Biotransformation of polybrominated diphenyl ethers and polychlorinated biphenyls in beluga whale (Delphinapterus leucas) and rat mammalian model using an in vitro hepatic microsomal assay

Melissa A. McKinney a, Sylvain De Guise b, Daniel Martineau c, Pierre Bélènd d, Augustine Arukwe e, Robert J. Letcher a,∗

a Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ont., Canada N9B 3P4
b Department of Pathobiology, University of Connecticut, Storrs, CT 06269, USA
c Département de Microbiologie et Pathologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Que., Canada J2S 7C6
d St. Lawrence National Institute of Ecotoxicology, 5040 Mentana, Montréal, Que., Canada H2J 3C3
e Department of Biology, Norwegian University of Science and Technology (NTNU), Høgskoleringen 5, 7491 Trondheim, Norway

Received 13 July 2005; accepted 30 August 2005

Abstract

Although polychlorinated biphenyls (PCBs) and polybrominated diphenyl ether (PBDE) flame retardants are important organic contaminants in the tissues of marine mammals, including those species from the Arctic, there is exceedingly little direct evidence on congener-specific biotransformation. We determined and compared the in vitro metabolism of environmentally relevant PCB (4,4′-di-CB15, 2,3′,5-tri-CB26, 2,4,5-tri-CB31, 2,2′,5,5′-tetra-CB52, 3,3′,4,4′-tetra-CB77, 2,2′,3,5,5′-penta-CB101, 2,3,3′,4,4′-penta-CB105 and 2,3,4,4′,5-penta-CB118), and PBDE (4,4′-di-BDE15, 2,4,4′-tri-BDE28, 2,2′,4,4′-tetra-BDE47, 2,2′,4,5,5′-pentra-BDE49, 2,2′,4,4′,5,6-hexa-BDE99, 2,2′,4,4′,5,5′,6-hexa-BDE100, 2,2′,4,5,5′,6-hexa-BDE153, 2,2′,3,4,4′,5,6-hepta-BDE154 and 2,2′,3,4,4′,5,6,7-octa-BDE183) congeners using hepatic microsomes of a beluga whale (Delphinapterus leucas) from the Arviat (western Hudson Bay) area of the Canadian Arctic. Ortho–meta bromine-unsubstituted BDE15, BDE28 and BDE47 were significantly metabolized (100%, 11% and 5% depleted, respectively) by beluga, whereas control rat microsomes (from pooled male Wistar Han rats) metabolized BDE28, BDE49, BDE99 and BDE154 (13%, 44%, 11% and 17% depleted, respectively). CB15 and CB77 (putative CYP1A substrates) were more rapidly metabolized (100% and 93% depleted, respectively) by male beluga than CB26 and CB31 (CYP1A/CYP2B-like) (25% and 29% depleted, respectively), which were more rapidly metabolized than CB52 (CYP1B1-like) (13% depleted). Higher chlorinated CB101 and CB105 showed no depletion. Rat control microsomes metabolized CB101 to a lesser extent (32% depleted) than beluga, but much more rapidly transformed CB52 (51% depleted, respectively). Within the 90 min in vitro assay time frame, the preference was towards metabolism of ortho–meta unsubstituted congeners (for both PCBs and PBDEs) in beluga whale, whereas for rat controls, meta–para unsubstituted congeners also substantially metabolized. For both beluga whale and rat, metabolic rates were inversely associated with the degree of halogenation. For the rapidly biotransformed CB15 and BDE15, water-soluble OH-metabolites were detected after incubation. These results indicate that CYP-mediated oxidative hepatic biotransformation is a metabolic pathway in the toxicokinetics of both PCB and PBDE congeners in beluga whales and in the rat model. This may suggest that the formation of potentially toxic oxidative PCB and PBDE products (metabolites), in addition to the parent pollutants, may be contributing to contaminant-related stress effects on the health of beluga whale.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Beluga whale (Delphinapterus leucas); In vitro biotransformation; Hepatic microsomes; Polychlorinated biphenyls; Polybrominated diphenyl ethers

1. Introduction

Cetaceans are exposed to persistent, bioaccumulative and potentially toxic substances through their diets, which they accumulate in lipid-rich tissues (blubber) throughout their long life spans (Houde et al., 2005). Currently used as flame retardant additives in polymeric materials, polybrominated diphenyl ethers (PBDEs) have been reported in blubber, and to a lesser
extent in hepatic, tissue of a limited number of marine mammal species and populations (Andersson and Wattanlan, 1992; Haglund et al., 1997; de Boer et al., 1998; Boon et al., 2002; Ikonomou et al., 2002a,b; Law et al., 2002; She et al., 2002; Wolters et al., 2004; Houde et al., 2005). In odontocetes cetaceans, PBDEs have been reported in the blubber and/or liver tissues of pilot whales (Lindstrom et al., 1999), killer whales (Rayne et al., 2004), and beluga whales from the St. Lawrence Estuary (Lebeuf et al., 2004; McKinney et al., 2006) and various Arctic regions (Law et al., 2002; McKinney et al., 2006; Wolters et al., 2004). PBDEs have also been reported in dolphin species from the Mediterranean Sea (Pettersson et al., 2004) and in deep-sea inhabiting, Atlantic sperm whales (de Boer et al., 1998). In these reports, BDE47 was generally found to be the dominant congener, with lesser amounts of BDE99, BDE100, BDE153, and BDE154.

Great Lakes and Pacific coast fish (Ikonomou et al., 2002a; Law et al., 2003), and beluga whales (both those inhabiting the St. Lawrence Estuary and the Canadian Arctic regions) show temporally increasing PBDE levels (Stern and Ikonomou, 2000; Law et al., 2003; Lebeuf et al., 2004). PBDE concentrations in tissue (liver and blubber) of beluga whales are lower, but at increasingly comparable levels relative to PCBs and organochlorine (OC) pesticides (Metcalfe et al., 1999; Letcher et al., 2000a; Hobbs et al., 2003; McKinney et al., 2006). Multiple pathologies exhibited in the threatened (COSEWIC, 2004) St. Lawrence beluga whale population have been associated with PCB and OC exposure (De Guise et al., 1995), however PBDE exposure may be contributing to these contaminant-related effects associations.

Phase I cytochrome P450 (CYP) and phase II conjugative enzymes, mainly operating in the liver, catalyze the metabolism of many endogenous and xenobiotic compounds in organisms including marine mammals (Goldsworthy and Forlin, 1992; Stegeman and Hahn, 1994; Lewis et al., 1998). A variety of CYPs, as well as epoxide hydrolase (EH) and UDP glucuronosyl transferase (UDPGT) enzymes, that have been found in beluga whale (White et al., 1994, 2000; McKinney et al., 2004), may influence the capacity of these cetaceans to detoxify and eliminate contaminants. As well, these catalytic processes can result in the formation of toxic, retained and/or persistent metabolites, e.g. methyl sulfonil- (MeSO2- ) and hydroxylated- (OH-) PCBs in the liver and/or adipose tissues (Letcher et al., 2000a; McKinney et al., 2006).

In general, the toxicokinetics of PBDEs and other BFRs of environmental importance is not nearly as well understood as that of PCBs in wildlife and in particular in marine mammals (Hakk and Letcher, 2003). Still, possible metabolites of PBDEs, the OH-PBDEs, have been found in the liver tissues of beluga whales (McKinney et al., 2006), in the blood plasma of a killer whale (Bennett et al., 2002), and in some fish and bird species (Asplund et al., 1999; Marsh et al., 2004; Valters et al., 2005). In addition, MeO-PBDEs have been reported in ringed seal, beluga whale and fish (Haglund et al., 1997; Asplund et al., 1999; Marsh et al., 2004; Wolters et al., 2004). Both OH-PBDEs and MeO-PBDEs may be of metabolic or natural origins (Malmvåm et al., 2005; Teuten et al., 2005).

Direct evidence of PBDE metabolism to OH-PBDE metabolites was reported in laboratory rodents after oral administration of BDE47, BDE99, BDE100 and BDE209 (Ön and Klasson-Wehler, 1998; Hakk et al., 2002; Morck et al., 2003). Catalytic debromination of higher brominated to lower brominated BDE congeners has also been recently reported. In the gut of diet-exposed common carp (Cyprinus carpio), within 2 h of exposure, debromination of BDE209 to octabromo- and nonabromo-BDE congeners, BDE183 to BDE154, and BDE99 to BDE27 was shown (Stapleton et al., 2004a,b,c). OH-PBDEs have shown estrogenic and thyroidogenic activities in experimental organisms (Meerts et al., 2000, 2001). It has yet to be definitively demonstrated for beluga whale, or any Arctic marine mammal, that OH-PBDE and OH-PCB congener residues found in tissue are formed in whole or part due to the metabolism of PBDEs and PCBs that have accumulated in tissues, as opposed to the direct ingestion of these metabolites.

Ethical rationale generally prevents organohalogen dosing studies in marine mammals, but in vitro assays provide a viable alternative. The contaminant(s) of interest can be incubated with prepared sub-cellular liver fractions and metabolic activity can be examined by monitoring depletion of the parent compound(s) and/or formation of metabolites (Murk et al., 1994; Boon et al., 1998; de Boer et al., 1998; Letcher et al., 1998; White et al., 2000; van Hezik et al., 2001; Li et al., 2003a). In vitro assays will not provide information on the metabolism of slowly transformed congeners, due to the time constraints of the assay. In addition, these assays may not be representative of what actually happens in vivo as, for instance, only one process (in this case, CYP-mediated oxidative biotransformation) can be studied, when there are many processes (e.g. reductive reactions, phase II reactions) that may be involved in the potential biotransformation of a single congener. However, when studying organisms for which dosing is not an ethically sound option, in vitro assays are a useful tool to provide information as to the metabolic potential towards the contaminant of interest. In the current study, we examine and compare the in vitro metabolism of PBDE and PCB congeners of environmental significance in order to determine whether OH-PBDEs and OH-PCBs previously detected in beluga whale (McKinney et al., 2006; Wolters et al., 2004) result from the metabolism of parent PCB and PBDE congeners present in beluga tissues. We address the hypothesis that biotransformation plays a role in the fate and bioaccumulation of PCBs and PBDEs in beluga whale via (1)
CYP-mediated oxidative biotransformation resulting in the formation of OH-PCB and OH-PBDE metabolites, and (2) catalytic debromination of higher brominated to lower brominated PBDE congeners.

2. Materials and methods

Mixed congener assays (MCAs) and individual congener assays (ICAs) were performed to study both PBDE and PCB metabolism (Table 1). PCBs used in the MCA were chosen as representative congeners of PCB metabolic groups, according to the metabolic classification proposed by Boon et al. (1997). As structure–activity relationships for the oxidative metabolism of PBDEs in marine mammals are unknown, the suite of PBDEs in the MCA represented environmentally relevant congeners ranging from di- to hepta-bromodiphenyl ethers. PBDE and PCB congeners that were either (1) significantly biotransformed in the MCA, (2) metabolized in rodent studies (e.g., Orn and Klasson-Wehler, 1998), and/or (3) were putative parent compounds of OH-PCB and OH-PBDE metabolites, and (2) catalytic activity determinations

Table 1

<table>
<thead>
<tr>
<th>Congener</th>
<th>Concentration (µg/ml)</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDE15 (4,4'-di-BDE)</td>
<td>5.3</td>
<td>24</td>
</tr>
<tr>
<td>BDE19 (2,3,5-tri-CB)</td>
<td>5.5</td>
<td>20</td>
</tr>
<tr>
<td>BDE11 (2,4,5-tri-CB)</td>
<td>5.3</td>
<td>20</td>
</tr>
<tr>
<td>BDE20 (2,2,5,5'-tetra-CB)</td>
<td>5.3</td>
<td>18</td>
</tr>
<tr>
<td>BDE21 (2,3,3,4,5,-hexa-CB)</td>
<td>5.3</td>
<td>16</td>
</tr>
<tr>
<td>BDE28 (2,4,4'-tri-CB)</td>
<td>5.3</td>
<td>16</td>
</tr>
<tr>
<td>BDE47 (2,2,4,4'-tetra-BDE)</td>
<td>4.9</td>
<td>10</td>
</tr>
<tr>
<td>BDE53 (2,2,3,4,4'-pentra-BDE)</td>
<td>4.9</td>
<td>8.7</td>
</tr>
<tr>
<td>BDE100 (2,2,4,4'-tetra-BDE)</td>
<td>4.9</td>
<td>10</td>
</tr>
<tr>
<td>BDE101 (2,2,3,4,4'-pentra-BDE)</td>
<td>4.9</td>
<td>7.8</td>
</tr>
<tr>
<td>BDE118 (2,3,3,4,5,5'-hexa-BDE)</td>
<td>5.3</td>
<td>16</td>
</tr>
<tr>
<td>BDE119 (2,2,4,4'-penta-BDE)</td>
<td>5.3</td>
<td>16</td>
</tr>
<tr>
<td>BDE123 (2,2,4,4'-penta-BDE)</td>
<td>5.3</td>
<td>16</td>
</tr>
<tr>
<td>BDE154 (2,2,4,4',5,5'-hexa-BDE)</td>
<td>5.3</td>
<td>16</td>
</tr>
<tr>
<td>BDE155 (2,2,4,4',5,5'-hexa-BDE)</td>
<td>5.3</td>
<td>16</td>
</tr>
</tbody>
</table>

2.1. Sampling, microsome preparation, and protein and catalytic activity determinations

A healthy adult male beluga whale (CA10, McKinney et al., 2004) was caught on 9 August 2003 during a native subsistence hunt near Arviat, Nunavut in western Hudson Bay, Canada. Hepatic tissues were removed within 1 h of death and immediately stored in a liquid N2 dry shipper. Once transported back to the laboratory (GLIER, University of Windsor), the hepatic tissues were stored for the long term at −80 °C. Beluga whale microsome preparation and protein content analysis were based on previously reported methods (Bradford, 1976) and described in McKinney et al. (2004). This beluga whale was selected for in vitro metabolism studies because it showed the most catalytically viable hepatic enzymes of all the whales captured near Arviat in the summers of 2002 and 2003 (Section 3.1). Due to experimental constraints and the fact that CA10 showed qualitatively similar phase I enzyme expression as other Canadian Arctic as well as St. Lawrence belugas (McKinney et al., 2004), biotransformation was investigated in this individual beluga whale. Liver microsomes from pooled male Wistar Han rats were purchased (Gentest, Woburn, MA, USA) and used as a model mammalian system for PCB and PBDE metabolism in vitro.

2.2. Reagents

Stock solutions of individual PBDE congeners were provided by Dr. Åke Bergman (Stockholm University, Sweden) and Wellington Laboratories (Guelph, Ont., Canada). Individual PCB (in isooctane, TMP) congener standards were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). NaH2PO4, H2O2 and MgCl2·6H2O were obtained from Sigma–Aldrich (St. Louis, MO, USA). Disodium ethylenediaminetetraacetate (Na2EDTA) was obtained from Bio-Rad Laboratories (Mississauga, Ont., Canada). NADPH regenerating system Solutions A (NADP+, glucose-6-phosphate, MgCl2) and B (glucose-6-phosphate dehydrogenase) were purchased from Gentest (San Jose, CA, USA). All other reagents and solvents were of analytical grade-quality. Water was supplied by a Milli-Q system (Millipore, San Jose, CA, USA) equipped with a 0.22 µm filter.

2.3. Preparation of PCB and PBDE mixtures

The beluga liver microsomes were incubated with either a mixture of PCBs or a mixture of PBDEs (MCA) or with individual PCB or PBDE congeners (ICA) (Table 1). Every mixed or individual standard contained CB153 as the internal standard because of its negligible metabolic depletion in the assay (van Hezik et al., 2001; Li et al., 2003a). Mixed standards were prepared by combining the required volume of individual standards (including CB153), evaporating the mixture to dryness under a gentle stream of nitrogen and re-dissolving it in analytical grade acetone. Individual standards were prepared in a similar fashion. The congeners in the mixed standard solutions and the individual standard solutions and their concentrations are given in Table 1:

<table>
<thead>
<tr>
<th>Congener</th>
<th>Concentration (µg/ml)</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDE15 (4,4'-di-BDE)</td>
<td>5.3</td>
<td>24</td>
</tr>
<tr>
<td>BDE19 (2,3,5-tri-CB)</td>
<td>5.5</td>
<td>20</td>
</tr>
<tr>
<td>BDE11 (2,4,5-tri-CB)</td>
<td>5.3</td>
<td>20</td>
</tr>
<tr>
<td>BDE20 (2,2,5,5'-tetra-CB)</td>
<td>5.3</td>
<td>18</td>
</tr>
<tr>
<td>BDE21 (2,3,3,4,5,-hexa-CB)</td>
<td>5.3</td>
<td>16</td>
</tr>
<tr>
<td>BDE28 (2,4,4'-tri-CB)</td>
<td>5.3</td>
<td>16</td>
</tr>
<tr>
<td>BDE47 (2,2,4,4'-tetra-BDE)</td>
<td>4.9</td>
<td>10</td>
</tr>
<tr>
<td>BDE53 (2,2,3,4,4'-pentra-BDE)</td>
<td>4.9</td>
<td>8.7</td>
</tr>
<tr>
<td>BDE100 (2,2,4,4'-tetra-BDE)</td>
<td>4.9</td>
<td>10</td>
</tr>
<tr>
<td>BDE101 (2,2,3,4,4'-pentra-BDE)</td>
<td>4.9</td>
<td>7.8</td>
</tr>
<tr>
<td>BDE118 (2,3,3,4,5,5'-hexa-BDE)</td>
<td>5.3</td>
<td>16</td>
</tr>
<tr>
<td>BDE119 (2,2,4,4'-penta-BDE)</td>
<td>5.3</td>
<td>16</td>
</tr>
<tr>
<td>BDE123 (2,2,4,4'-penta-BDE)</td>
<td>5.3</td>
<td>16</td>
</tr>
<tr>
<td>BDE154 (2,2,4,4',5,5'-hexa-BDE)</td>
<td>5.3</td>
<td>16</td>
</tr>
<tr>
<td>BDE155 (2,2,4,4',5,5'-hexa-BDE)</td>
<td>5.3</td>
<td>16</td>
</tr>
</tbody>
</table>
Table 1A and B, respectively. These concentrations represent a compromise between (1) approximating zero-order kinetics and (2) being low enough to detect metabolism (depletion) within the short time frame of the assay.

2.4. In vitro microsomal assay

For each contaminant standard mixture in the MCA, there were eight tubes: for samples with NADPH regenerating system (MCAs) and four controls (without NADPH regenerating system). For the ICA, three samples and three controls were run. Two blanks (containing only buffer and microsomes) were also run with each batch of samples. The microsomal assay followed the same procedure for the MCAs and the ICAs. A mixture (1 ml final volume) of buffer (80 mM NaH2PO4, 6 mM MgCl2, 1 mM Na2EDTA, pH 8.0), the appropriate PCB or PBDE standard (3 µl, thus only 0.3% acetone in the incubation mixture) and microsomal suspension (1 mg protein) were combined on ice. The mixtures were covered with solvent-washed foil and pre-incubated at 37 °C for 5 min. The assay was started by the addition of NADPH regenerating solution (50 µl of Solution A and 10 µl of Solution B) sequentially to all tubes and allowed to proceed for 90 min in a 37 °C, shaking (90rpm) water bath. To control tubes (containing buffer, PCB or PBDE standard and microsomes), 60 µl of buffer was added instead of NADPH.

2.5. Extraction of parent and metabolite compounds

2.5.1. MCA

For the mixed assays, depletion of the parent PCB or PBDE compounds was measured. The reaction was stopped after 90 min by the sequential addition of 1 ml of ice-cold MeOH to all tubes. The extraction procedure was based on previously published methods (Boon et al., 1998; Li et al., 2003a). In short, 90 min by the sequential addition of 1 ml of ice-cold MeOH to all tubes. The extraction procedure was based on previously published methods (Li et al., 2003b; Rozemeijer et al., 1997; White et al., 2000). After addition of the appropriate phenolic ISTDs, tubes were heated at 70 °C for 10 min. Then 2 ml of 6 M HCl and 1 ml of 2-propanol were added, followed by vortexing for 1 min to break down proteins. Next, 2 ml of 1:1 methyl-butyl ether (MIBE): hexanes were added. The tubes were vortexed for 1 min, then centrifuged at 2500 rpm for 5 min. The (upper) organic fraction was collected in a new tube, and the entire procedure was repeated two more times. The organic extract containing both neutral and phenolic contaminants was then concentrated to around 1 ml. A 2 ml aliquot of KOH was added, the tube vortexed, and the (bottom) aqueous phase containing the deprotonated phenolics was transferred to a clean tube. This KOH partitioning step was repeated three times. The KOH/aqueous (phenolic) fraction was rinsed with 3 ml × 1 ml hexanes and rinsings were transferred back to the organic (neutral) fraction. The neutral fraction was then eluted through hexane-saturated Na2SO4 into a clean tube. The phenolics in the aqueous fraction were reprotated by the addition of approximately 20 drops of concentrated H2SO4 (to a pH of 2). A 2 ml aliquot of 1:1 MIBE:hexanes was added, the tube vortexed, and the (upper) organic phase was transferred through MIBE:hexanes-saturated Na2SO4 into a new tube. This procedure was repeated twice more. The phenolic extract was then evaporated to around 0.5 ml, and treated with diazomethane as the methylating reagent. Both fractions were evaporated to around 1 ml, followed by the addition of 5 drops of concentrated H2SO4. After removal of the acid, the extract was neutralized with around 4 ml × 8 ml of H2O. The residual water was frozen and the hexane layer, followed by a 1 ml × 2 ml hexane rinsing, was transferred to a new tube. Extracts were transferred into TMP and into GC vials and made up to 250 µl by weight.

2.5.2. ICA

In the individual assays, it was possible to monitor depletion of the parent compound as well as formation of potential metabolites. The incubation reaction was stopped after 90 min by the sequential addition of 1 ml of ice-cold 0.5 M NaOH to all tubes. Internal standards (ISTDs) for the phenolic fractions were added at this point, because their susceptibility or resistance to enzymatic conversion in this in vitro system is not known. For PBDE assays, 2′-OH-BDE28 (5 µl, 206 pg/µl) was added as the internal standard. For PCB assays, 4-OH-CB72 (5 µl, 457.5 pg/µl) was used as the internal standard. The separation of neutral and phenolic contaminants is based on a combination of previously published methods (Li et al., 2003b; Rozemeijer et al., 1997; White et al., 2000). After addition of the appropriate phenolic ISTD, tubes were heated at 70 °C for 10 min. Then 2 ml of 6 M HCl and 1 ml of 2-propanol were added, followed by vortexing for 1 min to break down proteins. Next, 2 ml of 1:1 methyl-butyl ether (MIBE): hexanes were added. The tubes were vortexed for 1 min, then centrifuged at 2500 rpm for 5 min. The (upper) organic fraction was collected in a new tube, and the entire procedure was repeated two more times. The organic extract containing both neutral and phenolic contaminants was then concentrated to around 1 ml. A 2 ml aliquot of KOH was added, the tube vortexed, and the (bottom) aqueous phase containing the deprotonated phenolics was transferred to a clean tube. This KOH partitioning step was repeated three times. The KOH/aqueous (phenolic) fraction was rinsed with 3 ml × 1 ml hexanes and rinsings were transferred back to the organic (neutral) fraction. The neutral fraction was then eluted through hexane-saturated Na2SO4 into a clean tube. The phenolics in the aqueous fraction were reprotated by the addition of approximately 20 drops of concentrated H2SO4 (to a pH of 2). A 2 ml aliquot of 1:1 MIBE:hexanes was added, the tube vortexed, and the (upper) organic phase was transferred through MIBE:hexanes-saturated Na2SO4 into a new tube. This procedure was repeated twice more. The phenolic extract was then evaporated to around 0.5 ml, and treated with diazomethane as the methylating reagent. Both fractions were evaporated to around 1 ml, followed by the addition of 5 drops of concentrated H2SO4. After removal of the acid, the extract was neutralized with around 4 ml × 8 ml of H2O. The residual water was frozen and the hexane layer, followed by a 1 ml × 2 ml hexane rinsing, was transferred to a new tube. Extracts were transferred into TMP and into GC vials and made up to 250 µl by weight.

2.6. Analysis of neutral and phenolic fractions

Neutral fractions, containing either PCB or PBDE congeners, were separated using an Agilent 6890 GC (Palo Alto, CA, USA) equipped with a fused silica DB-5 column (0.5% phenylmethylene polysiloxane, 30 m × 250 µm i.d., 0.25 µm film thickness, J&W Scientific Inc., Folsom, CA, USA), an Agilent 7683 Series automatic liquid sampler and injector. Halogenated contaminants were monitored with a 63Ni eµECD detector. Helium and 5% methane in argon were used as the carrier and makeup gases, respectively. For PCBs, the temperature program was as follows: 90 °C for the first 1 min, increased to 200 °C at 15 °C/min and held for 2 min, then increased further to 280 °C at 2.5 °C/min and held for 9 min. The total run time was 51.5 min. For PBDEs, the oven temperature was held at 80 °C for 2 min, increased to 290 °C at 10 °C/min and held for 15 min, for a total run time of 38 min. HP ChemStation software was used to collect and process data from the GC-eµECD.
The phenolic fractions were analyzed for methylated OH-PCBs or OH-PBDEs on an Agilent 6890N GC system, with the same autosampler, injector and column as described for the GC-μECD, coupled to an Agilent 5973N MSD in the electron capture, negative ionization (ECNI) mode. The MSD was used in selected ion monitoring (SIM) mode. The carrier and reagent gases were helium and methane, respectively. The GC ramping program for methylated OH-PCBs started with an initial temperature of 80 °C for the first minute. It was then increased at a rate of 10 °C/min to 250 °C and held for 5 min. Another increase at 5 °C/min to 300 °C was held for a further 5 min. The total run time was 38 min. For possible formation of OH-PCB (as MeO-PCBs) metabolites, the [M]+, [M + 2]+ and [M – CH3]+ ions were selectively monitored. For CB15, di-chlorinated MeO-PCBs were monitored (ions of m/z: 252, 254, 237), as well as tetra-chlorinated MeO-PCBs (ions of m/z: 322, 324, 307) to detect the ISTD. Further attempts to identify CB15 metabolites involved monitoring for di-chlorinated di-MeO-PCBs (ions of m/z: 282, 284, 267). For CB105 and CB118, tetra- (ISTD) and penta-chlorinated MeO-PCBs (ions of m/z: 356, 358, 341) were monitored. For methylated OH-PBDEs, the same GC temperature profile chosen for the PBDEs was used. The isotopic 79Br− and 81Br− anions were monitored, as the Br− fragment ion has been shown to be highly abundant for PBDEs. A 1 μl injection, made in the splitless mode, was employed for all samples. The method limit of quantification (MLOQ, S/N = 10) for neutral compounds, based on the response of the ISTD CB153, was around 1 pg/μl. The MLOQs for derivatized phenolic metabolites, based on the appropriate ISTD, was around 4 pg/μl for MeO-PCBs and 1 pg/μl for MeO-PBDEs.

2.7. Data analysis

The results are reported as the fraction of PCB or PBDE congener depleted (metabolized) during the 90 min time frame of the microsomal assay (Boon et al., 1998; de Boer et al., 1998; Li et al., 2003a). For each incubation (MCA or ICA), the results for the parent PCB or PBDE congeners were first internal standard corrected:

\[
\text{ratio}_{\text{control}} = \frac{\text{peak area}_{x}}{\text{peak area}_{\text{CB153}}} 
\]

where \( x \) is a PCB or PBDE congener. The ratio \( \text{ratio}_{\text{control}} \) for the sample replicates (to which NADPH was added) was then compared to the ratio \( \text{ratio}_{\text{CB153}} \) for the corresponding control replicates (to which NADPH was not added), and the fraction of congener \( x \) remaining after metabolism is

\[
\text{fraction remaining} = \frac{\text{ratio}_{\text{CB153}}(\text{sample})}{\text{ratio}_{\text{CB153}}(\text{control})}
\]

Student’s t-tests were used to determine if the sample and control groups were significantly different from each other, assuming a two-tailed distribution and a maximum probability of a type-I error set to \( \alpha = 0.05 \).

The neutral fraction recoveries are reported as the ratio \( \text{ratio}_{\text{CB153}} \) in the control divided by the ratio \( \text{ratio}_{\text{CB153}} \) in the standard. The recoveries of PCB and PBDE congeners from the MCA extraction were thus all above 75%, except for CB15, which had a lower, but still reproducible, recovery of 70 ± 7%. The PCB and PBDE recoveries from the ICA extraction were thus >74% and >73%, respectively. For the phenolic fractions, the ratio of the peak area of either 4-MeO-CB72 or 2′-MeO-BDE28 in the control to that in the standard was used to calculate recoveries. Therefore, the OH-PCB and OH-PBDE recoveries were >73% and >67%, respectively.

3. Results

3.1. Microsomal protein content and enzyme activities

The hepatic microsomes of the western Hudson Bay male beluga whale showed the highest catalytic viability, as measured by CYP1A-mediated EROD activity (260 pmol/mg/min), of all beluga whales sampled during Arviat subsistence hunts in the summers of 2002 and 2003 (McKinney et al., 2004). Elevated protein levels and CYP1A activity in this male are likely, in part, due to the fact that most other belugas sampled were females, which generally exhibit lower expression of CYP1A than males (White et al., 1994). As well, a shorter time span between sacrifice and liver sampling for this individual than for some of the other animals may have contributed. Nonetheless, this individual more than likely represents the metabolic capacity of adult male (Arctic) beluga whales towards CYP1A-type substrates (e.g. ortho-meta unsubstituted PCBs and possibly PBDEs), as the EROD rate was similar to that of the only other adult male sampled from this population (180 pmol/mg/min) and well within the range found for eight other adult males from another Canadian Arctic population (413 ± 263 pmol/mg/min, White et al., 1994). Putative CYP1A-mediated testosterone 6β-hydroxylase and phase II UDPGT-mediated 1-naphthylglucuronidase rates in this individual were similar to rates among the other belugas sampled from the same population (McKinney et al., 2004). The testosterone 6β-hydroxylase rate was 560 pmol/mg/min in comparison to the means of 343 ± 235, 613 ± 304 and 450 ± 115 pmol/mg/min for juveniles, females and males sampled, respectively. In addition, the male in this study exhibited very comparable total CYP levels (240 pmol/mg protein) to that of the males published by White et al. (1994) (318 ± 73 pmol/mg protein). Therefore, this individual exhibited representative induced-type (CYP1A) and constitutive-type enzyme (CYP1A-like) hepatic enzyme activities and was an appropriate choice for the study of PBDE and PCB metabolism within the time constraints of a 90 min in vitro assay. Table 2 compares some CYP enzyme activities between this male beluga whale and the (pooled) male rat liver microsomes.

3.2. In vitro PBDE metabolism

Within the MCAs, beluga whale and rat liver microsomes were able to metabolize the PBDE mixture to different extents within the 90 min time frame of the in vitro assay (Fig. 1). Dibromo-BDE15 was completely depleted by the beluga, and 11% of BDE28 was also metabolized to a significant extent.
Table 2

<table>
<thead>
<tr>
<th>Enzyme activity (pmol/mg/min)</th>
<th>Male beluga (n = 1)</th>
<th>Male rat (pooled, n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total spectral P450 (pmol/ mg)</td>
<td>240</td>
<td>590</td>
</tr>
<tr>
<td>Ethoxyresorufin O-deethylase (CYP1A)</td>
<td>260</td>
<td>120</td>
</tr>
<tr>
<td>Androstenedione formation (from testosterone) (CYP2B/2C/3A)</td>
<td>70</td>
<td>NA</td>
</tr>
<tr>
<td>Testosterone 16α-hydroxylase (CYP2C)</td>
<td>ND</td>
<td>3400</td>
</tr>
<tr>
<td>Testosterone 6β-hydroxylase (CYP3A)</td>
<td>560</td>
<td>3700</td>
</tr>
</tbody>
</table>

ND: not detected, NA: not available.

a For belugas, specific CYP isozymes have not been confirmed to mediate particular testosterone metabolism activities. CYPs indicated in parentheses are involved in this conversion in laboratory rodents.

b McKinney et al. (2006).
c Gentest, Woburn, MA, USA.

Fig. 1. Fraction of BDE congener concentrations remaining after a 90 min in vitro metabolism assay using hepatic microsomes from adult male beluga whale (white bars, n = 1 individual) and adult male rat (black bars, 1 pool of n = 25 individuals). The dotted line signifies no depletion. Significant depletion (p < 0.05) is marked by an asterisk. Error bars represent ± S.D.

(p < 0.05). However, the beluga whale did not significantly metabolize the other PBDE congeners, specifically BDE49, BDE99, BDE100, BDE153, BDE154 and BDE183. In contrast, the control rat biotransformed BDE28, BDE49, BDE99 and BDE154 (13%, 44%, 11% and 17% depleted, respectively) to a significant extent. Thus, although both organisms showed comparable metabolic capacity towards tri-BDE28, the beluga more rapidly metabolized BDE15, and the rat exhibited more rapid metabolism of the higher brominated PBDE congeners (BDE49, BDE99 and BDE154). BDE47 was also included in the MCA; however, the MCA extraction procedure introduced either a peak to the chromatogram that interfered with BDE47 or BDE47 contamination itself. Although background correction was included, depletion results were inconsistent for BDE47 (data not shown). As shown later in the ICAs, the BDE47 contamination issue did not arise and therefore satisfactory results could be obtained for this congener.

In the ICAs, BDE15 was again rapidly metabolized, but to a lesser extent than in the MCA, which is probably a consequence of a higher treatment concentration in the ICA (Table 3). The concentration of BDE28 used in both conditions was nearly the same, and the fraction of BDE28 remaining was nearly identical under both assay conditions (89 ± 3% for MCA and 90 ± 1% for ICA). In the ICAs, BDE47 was slightly but significantly metabolized within the 90 min period. BDEs 99 and 154 were not significantly metabolized as individual congeners, which is in agreement with the MCA results.

Table 3

<table>
<thead>
<tr>
<th>PBDE congener</th>
<th>Fraction remaining ± S.D. (%)</th>
<th>Statistical significance (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDE15</td>
<td>37 ± 10</td>
<td>0.001*</td>
</tr>
<tr>
<td>BDE28</td>
<td>90 ± 1</td>
<td>0.007*</td>
</tr>
<tr>
<td>BDE47</td>
<td>95 ± 3</td>
<td>0.05*</td>
</tr>
<tr>
<td>BDE99</td>
<td>99 ± 3</td>
<td>0.8</td>
</tr>
<tr>
<td>BDE154</td>
<td>103 ± 5</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Statistical significance of the depletion was reported as the p-value obtained from a Student’s t test comparing ratio(CB153 between sample (n = 3) and control (n = 3) groups.

* Statistically significant depletion.

The presence of OH-PBDE metabolites was assessed as a result of in vitro PBDE metabolism. For all BDEs in the ICAs, any phenolic metabolites (e.g. mono- or di-OH-BDEs) potentially formed from the small amounts of BDE28 and BDE47 metabolism observed were below the MLOQ (1 pg/ml based on ISTD). The lone exception was BDE15, where a single phenolic metabolite of BDE15 was detected in the phenolic extract from the microsomal incubation (Fig. 2). In the neutral fractions from the ICAs of PBDEs, debrominated or methoxylated metabolite peaks were not observed in the ECD chromatograms (not shown). Even though 63% of BDE15 was depleted, the phenolic BDE metabolite had a S/N ratio of only 16, where the method limit of quantification is set to S/N = 10. Thus, it is unlikely that BDE28 or BDE47 metabolites could in fact be detected given the slower rates of biotransformation of BDE28 and BDE47 in comparison to BDE15.

Fig. 2. Fraction of BDE congener concentrations remaining after a 90 min in vitro metabolism assay using hepatic microsomes from adult male beluga whale (white bars, n = 1 individual) and adult male rat (black bars, 1 pool of n = 25 individuals). The dotted line signifies no depletion. Significant depletion (p < 0.05) is marked by an asterisk. Error bars represent ± S.D. for replicate assays (n = 4).
3.3. In vitro PCB metabolism

As for PBDEs, the beluga whale and the rat showed congener-dependent differences in the ability to deplete in vitro the PCB congeners present in the MCAs (Fig. 3). CB15 and CB77, substrates of CYP1A, were the most rapidly metabolized (0% and 7% remaining, respectively) PCB congeners in beluga whale, whereas 68% of CB15 and 100% of CB77 remained after incubation with the rat microsomes. CB52 was partially metabolized by both rat and beluga, but the rat more rapidly depleted this Group IV, putative CYP2B-type substrate (49% cf. 87% remaining). Beluga whale and rat comparably metabolized the Group VI, mixed CYP1A/2B-type substrate tri-CB31, at 29% and 33% depleted, respectively. Rat exhibited a greater metabolic capacity towards the other Group VI congener, CB26, than beluga whale (79% versus 25% depleted). Penta-CB101 and penta-CB105 (Groups IV and III, respectively) were not metabolized significantly by either beluga whale or rat.

<table>
<thead>
<tr>
<th>PCB congener</th>
<th>Fraction remaining ± S.D. (%)</th>
<th>Statistical significance (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB15</td>
<td>9 ± 2</td>
<td>0.000007*</td>
</tr>
<tr>
<td>CB105</td>
<td>100 ± 3</td>
<td>0.79</td>
</tr>
<tr>
<td>CB118</td>
<td>98 ± 1</td>
<td>0.05*</td>
</tr>
</tbody>
</table>

Statistical significance of the depletion was reported as the *p*-value obtained from a Student’s *t*-test comparing ratios between sample (*n* = 3) and control (*n* = 3) groups.

*Statistically significant depletion.

For beluga whale, we also ran ICAs for CB15, CB105 and CB118. Previous contaminant residue studies showed that some of the major OH-PCB residues found in the liver tissues of beluga whales were likely metabolites of CB105 and CB118 (McKinney et al., 2006). CB118 was slowly, but nonetheless significantly biotransformed by beluga whale (Table 4). Any...
pentachloro-OH-PCB metabolites that may have formed were below the MLOQ (4 pg/jg based on ISTD). As in the MCA, metabolism of CB105 was too slow to be detected in the assay (Table 4), and possible pentachloro-OH-PCB metabolite peaks were below the MLOQ in the phenolic fractions. Although CB15 was rapidly metabolized as in the MCA (although less depletion in the ICA is more than likely due to a higher starting concentration than in the MCA, as seen for BDE15), no metabolite peaks could be detected measuring for methylated derivatives of mono-OH-dichloro-CBs (m/z 252, 254, 237). However, when run for di-OH-dichloro-CBs, a small peak was found indicating the metabolic conversion of CB15 to a di-OH-di-chlorinated PCB metabolite.

4. Discussion

4.1. Biotransformation of PBDEs

For fish, there are published reports showing PBDE metabolism (oxidative and debromination) in vivo (Kierkegaard et al., 2001; Stapleton et al., 2004a,b). However, to our knowledge, this is the first report demonstrating direct evidence of PBDE metabolism in any mammalian wildlife species (Hakk and Letcher, 2003). Our findings suggest a role for liver CYP enzymes in the toxicokinetics of PBDEs in beluga whales. Previous studies demonstrated low, but measurable levels of OH-PCB metabolite and OH-PBDE residues in the liver tissues of beluga whales from two Canadian populations (McKinney et al., 2006). Both BDE47 and BDE49 contain two meta Br-atoms, but BDE47 contains two ortho-meta vicinal H-atoms, respectively, suggesting that the number of ortho-meta unsubstituted sites influences metabolic capacity towards PBDEs. In further support of this, tetra-BDE47 was slightly but significantly depleted by beluga, whereas metabolism of tetra-BDE49 was not detectable. Non-ortho-OH-PBDEs found previously in beluga liver tissues may be metabolites of the important PBDE congener, BDE47 (McKinney et al., 2006). Both BDE47 and BDE49 contain two ortho Br-atoms, but BDE47 contains two ortho-meta vicinal H-atoms pairs, whereas BDE49 contains one ortho-meta and one meta–para unsubstituted site. These results parallel PCB metabolism findings in that beluga whale, as well as in other cetacean species, show greater metabolic capacity towards congeners with (more) ortho-meta unsubstituted sites (Section 4.2; Tanabe et al., 1988; White et al., 2000). On the other hand, rat was able to rapidly metabolize BDE49, and also to metabolize the ortho-meta unsubstituted BDE99, containing 2 ortho H-atoms, and BDE154, containing no vicinal H-atom pairs and 3 ortho H-atoms. The PCB equivalents of these latter two congeners fall within the metabolic Groups II and I, respectively, and are considered to be recalcitrant congeners in a number of marine mammal species (Boon et al., 1997).

The differential capacity of the beluga whale to metabolize the PBDE congeners studied suggests unequal CYP enzyme affinity for different congeners or perhaps the involvement of multiple CYP isoforms. Given that metabolic rates of the two significantly depleted congeners, BDE15 and BDE28, in the MCA assay were not slower than in the ICA, these two congeners may not be substrates for the same CYP isoform. Other marine mammals show clear species-specific differences in the ability to metabolize individual congeners. This is likely due to differences in CYP isozyme activity and expression (Table 2; Boobis et al., 1990; Smith, 1991). Our study results indicate that laboratory animal models (in this case, rat) are not sufficiently useful predictors of PBDE (and PCB) metabolism, and the potential resulting toxicity, for beluga whale and probably other marine mammals. Although the direct study of organohalogen biotransformation and toxic effects in marine mammals is ethically objectionable, our findings show that risk assessment decisions should be based on appropriate data, such as those from marine mammal cellular or sub-cellular fractions, not those from other mammalian species.

It has been suggested that PBDEs are very resistant, more resistant than PCBs, to metabolic degradation in marine mammals (de Boer et al., 1998). The present study provides evidence that for lower halogenated congeners in the same isomer class (specifically, di-CBs cf. di-BDEs and tri-CBs cf. tri-BDEs), beluga whale show congener-specific, but nonetheless comparable capacities to biotransform PBDEs and PCBs. Also, both PBDE and PCB congeners of higher degrees of halogenation are metabolized much less rapidly, resulting in slow or negligible metabolism in the time frame of the in vitro assay. Harbour and ringed seals did not exhibit detectable metabolism of highly chlorinated PCBs in similar in vitro assays (Letcher et al., 1998; Li et al., 2003a). These results do not suggest the inability of beluga whale to biotransform higher brominated PBDE congeners, but rather likely suggest that slower metabolic transformation of such PBDEs cannot be detected within the time constraints of the microsomal assay. This was similar to the findings for PCB metabolism (Section 4.2). Indeed, evidence of metabolism of PBDEs of greater degrees of halogenation may be provided by tissue residue analyses. Although tetrabromo-OH-BDE congeners have been detected, but at very low levels in the liver tissues of beluga whales (McKinney et al., 2006), the present findings cannot confirm that these OH-PBDEs could be metabolites of BDE47, as OH-PBDE metabolites were not detectable in the in vitro metabolism assays. Mainly tetrabromo-mono-OH-BDE residues have been found in the blood plasma of a very limited number of other wildlife species, i.e. Detroit
Baltic salmon (Asphord et al., 1999) and Svalbard glaucous gull (Verreault et al., 2005b). Depending on the study, it was concluded that the source OH-PBDEs may be via PBDE metabolism and/or accumulation through diet from natural or anthropogenic sources. Additionally, direct evidence for the formation of OH-PBDE metabolites comes from dosing studies using experimental rodents, which were on scales of days and not minutes. Hydroxylated metabolites formed from tetra-BDE47 (Ostr and Klasson-Wehler, 1998; penta-BDE99, penta-BDE100 and even deca-BDE209 (Mørk et al., 2003) have been detected in rat and/or mouse.

In the only other known study of PBDE metabolism in marine mammal species, de Boer et al. (1998) did not detect depletion of BDE47, BDE99 or BDE209 congeners in sperm whale (Physeter macrocephalus), whitebeaked dolphin (Lagenorhynchus albirostris), minke whale (Balaenoptera acutorostrata) or harbour seal (Phoca vitulina) using a comparable in vitro assay. Consistent with their results, we did not measure in vitro metabolism of congeners with five or more bromine atoms. This was the case under both the MCA and ICA regimes in our study, suggesting that it is the slow or negligible biotransformation of such congeners in the assay and not competition with lower brominated congeners that prevented detection of parent depletion or metabolite formation, as found in analogous PCB studies with Baltic grey seal (Li et al., 2003a). Detectable metabolism of BDE47 in the present study of beluga whale, but not by organisms in the de Boer et al. (1998) study, may be due to a somewhat higher metabolic capacity of beluga towards this environmentally dominant PBDE, although variations in assay conditions may also be partially responsible. In vitro BDE47 metabolism may suggest that retained, hydroxylated forms of BDE47 (e.g., 3-OH-BDE47 and 4′-OH-BDE49) reported at low levels in liver tissues (McKinney et al., 2006) are the result of biotransformation of BDE47 in beluga whales.

4.2. Biotransformation of PCBs

Reports that demonstrate the metabolic biotransformation of PCBs in marine mammals are limited in number, generally focusing on only one or two specific congeners, i.e., CB177 and CB152 (Mørk et al., 1994; White et al., 2000) or PCB congener mixtures (Letcher et al., 1998; Li et al., 2003a). CYP isoforms have previously been implicated in the metabolism of CB177 and CB152 in beluga whale (White et al., 2000). We found that isozymes from the CYP family are also involved in the biotransformation of a number of other PCB congeners, representing different metabolic classes (Boon et al., 1997), as seen in grey and harbour seals (Letcher et al., 1998; Li et al., 2003a). However, the differential rates of metabolism of PCB congeners between and within metabolic groups (Fig. 3) imply the involvement of various isozymes (e.g. CYP1A, CYP2B, CYP3A) and differential enzyme affinities between PCB congeners, respectively. The use of CYP-specific chemical and/or antibody inhibitors would further clarify the involvement of specific CYP isozymes in the metabolism of individual congeners (Letcher et al., 1998; van Hezik et al., 2001; Li et al., 2003a).

Previously, penta- through hepta-chlorinated OH-PCB residues were detected in St. Lawrence beluga whale liver tissues, but only a single tetra-chlorinated OH-PCB congener (4′-OH-CB120) was found in western Hudson Bay animals (McKinney et al., 2006). In the present in vitro assay very slow to undetectable rates of metabolism were observed for higher chlorinated PCB congeners (CB101, CB105 and CB118). In contrast, the di- through tetra-chlorinated congeners all showed sufficient depletion in the 90 min assay, but such OH-PCB metabolites of these congeners are not generally found as tissue residues, suggesting that these metabolites are generally rapidly excreted, and do not show significant tissue retention. Significant metabolism of CB118 in the present study supports the hypothesis that the presence of higher-chlorinated, toxicologically active OH-PCBs as contaminant residues in beluga whales and in other marine mammals tissue is due to oxidative CYP biotransformation enzyme activities in liver tissues.

For the rapidly metabolized CB15, we were able to detect the formation of a di-OH-PCB metabolite. In a previous study of CB77 metabolism in beluga whale liver, only mono-OH-PCB type metabolites were found, even though di-hydroxy, sulfonated and (mono- and di-) hydroxylated mε were monitored (White et al., 2000). In addition, only mono-OH-PCBs were found in liver extracts of fish (White et al., 1997) and laboratory rodents (Klassen-Wehler et al., 1989). However, CB15 is co-planar and has four, ortho-meta- vicinal H atom pairs. It is likely that after a single (meta or para) hydroxylation, the mono-OH-dichloro-CB metabolites would still be somewhat coplanar (especially in comparison to higher chlorinated congeners used in the previous studies), and thus still good substrates for a second CYP1A-mediated hydroxylation step to occur.

5. Conclusions

Within the limited time frame of an in vitro microsomal assay, direct evidence of congener- and species-specific (beluga whale cf. rat) cytochrome P450 mediated metabolism of PBDEs and PCBs in beluga whale was found. Metabolic processes may influence not only the rates of accumulation and elimination of these contaminants in beluga whale, but also potential biological effects. Highly exposed populations, such as the St. Lawrence beluga whales, exhibit elevated, induced bio-transformation capacities, resulting in the formation of retained metabolites. As the body burdens of PBDEs rise in beluga whales and in other sensitive marine mammal populations, further research is imperative to better understand of the kinetics and potential toxicological outcomes of these flame retardant chemicals and their retained metabolites.

Acknowledgements

We would like to express our appreciation to those involved in sampling the Arviat beluga whale, specifically Milton Levin (University of Connecticut) and Mark Eetak (Arviat Hunters and Trappers Organization). Financial support for the sampling was provided as part of a grant from the US EPA STAR Program (to S. De Guise). In addition, this study was funded by the
Canada Research Chairs Program and the Natural Sciences and Engineering Research Council of Canada (to R.J. Letcher).

References


M.A. McKinney et al. / Aquatic Toxicology 77 (2006) 87–97


