9. Hardy ML, Banasik M, Stedeford T. 2009. Toxicology and human health assessment of decabromodiphenyl ether. *Crit Rev Toxicol* 39(S3):1–44.
10. Hardy ML, Margitich D, Ackerman L, Smith RL. 2002. The subchronic oral toxicity of ethane, 1,2-bis(pentabromophenyl) (Saytex 8010) in rats. *Int J Toxicol* 21:165–170.
11. Hardy ML, Mercieca MD, Rodwell DE, Stedeford T. 2010. Prenatal developmental toxicity of decabromodiphenyl ethane in the rat and rabbit. *Birth Defects Res B Dev Reprod Toxicol* 89:139–146.
12. Biesemeier JA, Beck MJ, Silberberg H, Myers NR, Ariano JM, Radovsky A, Freshwater L, Sved DW, Jacobi S, Stump DG, Hardy ML, Stedeford T. 2011. An oral developmental neurotoxicity study of decabromodiphenyl ether (DecaBDE) in Rats. *Birth Defects Res B Dev Reprod Toxicol* 92:17–35.

DOI: 10.1002/etc.1845

The authors' reply:

We respond to a commentary submitted by Hardy [1] concerning our *Environmental Toxicology and Chemistry* article, "Comparative Hepatic Microsomal Biotransformation of Selected Polybrominated Diphenyl Ethers (PBDEs), Including Decabromodiphenyl Ether, and Decabromodiphenyl Ethane Flame Retardants in Arctic-Marine Feeding Mammals" [2]. We thank her for the commentary, but presently discuss several aspects of the comments that are not fully correct and may mislead readers.

Hardy [1] states that our studies [2] showed that "in vitro hepatic microsomal preparations from polar bear (*Ursus maritimus*), beluga whale (*Delphinapterus leucas*), ringed seal (*Pusa hispida*), and rat did not metabolize decabromodiphenyl ethane (DBDPEthane), decabromodiphenyl ether (BDE-209), or the lower brominated polybrominated diphenyl ethers (PBDEs) BDE-99, -100 and -154." This is a partially inaccurate interpretation of our study results. In fact, and as we clearly reported, we did observe the depletion of BDE-209 (14–25% of 30 pmol) and DBDPEthane (44–74% of 90 pmol) in assays for individuals from all species relative to controls, but no depletion of the lower brominated BDE-99, -100 and -154 (0–3% of 30 pmol).

Hardy is incorrect in her interpretation that our in vitro results for BDE-209 in McKinney et al. [2] is consistent with the lack of depletion results for similar hepatic microsomal assays with BDE-209 for polar bear as reported in Gebbink et al. [3]. First, only partial details of the latter study were published, but were subsequently published in full in Letcher et al. [4]. In fact, the in vitro depletion results for BDE-209 reported in McKinney et al. [2] and Letcher et al. [4] were in contrast and expected to be different as a consequence of the enzyme activity of the microsomes used. In the earlier study [3,4], hepatic microsomes were from the liver of Canadian polar bears that had been collected between 1992 and 1994 near Resolute Bay, Northwest Territories (Canada) and had been stored continuously for more than 15 years at $< -80^{\circ}$C. At the time of these assays [3,4], a re-analysis of the ethoxyresorufin-*O*-deethylase (EROD) catalytic activity (representative of general cytochrome P450 monooxygenase activity) showed that the activity rate was 626 pmol/min/mg protein. In contrast, in McKinney et al. [2] we had access to extremely fresh (<60 min postmortem) liver specimens that were collected from a stranded polar bear from Iceland, and microsomes were prepared and used within weeks for the in vitro BDE-209 (and other BFR) assays. The liver microsomes for either study were prepared the same, with the exception, as detailed in McKinney et al. [2], that dithiothreitol (DTT; Cleland's reagent) was added to the microsomal buffer to preserve reductase and deiodinase enzyme activities and were added to the in vitro assay incubations for the same reason. Dithiothreitol is a nonnative cofactor for reductases and deiodinases. Regardless, the prepared microsomes used in McKinney et al. [2] were very catalytically active with EROD activity of 2,167 pmol/min/mg protein. This explains, in fact, why no in vitro depletion of BDE-209 was observed earlier for polar bears [3,4], and substantial depletion was observed as reported in McKinney et al. [2]. That is, EROD activity suggested that the catalytic rate of any BDE-209 metabolic depletion would be at least four times slower for microsomes used for the earlier [3,4] assays as compared to those in McKinney et al. [2]. For the earlier assays [3,4], the catalytic activity was far too low, and thus the catalytic rate was too slow to measure any BDE-209 depletion in the timeframe of the assay.

Hardy [1] points out that Albemarle Corporation has extensive experience with BDE-209 and DBDPEthane, and that both are highly insoluble in aqueous media and many organic solvents and prone to nonspecific binding to surfaces and particulates. Any such nonspecific binding loss in a given incubation vessel would render applicable analytes as unavailable substrates for any active (microsomal) enzymes suspended in solution. We fully agree with this opinion that non-specific surface losses of BDE-209 and DBDPEthane can occur. However, Hardy [1] goes on to state that from our study [2] "the low and variable recoveries of BDE-209 ($81 \pm 9\%$) and DBDPEthane ($49 \pm 23\%$) in controls and the 'depletion' observed in the test groups, 14 to 25% for BDE-209 and 44 to 74% for DBDPEthane, in the absence of identified metabolites suggest that other factors may be responsible." It is implied that our lower recoveries are consistent with non-specific losses of BDE-209 and DBDPEthane during the in vitro incubation and accounts for the "depletion" we observed in our assays.

Although we agree that non-specific losses occurred during the in vitro assays, our reported BDE-209 and DBDPEthane depletions are, in fact, clearly due to enzyme-mediated processes for the following reasons. First, we used BDE-153 as an internal standard during the in vitro assay incubations as we demonstrated that there was negligible metabolic depletion of BDE-153 under the assay conditions used. That is, there was no statistical difference ($p > 0.10$) for the lack of BDE-153 depletion between sample and control replicates for each individual animal. Second, PBDE, BDE-209 and DBDPEthane recoveries from the assays were calculated by comparing the analyte response in catalytically active assays versus non-catalytically active control assays. Thus, the 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. This approach and calculation inherently corrected for any variation in recoveries between samples.

Hardy [1] also comments that we [2] erroneously report our assay concentrations to be "pmol," without any explanation as to how she reached her conclusion. We would like to reiterate

what has already been stated, and correctly, in our paper. We noted that the PBDEs were prepared in acetone at a concentration of 10 μM. Then, 3μL of this 10 μM standard (i.e., 30 pmol) was spiked into the assay solution of final volume 1 μL, for a final amount of 30 pmol added to the assay solution, or restated, 30 nM (or in the case of DBDPE, 90 nM).

We discussed that apparent lack of detectable BDE-209 or DBDPEthane metabolites (e.g., debrominated or hydroxylated) after the in vitro assay as being due to a number to explanations, but in the context of perhaps being non-extractable and unavailable due to chemical binding (reactions) [2]. For example, unknown major metabolites, such as conjugated, water-soluble metabolites would have remained in the aqueous phase on extraction with hexane. Hakk et al. [5] noted that a characteristic of PBDE metabolism is the large proportion of non-extractable metabolites. Hardy noted [2] that formation of "reactive" metabolites is inconsistent with multiple mammalian repeated dose toxicity studies with no observable effects limits (NOELs) and no observable adverse effect levels (NOAELs) of $\geq$ 1,000 mg/kg/d for both BDE-209 and DBDPEthane. We agree that we hypothetically (and perhaps too speculatively) noted that formation of non-extractable metabolites might be a cause for toxicological concern, because they imply covalent-binding of reactive metabolites to macromolecules (proteins and/or lipids).

We agree with Hardy [1], and as we made clear in our paper [2], that our conclusions that BDE-209 undergoes significant metabolism were based on extraction profiles of the BDE-209 and not on structural identification of actual metabolites. We discussed in our paper that future studies should perhaps use radiolabeled BDE-209 and DBDPEthane to improve the ability to track loss of the parent compound in, for example, in vitro assay such as we used. We note for future reference such experiments have been performed [6] or are in progress by Albemarle Corporation.

Robert J. Letcher
Melissa A. McKinney
Environment Canada
Wildlife Research Centre, Ottawa, Ontario

REFERENCES

1. Hardy ML. 2012. Unrecognized causative factors for the lack of in vitro metabolism reported by McKinney et al. *Environ Toxicol Chem* 31:1184–1185 (this issue).
2. McKinney MA, Dietz R, Sonne C, De Guise S, Skirnisson K, Karlsson K, Steingrímsson E, Letcher RJ. 2011. Comparative hepatic microsomal biotransformation of selected polybrominated diphenyl ether, including decabromodiphenyl ether, and decabromodiphenyl ethane flame retardants in arctic marine-feeding mammals. *Environ Toxicol Chem* 30:1506–1514.
3. Gebbink WA, Sonne C, Kirkegaard M, Riget FE, Born EW, Letcher RJ. 2006. Chlorinated and brominated contaminants and their metabolic/degradation products: Bioaccumulation and distribution in East Greenland polar bears (*Ursus maritimus*) and ringed seals (*Phoca hispida*). *Organohalogen Compd* 68:336–339.
4. Letcher RJ, Gebbink WA, Sonne C, Dietz R, McKinney MA, Born EW. 2009. Bioaccumulation and biotransformation of brominated and chlorinated contaminants and their metabolites in ringed seals (*Pusa hispida*) and polar bears (*Ursus maritimus*) from East Greenland. *Environ Internat* 35:1118–1124.
5. Hakk H, Huwe J, Low M, Rutherford D, Larsen G. 2006. Tissue disposition, excretion and metabolism of 2,2′,4,4′,6-pentabromodiphenyl ether (BDE-100) in male Sprague-Dawley rats. *Xenobiotica* 36:79–94.
6. Hardy ML, Banasik M, Stedeford T. 2009. Toxicology and human health assessment of decabromodiphenyl ether. *Crit Rev Toxicol* 39(S3):1–44.

DOI: 10.1002/etc.1846