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COMPARATIVE HEPATIC MICROSOMAL BIOTRANSFORMATION OF SELECTED PBDES, INCLUDING DECABROMODIPHENYL ETHER, AND DECABROMODIPHENYL ETHANE FLAME RETARDANTS IN ARCTIC MARINE-FEEDING MAMMALS

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Abstract—The present study assessed and compared the oxidative and reductive biotransformation of brominated flame retardants, including established polybrominated diphenyl ethers (PBDEs) and emerging decabromodiphenyl ethane (DBDPE) using an in vitro system based on liver microsomes from various arctic marine-feeding mammals: polar bear (*Ursus maritimus*), beluga whale (*Delphinapterus leucas*), and ringed seal (*Pusa hispida*), and in laboratory rat as a mammalian model species. Greater depletion of fully brominated BDE209 (14–25% of 30 pmol) and DBDPE (44–74% of 90 pmol) occurred in individuals from all species relative to depletion of lower brominated PBDEs (BDEs 99, 100, and 154; 0–3% of 30 pmol). No evidence of simply debrominated metabolites was observed. Investigation of phenolic metabolites in rat and polar bear revealed formation of two phenolic, likely multiply debrominated, DBDPE metabolites in polar bear and one phenolic BDE154 metabolite in polar bear and rat microsomes. For BDE209 and DBDPE, observed metabolite concentrations were low to nondetectable, despite substantial parent depletion. These findings suggested possible underestimation of the ecosystem burden of total-BDE209, as well as its transformation products, and a need for research to identify and characterize the persistence and toxicity of major BDE209 metabolites. Similar cause for concern may exist regarding DBDPE, given similarities of physicochemical and environmental behavior to BDE209, current evidence of biotransformation, and increasing use of DBDPE as a replacement for BDE209. Environ. Toxicol. Chem. 2011;30:1506–1514. © 2011 SETAC

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INTRODUCTION

Brominated flame retardants (BFRs) comprise a diverse suite of commercial chemicals used in consumer products to meet fire safety standards. Some BFRs, such as polybrominated diphenyl ethers (PBDEs), are established environmental contaminants, including within Arctic ecosystems [1]. Environmental behavior as well as toxicological research has largely focused on the lower brominated congeners, demonstrating bioaccumulation, long-range transport, and neurotoxic and endocrine-disrupting properties [2]. The pentaBDE and octaPBDE products have been phased out of use in the European Union, and North American production voluntarily ceased in 2004. In 2009, tetra- to hepta-brominated PBDEs were listed under the Stockholm Convention on Persistent Organic Pollutants (http://www.pops.int). The fully brominated 2,2′,3,3′,4,4′,5,5′,6,6′-decaBDE (BDE209), which constitutes >90% of the high production volume decaBDE products [3], remains unregulated, although phase-out of decaBDE production in the United States is scheduled for 2012 (http://www.bsef.com). BDE209 has been detected in nonsource regions [4] and may degrade to more persistent, lower brominated PBDEs by photolysis [5] and biotransformation [6–8].

As market demand for PBDE alternatives increases, other current-use BFRs are also being reported in environmental matrices. Notable is decabromodiphenyl ethane (DBDPE), marketed as an alternative to decaBDE. Available in the mid-1980 s, limited production data indicates increasing DBDPE use in the past two decades in Japan, with the reverse trend observed for decaBDE [9]. In 2006, estimated DBDPE production volume (12,000 metric tonnes) in China was similar to that of decaBDE formulations (20,000 metric tonnes) [10]. Decabromodiphenyl ethane has recently been reported in North American herring gulls and freshwater fish, and in Chinese waterbirds and captive pandas [10–14]. In some instances, DBDPE levels (on the order of parts per billion) were similar or higher than the levels of BDE209 and/or found in nonsource regions.

Within arctic environments and ecosystems, BFR patterns appear highly variable. In arctic air, BDE209 is a major congener [15]. However, BDE209 and DBDPE were very infrequently detected in spatial and temporal contaminant monitoring studies of the top marine-feeding arctic mammal, the polar bear (*Ursus maritimus*) [16,17]. In these bears, the congener patterns were very simple, with 2,2′,4,4′-tetraBDE (BDE47) and 2,2′,4,4′,5,5′-hexaBDE (BDE153) predominating. More complex congener patterns were observed in a

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separate study examining ringed seals (*Pusa hispida*) and beluga whales (*Delphinapterus leucas*), although BDE47 proportions were still high [18]. Contrasts of the BFR patterns between abiotic matrices (for example, air, ocean waters) and high trophic mammals may be due, among other factors, to the varying ability of these species to biotransform BFRs. Variation in BFR levels and patterns between these mammal species may, in part, be related to interspecies differences in biotransformation capacities and substrate-selectivities towards individual BFR contaminants. High phase I cytochrome P450 (CYP) activities and blood levels of hydroxylated-polychlorinated biphenyls (OH-PCBs) in polar bears have implied an elevated ability to biotransform PCB congeners compared to belugas and ringed seals [19,20]. In contrast, low levels of OH-PBDEs and slow to nondetectable turnover of certain environmentally relevant PBDE congeners during oxidative hepatic microsomal assays has suggested, at least from a CYP perspective, a low biotransformation potential towards PBDEs in these three high-trophic marine-feeding mammals [21–24].

These CYP-oxidative assay designs may not have adequately captured the metabolic potential towards, in particular, highly brominated BFRs, given that varying rates and degrees of reductive debromination of BDE209 (and some other PBDEs) to lower brominated congeners have been found in dosed fish, birds, and rats [6,8,25]. Dosed rats metabolized BDE47, 2,2′,4,4′,5-pentaBDE (BDE99), 2,2′,4,4′,6-pentaBDE (BDE100), 2,2′,4,4′,5,6′-hexaBDE (BDE154), and especially BDE209 [25–31]. Oxidation and oxidative debromination were predominant biotransformation pathways, the first step likely being CYP-mediated arene epoxide formation [32]. The fully brominated BDE209 is not likely metabolized directly by this pathway. Varying rates and degrees of reductive debromination of BDE209 (and some other PBDEs) to lower brominated congeners have been found in dosed fish, birds, and rats [6,8,33]. Results using hepatic microsomes from Chinook salmon (*Onchorhynchus tshawytscha*) and common carp (*Cyprinus carpio*) demonstrated that dithiothreitol (DTT; Cleland's reagent) but not nicotinamide adenine dinucleotide phosphate (NADPH) was required for PBDE debromination, discounting CYP and suggesting involvement of other enzymes, possibly iodothyronine deiodinases [34,35]. It is unknown whether DBDPE may undergo transformations similar to those of BDE209, but it is possible given their physicochemical similarities. Greater understanding of the metabolic capacity towards highly brominated BFRs in wildlife species would contribute to assessing risks associated with oxidative and reductive biotransformation to potentially more toxic, lower brominated metabolites. In this study, we investigated metabolism of BDE209 and DBDPE in high-trophic feeding arctic marine wildlife, in comparison to relatively lower brominated PBDEs (BDE99, BDE100, and BDE154; Fig. 1) using an in vitro assay design that expanded the range of possible first step enzymatic transformations. We compared the BFR biotransformation capacity across arctic species including polar bear, beluga whale, and ringed seal, as well as the laboratory rat as a mammalian model species.

MATERIALS AND METHODS

Chemicals and reagents

Dithiothreitol (DTT; Cleland's reagent) and NADPH regenerating systems were purchased from Sigma-Aldrich and BD Gentest, respectively. The PBDE and DBDPE standards were from Wellington Laboratories. OH-PBDE and MeO-PBDE standards were generously provided by G. Marsh and Å. Bergman (Stockholm University, Sweden). Water was supplied by a Milli-Q system (Millipore) using a 0.22 μm filter. All other reagents and solvents were of at least analytical grade purity.

Collection, microsome preparation, and analysis

Fresh (<60 min postmortem) liver specimens were collected from a stranded polar bear from Iceland and from subsistence-hunted beluga whales and ringed seals from Canada (Table 1). The liver tissues were temporarily stored in a liquid nitrogen dry shipper and subsequently transferred to a $\leq -80^{\circ}$C freezer once received at Environment Canada's Wildlife Specimen Bank. Hepatic microsomes were prepared and biotransformation assays performed in summer 2009. Microsome preparation

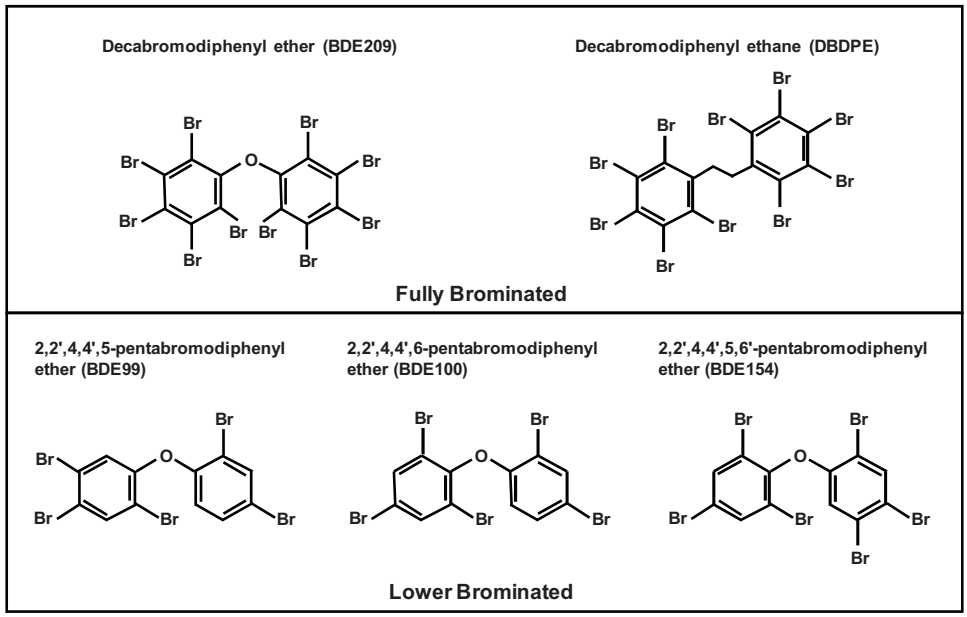

Fig. 1. Structures of the brominated flame retardants (BFRs) investigated in the current biotransformation study. Hydrogen atoms have been omitted for clarity.

Table 1. Sample collection location and date and biological data

Sample ID[a]	Species	Collection region	Collection date (YYYY/MM)	Sex	Age class
PB1	Polar bear	Iceland[b]	2008/06	F	Adult
BW1	Beluga whale	Western Hudson Bay, Canada	2003/08	M	Adult
BW2	Beluga whale	Western Hudson Bay, Canada	2003/08	F	Adult
RS1	Ringed seal	Cumberland Sound, Canada	2001/07	F	Adult
RS2	Ringed seal	Cumberland Sound, Canada	2001/07	F	Adult

[a] Samples used in previous studies, under different sample ID: BW1 = CA10 and BW2 = CA6 [24,26].
[b] PB1 was stranded in Iceland as the sea ice retreated during the summer. It is thus likely to be an individual from the East Greenland subpopulation.

has been described previously [26], except that the buffers used here had a higher DTT concentration (10 mM) to preserve reductase and deiodinase enzyme activities. The CYP1A-catalyzed 7-ethoxyresorufin-*O*-deethylase (EROD) activity was used as an overall indicator of microsomal enzymatic capacity. The EROD and protein content were simultaneously determined using the fluorescence microplate assay of Kennedy and Jones [27], but the reaction was terminated after 2 min to ensure resorufin concentrations fell within the calibration curve range. Calibration curve stock standard concentrations were determined spectrophotometrically immediately prior to the assay. Bovine serum albumin was used as the protein standard. Resorufin was the standard for EROD measurements. Hepatic microsomes from pooled ($n = 17$) adult male Wister Han rats (herein denoted RAT) were purchased from BD Gentest.

BFR standards for in vitro assays

In the Organic Contaminants Research Laboratory (OCRL) at the National Wildlife Research Centre of Canada (NWRC), microsomes were incubated with either individual BFRs or a mixture of the BFRs. Without an internal standard to correct for small variations in spike volume, recovery, and run-to-run instrument response, it may be difficult to conclusively detect slow depletion of a substrate in samples compared to controls. We chose BDE153 as the internal standard to include in both the mixed and individual spiking standards, because it is monitored by isotopic $[Br]^-$ in electron capture negative ionization mass spectrometry (ECNI MS) as are the other analytes, and preliminary work demonstrated negligible metabolic depletion of BDE153 under the present assay conditions. Two-tailed *t* tests of BDE153 levels showed no statistical difference ($p > 0.10$) between sample and control replicates for each individual animal. This is supported by previous in vitro and in vivo biotransformation studies on BDE153 [21,24,28,29].

The standard mixture contained the BFRs BDE99, BDE100, BDE154, BDE209, and DBDPE, as well as the BDE153 IS. The original solvent (nonane for PBDEs; toluene for DBDPE) was evaporated under nitrogen flow and the mixture was redissolved in acetone for a final 10 μM concentration (30 pmol in the assay) for each BFR, except DBDPE was set to 30 μM (90 pmol in the assay) due to its low ECNI $[Br]^-$ response. The concentrations of BFR substrate and microsomal protein (discussed further below) were chosen based on similar studies of CYP-mediated metabolism of PCBs and toxaphene [30,31].

In the mixed BFR assay, possible debromination products could be masked by the presence of PBDEs of differing degrees of bromination in the original mixture and potential metabolites could not be unequivocally linked to a particular parent compound. Therefore, individual BFR standards containing BDE153 were prepared at the same concentrations as in the standard mixture for use in individual assays. These assays further served as replicates to confirm the depletion results from the mixed assays.

In vitro biotransformation assay

For assays using the BFR mixture, there were four control and four sample replicate tubes for one specimen of each species (polar bear [PB1], beluga whale [BW1], ringed seal [RS1], and RAT). For assays using the individual BFRs, there were three control and three sample replicates for all specimens (including BW2 and RS2 as well). Control tubes contained the BFR spike (3 μl) and buffer (80 mM NaH_2PO_4, 6.0 mM $MgCl_2$, 1.0 mM disodium ethylenediaminetetraacetate [Na_2EDTA], pH 8.0). Sample tubes were prepared similarly, but also contained 10 mM DTT (nonnative cofactor for reductases and deiodinases) and 1.6 mM NADPH regenerating system (cofactor for oxidative CYPs). Preliminary tests on PB1 demonstrated that postassay extracts from controls with and without cofactors showed no concentration differences; therefore, cofactors were only added to the samples in subsequent assays. External standards were prepared containing the appropriate 3-μl BFR solution to monitor recoveries. Tubes were preincubated at 37°C for 5 min. During this time, 1 ml of ice-cold methanol (MeOH) was added to all control tubes to destroy enzyme activity upon addition of the microsomes. To start the assay, 1 mg of microsomal protein was then added sequentially to all sample and control tubes (1 ml final volume). Blanks containing only buffer and microsomes were also run, as well as a buffer-only blank. After 90 min in a shaking (120 rpm), 37°C water bath, 1 ml of ice-cold MeOH was added sequentially to all sample and blank tubes to stop the reaction. Therefore, at this point and prior to extraction, samples and controls were of identical composition.

BFR extraction and analysis

After addition of hexane (3 ml), tubes were mixed by vortex and centrifuged (10 min, 3,000 rpm / 1,400 RCF) and then the hexane layer was collected. Two further extractions with hexane (3 ml each) were performed. Lipids were removed by treatment of the combined hexane extract with concentrated H_2SO_4, agitation, centrifugation, and removal of the hexane layer. A further 3-ml hexane rinse followed to ensure quantitative transfer of the analytes. The extract was evaporated and solvent-exchanged into a final 200-μl volume of 2,2,4-trimethylpentane (isooctane).

After analysis of parent compound depletion and any neutral debrominated metabolite formation, some of the final extracts from the individual BFR assays were then derivatized to additionally investigate the possibility of oxidative metabolite formation. Treatment with diazomethane allowed for analysis of the extracts for methoxylated derivatives of any phenolic and/or phenolic debrominated metabolites [24]. A separate test sample spiked with 2′-OH-2,4,4′-triBDE (2′-OH-BDE28) and 6-OH-

2,2′,4,4′,5-pentaBDE (6-OH-BDE99) and taken through the entire assay, extraction, and derivatization procedures, showed acceptable recoveries of 56 and 87%, respectively.

Separation and quantification of all neutral BFR analytes by gas chromatography-mass spectrometry (GC-MS) in ECNI mode was previously described [16]. Derivatized fractions were also analyzed for methoxylated BFRs under the same conditions and by monitoring $[Br]^-$ isotopes. Extracts were compared to available standards containing a suite of congeners that generally encompass the majority of known and environmentally relevant congeners, including a 49 PBDE congener plus DBDPE standard, and for derivatized phenolics, a 15 congener MeO-PBDE standard. The PBDE standard contained BDEs 1, 2, 3, 10, 7, 15, 30, 17, 28, 54, 49, 71, 47, 66, 77, 100, 119, 99, 85, 155, 154, 153, 139, 140, 138, 156, 188, 184, 179, 183, 182, 191, 180, 181, 190, 171, 170, 202, 201, 197, 203, 196, 205, 194, 195, 208, 207, 206, and 209. The MeO-PBDE standard contained 6′-OH-BDE17, 2′-OH-BDE28, 4′-OH-BDE17, 6′-OH-BDE49, 2′-OH-BDE68, 6-OH-BDE47, 3-OH-BDE47, 5-OH-BDE47, 4′-OH-BDE49, 4-OH-BDE42, 6-OH-BDE90, 6-OH-BDE99, 2-OH-BDE123, 6-OH-BDE85, and 6-OH-BDE137.

Based on a signal-to-noise ratio of 10 and the external standard response and correcting for nonquantitative recoveries where appropriate, the method limit of quantification (MLOQ) was 0.04 to 0.09 nM for brominated diphenyl ether compounds, from responses of BDEs 99, 100, 154, and 209, in the assay solution and 8 nM for brominated diphenyl ethane compounds, from the DBDPE response. The MLOQ for derivatized phenolic brominated diphenyl ethers was 0.2 to 0.7 nM. In terms of metabolite formation, these MLOQs indicated that a single extractable debrominated BDE metabolite should have been quantifiable if around $\geq$0.3% of the parent was converted. For debrominated BDPEs, around $\geq$9% conversion would have resulted in a quantifiable metabolite and for phenolic BDEs around $\geq$ 2% conversion would have been required. If two or more metabolites were formed, of course, greater conversion of the parent would have been required to detect a given metabolite.

Quality control

Recoveries were calculated by comparing the analyte response in the controls to that in the appropriate spiked external standard. Recoveries for BDEs 99, 100, 154, 209, and DBDPE were $115 \pm 6\%$, $106 \pm 11\%$, $103 \pm 15\%$, $81 \pm 9\%$, and $49 \pm 23\%$, respectively.

The buffer blanks had trace levels of BDEs 47, 99, and/or 100 that were below the MLOQ (signal-to-noise ratio of 10). Some of the microsome blanks had trace levels of BDEs 47, 99, 100, 153, and/or 154, but most were below the MLOQ, and the highest level (0.12 nM) was still 260-fold less than the concentration of the spike in the assay samples and controls.

With respect to quality assurance and quality control (QA/QC) for PBDE analysis, PBDE concentrations in repeated analyses of the National Institute of Standards and Technology (NIST) pilot whale blubber, standard reference material (SRM)1945 in our lab showed percent relative standard deviation to be $14 \pm 7\%$ of the NIST certified values. The OCRL at NWRC has also achieved similarly high accuracy and precision for PBDEs in analysis of SRM 1945 as part of the 2007 NIST/National Oceanic and Atmospheric Administration Interlaboratory Comparison Exercise Program for Organic Contaminants in Marine Mammal Tissues, and of fish homogenate matrices as part of a 2009 interlaboratory exercise of PBDEs and other BFRs for the Canadian Northern Contaminants Program (NCP). Interlaboratory QA/QC programs, to our knowledge, do not currently exist for DBDPE.

Data analysis

Extent of depletion was reported as the fraction of the BFR remaining in the samples compared to the controls after the assay (the unmetabolized fraction), using internal standard normalization [24]:

$$\text{fraction remaining} = \frac{\text{ratio}_{\text{BDE153}}(\text{sample})}{\text{ratio}_{\text{BDE153}}(\text{control})}$$

where $\text{ratio}_{\text{BDE153}}$ is the ratio of the peak area of the BFR of interest to the peak area of BDE153 in a given chromatogram. This calculation inherently corrects for any variation in recoveries between samples. Statistical significance of the depletion was considered by comparison of the sample and control groups using Student's *t* test. By considering the average unmetabolized fraction values of all specimens as a group, the difference in the extent of depletion that occurred in the individual assay versus the mixed assay for each compound was compared using a paired *t* test. Again considering all specimens together, differences in the extent of depletion among the different BFRs were tested using analysis of variance (ANOVA) (followed by post hoc Tukey's honestly significantly different [HSD]). All tests were two tailed with results considered significantly different at $p \leq 0.05$ using Statistica (v. 6.0, StatSoft, 2003).

RESULTS AND DISCUSSION

Microsomal enzyme viability

The EROD activity among the arctic marine wildlife microsomes was highly variable, from 200 to 2,200 pmol/mg/min turnover rate (Table 2). For all specimens, these activities fell within the expected ranges for the individual species [24,32,33], indicating viability for use in the depletion assays. Elevated EROD activity of PB1 was in good agreement with higher CYP protein expression [32], higher levels of OH-PCB and $MeSO_2$-PCB and -DDE metabolites [19], and simpler PCB congener patterns [20] in polar bears relative to beluga whales and ringed seals. In addition to interspecies differences, large variation in EROD activity between the specimens may also have been related to sex, age, health status, differential exposure to enzyme-inducing chemicals, time span between death and liver sampling, and length of time the liver tissues were in long-term storage prior to microsome extraction and analysis [26].

Table 2. Polar bear (PB), beluga whale (BW), ringed seal (RS), and rat (RAT) liver microsomal protein content and ethoxyresorufin-*O*-deethylase (EROD) activity

Sample ID	Microsomal yield[a] (mg protein/g tissue)	EROD[a] (pmol/mg protein/min)
PB1	15 (2)	2167 (99)
BW1	7.3 (0.3)	694 (41)
BW2	9.0 (0.3)	309 (6)
RS1	18 (1)	397 (15)
RS2	19.9 (0.4)	199 (10)
RAT	NA[b]	120

[a] Mean (± range) of interday duplicate assays ($n = 3$ replicates/assay), except RS2 microsomal yield is mean (±SD) of single day replicates and RAT EROD data is from microsome provider (BD Gentest).
[b] Not available.

In vitro depletion of BFRs

Generally, the polar bear (PB), beluga whale (BW), ringed seal (RS), and rat (RAT) microsomes substantially depleted the two fully brominated BFRs, BDE209, and particularly DBDPE, within the limited time frame of the assay (Fig. 2). In the BFR mixture assays, between 6 to 17% of BDE209 was depleted and between 27 to 59% of DBDPE was depleted. In the single BFR assays, BDE209 was depleted between 14 to 25% and DBDPE between 44 to 74%. A single published study on DBDPE metabolism also found clear evidence of its metabolism in dosed rats [34]. Evidence of substantial BDE209 metabolism is more extensive, both in vitro and in vivo in mammals, including humans, and fish and birds. Rapid metabolism of hexabromobenzene has also been documented in a rat toxicokinetic dosing study [35]. Taken together, these findings suggest that such metabolism might be characteristic of fully brominated aromatic compounds.

Considering all specimens together, significant differences in the extent of depletion between BFRs occurred in both the mixture and individual assays ($p < 0.00001$). Greater depletion of DBDPE than all PBDEs including BDE209 drove the differences in both the mixture assays and the individual assays, except that greater depletion of BDE209 than the lower brominated PBDEs also reached statistical significance in the individual assays (probably due to greater n). Greater depletion of DBDPE compared to BDE209 may be related to the ethane linkage in DBDPE versus the ether linkage in BDE209 (Fig. 1). The saturated bonds of the DBDPE ethane linkage and the lack of unsaturated conjugation result in a low energetic barrier to rotation around these carbon–carbon bonds, whereas the ether linkage of BDE209 is more energetically constrained and the molecular structure more rigid. Such molecular flexibility for DBDPE may permit structural orientations that better facilitate enzyme-mediated metabolism. Further investigation of the comparative metabolic capacity towards these two BFRs, the mechanisms of their biotransformation, and study of their metabolites in experimental animals is warranted, particularly in light of their large, and in the case of DBDPE likely growing, production volumes [9].

Consistent with the current findings, much more extensive metabolism of BDE209 [25,36] than BDE47, BDE99, BDE100, BDE153, and BDE154 [28,37–41] has been observed in rat dosing studies. In contrast, no detectable depletion of BDE209 was observed with phenobarbital-treated (CYP2B-induced) rat microsomes [29], archived polar bear microsomes [21], and various marine mammal microsomes [42] incubated with only NADPH as cofactor, suggesting the possibility that CYPs may not be involved at least in the initial biotransformation step of BDE209. In vitro microsomal (dependent on DTT but not NADPH) and in vivo studies in fish found substantial debromination of BDE209 in carp and, to a much lesser extent, rainbow trout [6]. Taken together with the current findings, these results suggest that the metabolism of BDE209 and possibly DBDPE may be highly species-specific and/or possibly mechanistically different than that of the lower brominated compounds. Supporting this, the extent of BDE209 depletion was not statistically distinguishable between the individual and the mixed assays, although it was just significant ($p = 0.05$) for DBDPE. This finding may suggest that BDE209 and the lower brominated PBDEs are not substrates for the same isoenzyme.

This substantial depletion of BDE209 relative to the lower brominated PBDEs has implications for the understanding of

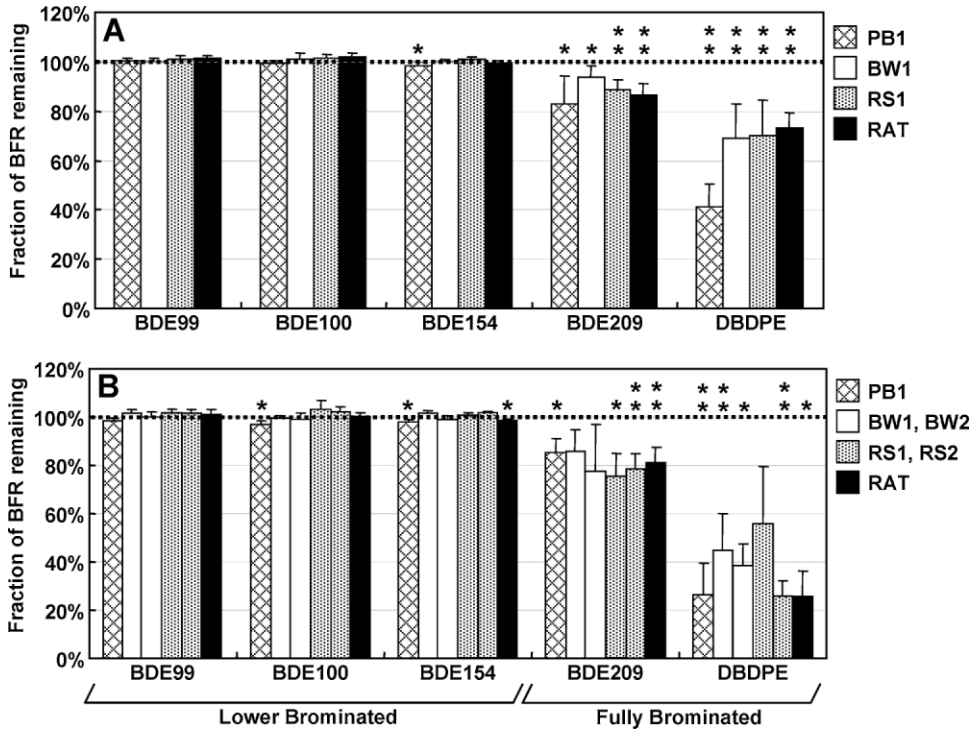

Fig. 2. Percent fraction of brominated flame retardant (BFR) concentrations, dosed as a mixture (**A**) or dosed as individual chemicals (**B**), remaining after 90 min incubation with hepatic microsomes (with dithiothreitol [DTT] and nicotinamide adenine dinucleotide phosphate [NADPH] added) from individual polar bear (PB1; cross-hatched bars), beluga whale (BW1 and BW2; white bars), and ringed seal (RS1 and RS2; dotted bars) and pooled ($n = 17$) rat (RAT; black bars). Error bars represent ± standard deviation (SD) for the mixed BFR ($n = 4$ sample and control replicates) and individual BFR ($n = 3$ replicates) assays. The heavy dotted line denotes no depletion. Significant depletion at the 95% ($p \leq 0.05$) and the 99% ($p \leq 0.01$) confidence levels are indicated by single and double asterisks, respectively.

BDE209 contamination in the environment. The extent of wildlife, and likely human, exposure to BDE209 may not be fully realized by measurement of tissue levels of the parent compound, which may be very low and highly affected by metabolism/degradation, thus leading to underestimation of the ecosystem burden of total-BDE209 (that is, BDE209 and its transformation products). As Ross et al. [43] have pointed out, increasing and substantial abiotic reservoirs of BDE209, despite possibly lower bioavailability than for other PBDEs, represent a serious concern for marine ecosystems. The same case may be made for DBDPE, given its obvious similarities to BDE209, the present results demonstrating depletion, and the apparently increasing use of DBDPE as a replacement for BDE209.

The in vitro assay results indicated less depletion, either oxidatively or by reductive debromination, of the lower brominated, environmentally relevant BDE99, BDE100, and BDE154, in the studied species in comparison to the highly brominated BFRs. For PB1, 1% of BDE154 was depleted in the BFR mixture, and 2%, 3%, and a marginally significant 1% of BDE154, BDE100, and BDE99, respectively, were depleted in the individual BFR assays. In addition, 2% of BDE154 was depleted by RAT in the single BFR assay. Slow but significant depletion of BDE154 was also observed in the archived polar bear study [21]. The BW and RS microsomes did not significantly deplete any of the lower brominated PBDEs. This is in agreement with previous NADPH-dependent microsomal assays showing nondetectable penta- and hexa-BDE depletion in beluga whale, archived polar bear, and various other marine mammal microsomes [21,24,42]. Studies on other species using different experimental designs have nonetheless demonstrated varying congener-specific PBDE biotransformation results. Dosed rats metabolized BDE47, 99, 100, and 154, but did not appear to metabolize BDE153 [28,36,37,39–41,44]. Oxidation and oxidative debromination were predominant biotransformation pathways, the first step likely CYP-mediated arene epoxide formation [38]. Certain fish species appear to rapidly metabolize not only BDE209 [6], but also lower brominated PBDEs. For instance, carp liver microsomes completely debrominated BDE99 to BDE47 within 60 min [45], and the transformation was dependent on the presence of DTT but not NADPH, suggesting the involvement of enzymes other than CYPs, possibly iodothyronine deiodinases. Metabolism of BDE99 was very slow in Chinook salmon hepatic microsomes, however, and BDE49 rather than BDE47 was the observed metabolite [46].

Because of the low number of available individuals from each species, it was not possible to quantitatively assess interspecies differences in metabolic capacity towards the various BFRs. Nonetheless, the present results indicated that all studied species were able to considerably deplete BDE209 and DBDPE relative to the lower brominated PBDEs. Differences in the rate of metabolism may also exist among species, with a tendency for slower transformation in beluga whale, and faster transformation in rat and polar bear. This conclusion is tentative based on the small dataset and other potentially confounding variables (e.g., sex, age, tissue preservation), but is consistent with what is known of these species' metabolic capacities towards other xenobiotics. Differences in biotransformation potential towards these BFRs may be related to differences in induced or constitutive enzyme expression and activity between species, as well as variation in substrate-specificity of particular enzymes among species. At least for CYP isozymes, it is well documented that such differences lead to variation in biotransformation potential towards various xenobiotics, which results in differences in levels and patterns of parent compounds as well as metabolites (for example, for PCBs [47]).

Formation of metabolites

The PB, BW, and RS microsomes were prepared to preserve, and the biotransformation assays were designed to optimize, multiple enzyme systems. This approach was used to hopefully allow for both reductive and oxidative pathways to be substantial enough to observe BFR metabolism in vitro. This was particularly critical to facilitate the metabolism of fully brominated BFRs, wherein a CYP-mediated direct hydroxy group insertion or arene-epoxide formation on a fully halogen-substituted aromatic as an initial metabolic step seems unlikely [36]. Initially, the present study focused on possible debrominated metabolites, given their apparently greater toxicity and environmental persistence, at least in the case of PBDEs [2]. However, no clear evidence for formation of any debrominated PBDEs or debrominated DBDPE metabolites was observed (see Fig. 3 for BDE154, BDE209, and DBDPE metabolism in PB1; chromatograms not shown for BDE99 and BDE100). For all BFRs studied, no peaks indicative of metabolites present in the sample chromatograms were observed (Fig. 3; third chromatogram in each series) that were not also present in the control chromatograms (Fig. 3; fourth chromatogram in each series) at similar concentrations. Nonetheless, all three nona-BDEs (BDE206, BDE207, and BDE208) appeared in the BDE209 chromatograms, as well as two likely nona-BDPEs with retention times between 17.6 to 18.0 min in the DBDPE chromatograms. No peaks were detected in the range expected for octa- or hepta-BDE or -BDPE metabolites. The debrominated peaks observed may be present due to minor contamination of the original standard, breakdown of these BFRs in the GC injection port during analysis of the extracts, and/or precursor BFR metabolism in the assays. Metabolism alone cannot fully explain these findings, because these peaks were also found in the controls. If they were present solely due to degradation in the injection port, their response would likely be proportional to the response of the BDE209 (or DBDPE). However, as is particularly noticeable in the case of DBDPE, wherein generally greater than half of the original concentration was metabolized, the response of the first eluting apparent nona-BDPE in the sample extracts was still similar to that in the controls (Fig. 3C). Thus, no definitive conclusion can be reached, but these observations suggest that both slight contamination of the original standard and metabolic debromination in the assays may explain the presence of nona-BDPEs and -BDEs in the case of DBDPE and possibly BDE209, respectively.

Regardless, given the apparent lack of substantial debrominated metabolites which would account for the extensive depletion of DBDPE and to a lesser extent BDE209, attempts to identify metabolites were expanded by derivatizing the extracts from some individuals, PB1 and RAT, to examine phenolic and/or phenolic debrominated metabolite formation. No derivatized phenolic peaks were observed for BDE209 (Fig. 3B, first and second chromatograms in the series), BDE99, or BDE100 (data not shown).

Despite limited depletion of BDE154, a single derivatized phenolic peak was observed in both PB1 (Fig. 3A, first chromatogram in the series) and RAT (data not shown) samples, that was not detectable in the controls (Fig. 3A, second chromatogram in the series). Although it did not match the retention time of any of the MeO-PBDE congeners in the standard, it was in the retention time range expected for a MeO-hexaBDE (deriv-

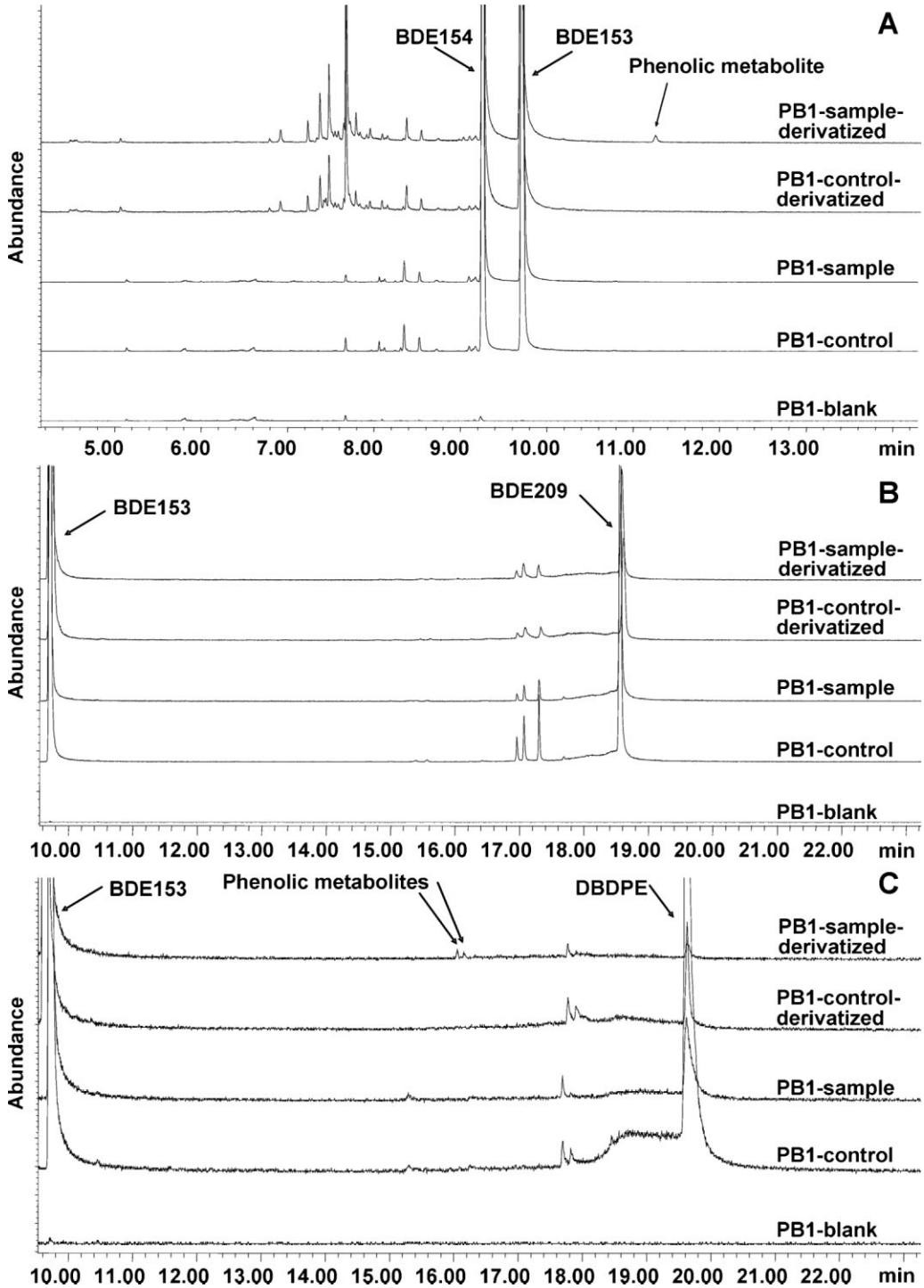

Fig. 3. Representative electron capture negative ionization, selected ion monitoring ($[Br]^-$, *m/z* 79) mass chromatograms of extracts from an in vitro biotransformation assay using polar bear (PB1) hepatic microsomes incubated with individual brominated flame retardants (BFRs): (**A**) BDE154, (**B**) BDE209, and (**C**) DBDPE. Control microsomes were inactivated by MeOH treatment prior to the assay, whereas sample microsomes were viable and were incubated with the cofactors nicotinamide adenine dinucleotide phosphate (NADPH) and dithiothreitol. Subsequent to initial screening for parent compound and possible neutral (debrominated) metabolites, controls and samples were derivatized to detect possible phenolic metabolites as their methoxylated analogs. Replicate control and sample *m/z* 79 chromatograms as well as all *m/z* 81 chromatograms gave the same results.

atized OH-hexa-BDE). Based on the response of the single hexa-brominated congener in the standard, 2′-MeO-BDE123, the concentration of this metabolite after 90 min was 0.5 ± 0.1 nM in PB1. Given that 2% of BDE154 (30 nM original concentration) was transformed by PB1, nearly all of the BDE154 metabolized was accounted for by this single metabolite. The concentration of this metabolite was 0.11 ± 0.01 nM in the RAT, accounting for around one-fifth of the portion of metabolized BDE154. This phenolic metabolite eluted after BDE154, suggesting either no concomitant debromination of BDE154 in this transformation pathway or possibly debromination with multiple hydroxylation. Based on monitoring of the $[Br]^-$ anions only, the metabolite could be singly or multiply hydroxylated. Mono-OH-tetra- to hexa-BDEs, as well as

di-OH-penta- and hexa-BDEs were found in BDE154-dosed rats, yet high amounts of nonextractable metabolites were also noted [41].

In PB1, but not in RAT, two phenolic metabolites, likely multiply debrominated based on much earlier elution than DBDPE, were detected in the derivatized DBDPE samples (Fig. 3C, first chromatogram in the series) that were not detected in the derivatized controls (Fig. 3C, second chromatogram in the series). These metabolites were below the estimated MLOQ of 8 nM for a BDPE-derivative. However, if generously assumed to each be at the MLOQ of 8 nM, these metabolites would still only account for around one-fifth of the 74% of the DBDPE depleted by PB1 microsomes. An earlier rat dosing study found seven DBDPE metabolites, which also did not appear to be formed from reductive debromination [34].

We did not detect debrominated and/or phenolic metabolites of BDE209, and could only account for a limited fraction of the largely depleted DBDPE by the detection of two phenolic metabolites. Previous BDE209 rat dosing studies identified traces of octa- and nona-BDEs and 13 debrominated phenolic metabolites, including an OH-octa-BDE, an OH-nona-BDE, and an OH-MeO-hexa-BDE [25,36,48]. Nonetheless, these studies also found that despite extensive metabolism the majority of the original concentration could not be accounted for by the identified metabolites. Hakk et al. [40] noted that a characteristic of PBDE metabolism is the large proportion of nonextractable metabolites. This was also the case for another BFR, 1,2-*bis*(2,4,6-tribromophenoxy)ethane (BTBPE), in dosed rats [49]. Our results may be interpreted similarly or in combination with other possible explanations. For example, several metabolites could have formed at concentrations below the limit of detection, as a large number of debrominated/phenolic metabolites could be formed from BDE209 or DBDPE. As well, highly brominated phenolic metabolites may not have been effectively derivatized by the diazomethane treatment. Another alternative is low recovery of unknown major metabolites, such as conjugated, water-soluble metabolites that would have remained in the aqueous phase on extraction with hexane. Substantial conjugated metabolite formation is unlikely, but nonetheless possible because the microsomal fraction could contain residual cofactors for phase II reactions, for example, glutathione (GSH) or glucuronic acid. From a toxicological perspective, the first-mentioned explanation of high rates of formation of nonextractable metabolites may be a cause for concern, because they imply covalent-binding of reactive metabolites to macromolecules (proteins and/or lipids) [36,40]. Future studies should perhaps use radiolabeled BDE209 and DBDPE to improve the ability to track loss of the parent compound.

Overall, this study found substantial depletion of the fully brominated BDE209, and especially DBDPE, in comparison to the lower brominated PBDEs across a range of Arctic marine-feeding mammals. Given the large environmental reservoirs of BDE209 and possibly DBDPE, and their apparently susceptibility to biotransformation, it is possible that despite low to moderate bioavailability, wildlife may be more or less continuously exposed to these BFRs. Our findings indicate that for highly brominated BFRs, further research into their environmental fate, abiotic and metabolic transformation products, and interactions with biomacromolecules is required to understand their environmental risks.

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