubated for 2 minutes at 37° C with the shaker set to 80 RPM prior to adding 50 μL of microsome. The reaction contained potassium phosphate buffer (0.5 M, pH 7.4), NADPH regenerating solution, and OP triester dosing solution (2 μM). The reaction was vortexed well and 100 μL aliquots were taken at designated time intervals (0, 1, 2, 5, 10, 40, 70, 100 minutes). These aliquots were placed in a quenching solution containing 25 ppb mixture of internal standard in methanol. Solutions were capped to prevent evaporation. The solution was then microcentrifugated in prewashed tubes at 10,000 RPM for five minutes. To ensure replicable results, Wistar-Han rat negative controls, methanol blanks, and intra-day triplicates were conducted.

Chemical Analysis: Quantification of OP triesters and their diester metabolites was conducted utilizing a Waters Acquity UPLC coupled to a Waters Xevo triple quadrupole mass spectrometer (TQ-S) operated in the ESI+ mode.[16,17] 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). A dicationic reagent was mixed with the aqueous mobile phase at a constant flow of 10 μL/min (decamethonium hydroxide, 0.1 mM).

Statistics: R version 0.99.903 and analysis of variance (ANOVA) were used to determine significant depletion of OP triesters from the greatest initial quantity measured (at one minute)[18]. Statistical significance of $p<0.05$ or $p<0.01$ will be used where appropriate. Time points indicating significance will be compared to begin to contrast rates between polar bears and ringed seals as well as between OPEs.

Results and Discussion:

Preliminary *in vitro* metabolism data from this study strongly suggests that the OP triester metabolic rates are rapid in East Greenland polar bears and comparable to recently reported rates in Great Lakes herring gulls (Figure 1)[14]. Additionally, metabolic rate is more rapid in polar bears than in ringed seals. This is consistent with EROD assay data where specific activity at an eight times dilution factor for polar bear microsomes was 1784 pmol/min mg and ringed seal microsomes was 392 pmol/min mg. Thus, the representative enzymatic activity for polar bears was measured to be over four times more active than ringed seals. This difference in biotransformation capacity provides evidence and mechanism for the observed differences measured in metabolism. This is striking when comparing tris(1,3 – dichloro-2-propyl) phosphate (TDCIPP) metabolism between the two species (Figure 1). Polar bear metabolism shows significant depletion at 70 minutes ($p < 0.05$), whereas ringed seal depletion was not observed (not statistically significant) over 100 minutes. When comparing tri (n-butyl) phosphate (TNBP), polar bear metabolism is significant at 2 minutes ($p<0.001$) and ringed seal metabolism is significant at 10 minutes ($p < 0.05$). Thus, it appears possible that polar bear exposure to OPEs could potentially occur from the diet as well as environmental matrices. Further analysis between polar bear and ringed seal OPE metabolism will allow for estimates of trophic transfer and biomagnification between polar bears (the apex predator of the Arctic food web) and their primary dietary item (blubber of ringed seals).

Further comparisons can be made between compounds. TNBP depletion is much more rapid than TDCIPP depletion regardless of the wildlife liver microsomes studied thus far (herring gull, polar bear, and ringed seal). The difference in rate of OP triester depletion can be used to better describe the role of structure on the fate of OPEs. This research will provide toxicokinetic data on priority OPEs in Arctic marine predators. This information can then be applied to current regulatory decision-making through risk assessments and food web modeling.

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