



## **Quality Assurance Project Plan**

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### **PBDE Flame Retardants in Spokane River Fish Tissues and Osprey Eggs**

April 2009

Publication No. 09-03-108



## Publication Information

This plan is available on the Department of Ecology's website at [www.ecy.wa.gov/biblio/0903108.html](http://www.ecy.wa.gov/biblio/0903108.html).

Data for this project will be available on Ecology's Environmental Information Management (EIM) website at [www.ecy.wa.gov/eim/index.htm](http://www.ecy.wa.gov/eim/index.htm). Search User Study ID, CFUR0005.

Ecology's Project Tracker Code for this study is 09-502.

Waterbody Numbers: WA-57-1010, WA-54-1010, WA-54-1020, WA-54-9040, WA-34-9480, WA-34-9060, and WA-34-9290.

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# Quality Assurance Project Plan

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## PBDE Flame Retardants in Spokane River Fish Tissues and Osprey Eggs

April 2009

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EAP - Environmental Assessment Program.  
EIM - Environmental Information Management system.  
USGS – U.S. Geological Survey.

# Table of Contents

	<u>Page</u>
Abstract.....	3
Background.....	4
Project Description.....	6
Organization and Schedule.....	7
Quality Objectives.....	9
Sampling Process Design (Experimental Design).....	11
Sampling Procedures.....	14
Fish.....	14
Osprey Eggs.....	14
Measurement Procedures.....	15
Preparation of Samples.....	15
Chemical Analyses.....	16
Quality Control Procedures.....	18
Field.....	18
Laboratory.....	18
Budget.....	19
Data Management Procedures.....	19
Audits and Reports.....	20
Data Verification.....	20
Data Quality (Usability) Assessment.....	20
References.....	21
Appendices.....	

Appendix A. Possible sources and effects of Polybrominated Diphenyl Ethers (PBDEs) on Ospreys nesting along the Spokane River.

Appendix B. Method for the Determination of Multiple Brominated Flame Retardants (BFRs) and Isomers of the Chlorinated Flame Retardant, Dechlorane Plus, in Biological Matrices of Wildlife Species.

Appendix C. Protocol for Bird Egg Collection, Measurement, Preparation, and Shipment for Contamination Residue Analysis.

Appendix D. Acronyms and Abbreviations.

## Abstract

Each study conducted by the Washington State Department of Ecology (Ecology) must have an approved Quality Assurance (QA) Project Plan. The plan describes the objectives of the study and the procedures to be followed to achieve those objectives. After completion of the study, a final report describing the study results will be posted to the Internet.

This QA Project Plan describes a cooperative effort by Ecology and the U.S. Geological Survey to sample and analyze polybrominated diphenyl ether (PBDE) flame retardants in resident fish and osprey eggs along the Spokane River.

Goals of the study are to:

1. Track changes in PBDE levels since fish were last sampled in 2005.
2. Assess PBDE concentrations in the osprey diet.
3. Determine if reproductive success of osprey nesting on the Spokane River is negatively affected by PBDE exposure.

Twenty eggs will be collected along the river from the Idaho border through Long Lake. Thirty-eight fish tissue composites will be analyzed from the same stretch of river. PBDE concentrations in eggs and fish tissues will also be determined at reference sites near the Spokane River. The study will be conducted in 2009.

## Background

PBDEs have been used as fire-retarding additives to paint, plastics, textiles, and electronics for about three decades. There is increasing experimental evidence that PBDE exposure may be detrimental to wildlife health including the effects on sex and thyroid hormones as well as the effects on the modulation of liver enzyme activity, immunotoxicity, and neurological development (Kierkegaard et al., 2006; Darnerud et al., 2001; Birnbaum and Staskal, 2004). The Washington State Department of Ecology (Ecology) and Washington State Department of Health (DOH) finalized a Chemical Action Plan for PBDEs in 2006 that outlines future steps to reduce the threat of PBDEs in the environment (Ecology and DOH, 2006). In 2007, the Washington State legislature passed legislation banning penta- and octa- BDEs.

PBDEs are distributed globally, environmentally persistent, and bioaccumulative. In contrast to organochlorine pesticides and PCBs, their levels have been increasing in biota since the 1970s (Norstrom et al., 2002; Law et al., 2006). In North America, several studies have shown PBDE concentrations to be increasing at an exponential rate in fish, bird, and mammal species in the following locations: (1) the Arctic (Ikonomou et al., 2002), (2) the St. Lawrence Estuary (Lebeuf et al., 2004), (3) the Great Lakes ecosystem (Norstrom et al., 2002), and (4) the Columbia River (Rayne et al., 2003). PBDE levels are greatest in predator fish and fish-eating wildlife in aquatic food webs (Alaee and Wenning, 2002). Reports of PBDEs in the tissues of avian species, particularly fish-eating birds, remain very limited.

The osprey is a large fish-eating bird of prey, which has a nearly world-wide breeding distribution. The osprey has been shown to be a useful sentinel species for monitoring contaminants in waterbodies due to its position at the top of the aquatic food web and its ability to accumulate lipophilic contaminants (Grove et al., 2009). The U.S. Geological Survey (USGS) analyzed 190 osprey eggs from Oregon and Washington between 2002 and 2007, all of which contained PBDEs (Henny et al. In press).

Several studies have examined PBDE concentrations in Washington state fish (Johnson and Olson, 2001; Seiders and Yake, 2002; Johnson et al., 2006) and surface waters (Johnson et al., 2006; Sandvik et al., in prep.). In 2005, Ecology conducted a study measuring PBDE concentrations in fish tissues from six different reaches of the Spokane River (Serdar and Johnson, 2006). The Spokane was identified as having the highest PBDE levels in both water and tissue samples statewide (Figure 1). Currently, sources of PBDEs to the Spokane River are unknown.

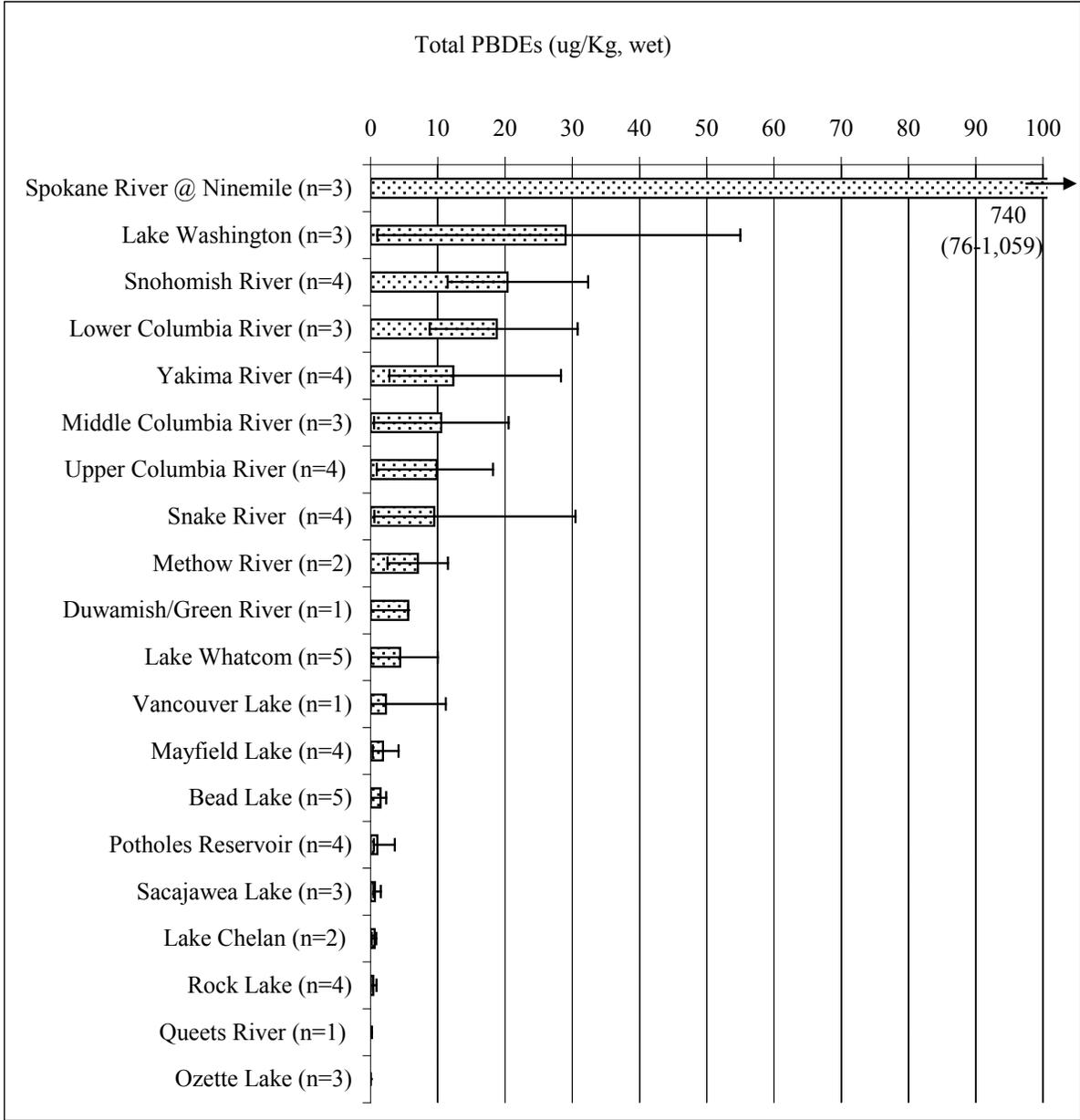


Figure 1. Mean and Range of Total PBDE Concentrations in Fish Fillets Analyzed for Ecology’s Statewide Survey (from Johnson et al., 2006).

In view of the limited data that has been collected and the potential adverse effects of PBDEs on wildlife relying on the Spokane River food web, this study will provide data on concentrations found in osprey eggs and fish tissues. The information can be used in the future to evaluate the effectiveness of Washington State’s Chemical Action Plan for PBDEs and other efforts to reduce PBDE inputs to the environment.

## Project Description

In conjunction with USGS, the Ecology Environmental Assessment (EA) Program will conduct a one-time study to measure PBDE concentrations in fish tissues and osprey eggs in six reaches of the Spokane River. The area is from the Idaho border through Long Lake. Osprey egg collections will be carried out by USGS staff to determine PBDE levels in eggs and evaluate whether reproductive success of ospreys nesting on the Spokane River is negatively affected by PBDE exposure.

The project plan describing egg collections and data interpretation is included in Appendix A. Fish tissue sampling will be conducted by the EA Program. The goals of the fish collections are to assess PBDE concentrations in the osprey diet and to track changes in concentrations since the river was last sampled in 2005. A shared objective of both studies will be to determine PBDE biomagnification factors from fish to osprey eggs.

Specific EA Program study objectives will be to:

1. Measure PBDE concentrations in composite fish tissue samples for two to three species at six Spokane River sites and reference sites near Spokane.
2. Measure PBDE concentrations in five osprey eggs to determine inter-laboratory differences between Manchester Environmental Laboratory (MEL) and the National Wildlife Research Centre (NWRC), Science and Technology Branch, Carleton University, Ottawa, Ontario.
3. Identify spatial, species, and temporal patterns in the environmental distribution and accumulation of PBDEs.

Field work for the study will be conducted during March - July 2009. Eggs are being analyzed at MEL to assess PBDE data comparability between the two labs. The following congeners will be analyzed using gas chromatography/mass spectrometry select ion monitoring (GC/MS SIM:) BDE-47, -66, -71, -99, -100, -138, -153, -154, -183, -190, and -209.

Thirty osprey egg samples will be analyzed by NWRC in Ottawa, Ontario, using gas chromatography/high-resolution mass spectrometry (GC/HRMS). The following list of congeners will be analyzed by NWRC: BDE -17, -28, -47, -49, -66, -85, -99, -100, -101, -138, -153, -153/154, -183, -190, -209, and hexabromocyclododecane (HBCDD).

## Organization and Schedule

The following people are involved in this project.

Table 1. Organization of Project Staff and Responsibilities.

Staff (EAP unless otherwise noted)	Title	Responsibilities
Chad Furl Toxics Studies Unit, SCS Phone (360) 407-6060	Project Manager	Writes the QAPP; oversees fish collections, processing, and transportation of samples to the laboratory; conducts QA review of data; analyzes and interprets data; and writes the draft and final reports detailing fish tissue results.
Chuck Henny Forest & Rangeland Ecosystem Science Center, USGS Phone (541) 757-4840	Principal Investigator	Writes the osprey egg QAPP (Appendix A), conducts QA review of data, analyzes and interprets data, and writes the draft and final reports detailing osprey egg results.
James Kaiser Forest & Rangeland Ecosystem Science Center, USGS Phone (541) 757-4840	Osprey Lead Scientist	Reviews and clarifies study plan, budget, and QAPP; prepares osprey work plan and conducts field sampling; records field information; processes samples; oversees sample shipment to laboratories; summarizes data; and assists with data analysis and report writing.
Callie Meredith Toxics Studies Unit, SCS Phone (360) 407-6965	Co-Author, Field Assistant, EIM Data Engineer	Helps collect samples, records field information, and enters data into EIM.
Michael Friese Toxics Studies Unit, SCS Phone (360) 407-6737	Co-Author, Field Assistant	Helps collect samples and record field information.
Dale Norton Toxics Studies Unit, SCS Phone (360) 407-6765	Unit Supervisor	Provides internal review of the QAPP, approves the budget, and approves the final QAPP.
Will Kendra SCS Phone (360) 407-6698	Section Manager	Reviews the project scope and budget, tracks progress, reviews the draft QAPP, and approves the final QAPP.
Carol Kraege SWFAP Phone (360) 407-6906	EAP Client	Clarifies the scope of the project, provides internal review of the QAPP, and approves the final QAPP.
Stuart Magoon Manchester Environmental Laboratory Phone (360) 871-8801	Director	Approves the final QAPP.
William Kammin Phone (360) 407-6964	Ecology QA Officer	Reviews the draft QAPP and approves the final QAPP.

EAP – Environmental Assessment Program.

EIM – Environmental Information Management system.

SCS – Statewide Coordination Section.

SWFAP – Solid Waste & Financial Assistance Program.

QAPP – Quality Assurance Project Plan.

QA – Quality Assurance.

Table 2. Proposed Schedule for Completing Field and Laboratory Work, Data Entry into EIM, and Reports Detailing Fish Findings.

Field and laboratory work	
Field work completed	June 2009
Laboratory analyses completed	August 2009
Environmental Information System (EIM) system	
EIM data engineer	Callie Meredith
EIM user study ID	CFUR0005
EIM study name	PBDE Flame Retardants in Spokane River Fish Tissues and Osprey Eggs
Data due in EIM	December 2009
Final report	
Author lead	Chad Furl
Schedule	
Draft due to supervisor	September 2009
Draft due to client/peer reviewer	October 2009
Draft due to external reviewer(s)	November 2009
Final report due on web	December 2009

## Quality Objectives

MEL and NWRC are expected to meet all quality control requirements of the analytical methods being used for this project. Recoveries of the decachlorobiphenyl (DCB) surrogate will determine whether measurement quality objectives (MQOs) for estimating the accuracy of the PBDE analysis in MEL tissue samples have been met. The accuracy of NWRC's PBDE analysis in osprey egg samples will be based on surrogate recoveries of BDE-30, BDE-156, <sup>13</sup>C<sub>12</sub> BDE-209 (Gauthier et al., 2007; Gauthier et al., 2008). Results from the NWRC are recovery corrected. Laboratory analysis of osprey egg samples by the NWRC follow quality assurance procedures described by Letcher (2008) (Appendix B).

The MQOs for all analyses being conducted for this project are shown in Table 3. MQOs for this project were established so that (1) uncertainties in contaminant concentrations were minimized and (2) results are comparable to existing studies.

Table 3. Measurement Quality Objectives.

Matrix	Analysis	Measurement Quality Objectives	Laboratory
Tissue*	PBDEs	50-150% surrogate recovery 50-150% LCS** recovery ≤50% duplicate RPD*** 50-150% matrix spike recovery	MEL
	Lipids	80-120% LCS recovery ±20% duplicate precision	
Osprey Egg	PBDEs	80-110% surrogate recovery 90 ± 10% LCS recovery ≤50% duplicate RPD 90 ± 10% matrix spike recovery	NWRC
	Lipids	80-120% LCS recovery ±20% duplicate precision	

\* Fish tissue and egg.

\*\* Laboratory Control Sample.

\*\*\* Relative Percent Difference.

A subset of five eggs will be homogenized and analyzed by Ecology for inter-laboratory comparison between MEL and NWRC. This has application for calculating biomagnification factors from fish to eggs, especially with regard to recovery of individual congeners. Fish tissue and egg results from MEL will not be recovery corrected. A final assessment to see if the results can be used to calculate biomagnifications factors will be made, once all data has been received.

The lowest concentrations of interest for each matrix are listed in Table 4. Based on past fish tissue and osprey egg studies conducted within Washington State, analyzing down to these levels should be sufficient to quantify the dominant PBDE congeners and percent lipids in the majority of samples.

Table 4. Lowest Concentrations of Interest.

Matrix	Analysis	Lowest Concentration of Interest	Laboratory
Tissue*	PBDEs	1 ug/Kg ww	MEL
	Lipids	0.10%	
Osprey Egg	PBDEs	0.02-0.06 (0.07-0.12 BDE-209) ug/Kg ww	NWRC
	Lipids	0.10%	

\*Fish tissue and egg.  
ww = wet weight.

# Sampling Process Design (Experimental Design)

The locations proposed for fish and osprey egg collections are displayed in Figure 2.

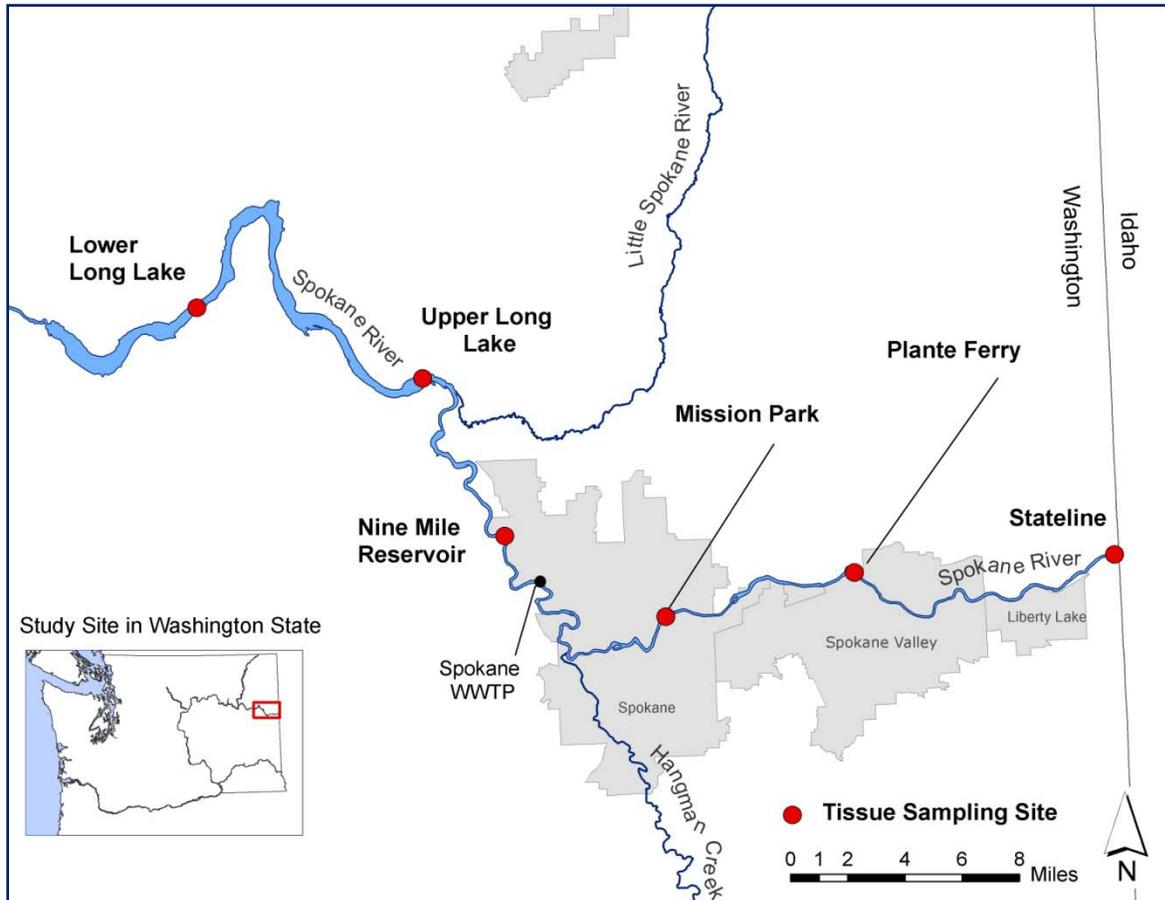


Figure 2. Fish and Egg Sampling Locations.

Fish collections will be focused on the same river reaches where tissue sampling occurred in 2005 (Serdar and Johnson, 2006). The selected locations provide sampling coverage of nearly the entire river between the Idaho border through Long Lake. Twenty nests will be strategically selected for egg collection, with 10 located above and 10 below the city of Spokane Wastewater Treatment Plant at river mile (RM) 67.5. Optimally, one egg will be collected for PBDE analysis from at least three nests within each of the six reaches along the Spokane River where fish collections will occur.

In addition to the Spokane River, fish and osprey eggs will be sampled from Rock, Williams, and Badger Lakes, approximately 25 miles southwest of Spokane. A total of 10 eggs will be collected among the lakes along with 2 different species of fish at each lake. These lakes will serve to establish the PBDE background for the study area. Fish tissue samples analyzed from

Rock Lake in 2005 had very low total PBDE concentrations (Johnson et al., 2006). Tissue and egg samples will be collected from March through May 2009.

An effort will be made to collect the same fish species as in the 2005 study to bolster the temporal analysis. Table 5 lists the species obtained from each sampling location during 2005. A combination of samples will be prepared in order to (1) match sample type (fillet or whole body) from the 2005 study and (2) mimic osprey feeding habits (whole body). Whole fish PBDE and lipid concentrations will be calculated from the relative weights of the fillet and carcass when necessary.

Ospreys are opportunistic foragers and their diet often consists of 2-3 species regardless of fish communities (Poole et al., 2002). Johnson et al. (2008) found largescale suckers to be the major component of osprey diets along the Columbia and Willamette rivers representing 84.3% and 92.7% of biomass consumed, respectively. Three composite samples of whole largescale suckers from each Spokane River station will be analyzed in an effort to provide representative data for this species. Two composite samples (1 fillet and 1 carcass) of each additional species encountered at each sampling site will be analyzed.

Table 5. Locations and Fish Species Sampled in 2005 and Proposed Sampling for 2009.

Location	Approximate River Mile	Species Recovered in 2005	Proposed No. of Tissue Samples*
Stateline	96.1 - 95.5	Largescale Sucker	3
Plante Ferry	86.0 - 85.0	Rainbow Trout	2
		Largescale Sucker	3
Mission Park	78.5 - 74.5	Rainbow Trout	2
		Mountain Whitefish	2
		Largescale Sucker	3
Ninemile	64.5 - 63.5	Rainbow Trout	2
		Mountain Whitefish	2
		Bridgelip Sucker	3
Upper Long Lake	56.3 - 50.6	Mountain Whitefish	2
		Brown Trout	2
		Smallmouth Bass	2
		Largescale Sucker	3
Lower Long Lake	40.1 - 33.9	Mountain Whitefish	2
		Smallmouth Bass	2
		Largescale Sucker	3
Williams Lake	NA	NA	2
Badger Lake	NA	NA	2
Rock Lake	NA	NA	2
		Total No. of Proposed Samples	44

\* Sucker composites will consist of whole fish only. Non-sucker composites will consist of 1 fillet and 1 carcass sample. Tissue samples from Williams, Badger, and Rock will be whole fish only.

All tissue samples performed by MEL will be analyzed for BDE-47, -49, -66, -71, -99, -100, -138, -153, -154, -183, -184, -191, and -209. Tissue samples will also be analyzed for percent lipids, as this parameter may be useful for normalizing the data. Osprey egg contents analyzed by NWRC will be measured for lipids and BDE -17, -28, -47, -49, -66, -85, -99, -100, -101, -138, -153, -153/154, -183, -190, -209, and HBCDD.

# Sampling Procedures

## Fish

The collection, handling, and processing of fish tissue samples are guided by methods described in the (1) U.S. Environmental Protection Agency's (EPA) *Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories* (EPA, 2000) and (2) EA Program's *Standard Operating Procedures for Field Collection, Processing, and Preservation of Finfish Samples at the Time of Collection in the Field* (Sandvik, 2006a). Fish will be collected using boat electrofishing, netting (gill or fyke nets), or hook and line.

Fish will be inspected to ensure that they are acceptable for further processing (e.g., no obvious damage to tissues, skin intact). Acceptable fish are killed by a blow to the head, rinsed in ambient water to remove foreign material, weighed to the nearest gram, and their total lengths measured to the nearest millimeter. Individual fish are double wrapped in foil and placed in a plastic zip-lock bag along with a sample identification tag. The bagged specimens will be placed on ice in the field. Fish will remain on ice until frozen at  $-20^{\circ}\text{C}$  at Ecology's facilities in Lacey, Washington, for processing at a later date.

## Osprey Eggs

Partially incubated osprey eggs will be collected during spring 2009 by USGS field staff following *Protocol for Avian Egg Collection and Removal of Contents for Contaminants Analysis*. Eggs will be collected from accessible nests (generally nesting platforms on non-energized poles) along the Spokane River and at a reference area (USGS Study Plan, Appendix A) following *Protocol for Bird Egg Collection, Measurement, Preparation, and Shipment for Contaminant Residue Analysis* (Buck, 2008) (Appendix C).

It is anticipated that Avista Utilities and Inland Power and Light Company will provide technical assistance during sample collection (i.e., bucket truck and crew to access nests). A single egg is removed from each nest and wrapped in chemically-cleaned aluminum foil (dull side in). Eggs are clearly labeled, placed in protective materials, and cooled to  $4^{\circ}\text{C}$  until further processing.

# Measurement Procedures

## Preparation of Samples

Fish tissue samples will be prepared following adapted guidelines from the EA Program's *Standard Operating Procedures for Resecting Finfish Whole Body, Body Parts, or Tissue Samples* (Sandvik, 2006b). Fish will be partially thawed before processing. Slime and scales will be removed, with a subset of scales retained for age determination. Fish will be rinsed with tap water followed by a deionized water rinse. Skin-on fillets will be removed from non-sucker species.

All samples will be composites consisting of three to five fish. Fillets will be passed two times through a Kitchen-Aid food grinder. Equal weights of fillet tissue will be mixed together and homogenized a third time using a sonicator. Whole fish composite samples will be prepared the same as fillet composites. A Hobart commercial meat grinder will be used to homogenize whole fish. Carcass samples will be processed as whole fish samples after the fillet has been removed.

The weight of the whole fish, fillet, and carcass will be recorded in order to back calculate whole body concentrations. Subsamples of the homogenates will be placed into laboratory-provided, clean glass jars. Samples will be refrozen, assigned a MEL identification number, and shipped to the laboratory for analysis. Excess homogenate will be labeled and archived at -20° C at Ecology Headquarters.

All utensils will be cleaned before using in order to prevent contamination of samples. Utensils include resecting tools; scalpels; bowls; spoons; and blender parts having plastic, wood, bronze, and stainless steel parts. The cleaning procedure will include: hand-wash with soap (Liquinox) and hot tap water, hot tap water rinse, deionized water rinse, and finally a pesticide-grade acetone rinse. Fish processing will be carried out on a nylon cutting board, covered with aluminum foil (dull side up). All staff will wear nitrile gloves during tissue processing.

The sex of the fish will be determined after tissue samples have been processed. Otoliths and scales will be sent to the Washington State Department of Fish and Wildlife biologists to determine age.

Individual osprey eggs will be processed following *Protocol for Bird Egg Collection, Measurement, Preparation, and Shipment for Contaminants Residue Analysis* (Buck, 2008; Appendix C). Eggs will be gently cleaned, dried, and weighed prior to processing. Volume and length measurements will also be taken. Eggs will be scored around the equator of the egg until the membrane is visible. The membranes will then be cut with a scalpel and egg contents transferred to a pre-cleaned stainless steel jar for homogenizing. Eggs will be homogenized using a sonicator until they are of consistent color and texture. Samples will then be placed in the proper pre-cleaned jars and sent frozen on dry ice to NWRC, Ottawa, and MEL for chemical analysis. Sample preparation of osprey eggs and fish tissues will occur at Ecology headquarters.

## Chemical Analyses

Table 6 displays the expected range of results, required reporting limits, and sample preparation and analysis methods for each sample type. Methods were chosen to give reporting limits equal to, or less than, the lowest concentrations of interest.

Table 6. Expected Range of Results, Reporting Limits, and Methods.

Analysis	Matrix	Number of Samples	Expected Range of Results	Reporting Limits	Preparation Method	Analytical Method
PBDEs	Tissue*	49	1 - 1500 ug/Kg	0.5 - 1.0 ug/Kg	EPA 3450	EPA 8270
Lipids			1 - 15%	0.10%	extraction	EPA 608.5
PBDEs	Egg	30	$\Sigma$ PBDE range from above detection limit to over 1.0 ppm	0.02-0.06 (0.07-0.12 BDE 209) ug/Kg	See Appendix B	See Appendix B
Lipids			5 – 15%	0.10%	extraction	gravimetric

\*Fish tissue and egg.

BDE = Brominated diphenyl ether

Table 7 displays the PBDE congeners analyzed by both laboratories. MEL analyzes 11 congeners while the NWRC analyzes 15 congeners along with HBCDD. HBCDD is a brominated flame retardant preferred for its ability to meet fire safety regulations when used in low concentrations (Law et al., 2005).

Table 7. PBDE congener lists for laboratories involved with the study.

Manchester Environmental Laboratory (MEL)	National Wildlife Research Centre (NWRC)
47	17
66	28
71	47
99	49
100	66
138	85
153	99
154	100
183	101
190	138
209	153
	153/154
	183
	190
	209

The two congener lists are expected to result in similar total PBDE values. BDE- 47, 100, 99, 153, 153/154 represented > 95% of total PBDEs in osprey eggs from Washington and Oregon (personal communication, James Kaiser).

## Fish Tissue

Fish tissue samples will be analyzed following MEL's standard operating procedure for PBDEs. The samples will be extracted with methylene chloride/acetone by EPA SW-846 Method 3540 then solvent exchanged to iso-octane. The extracts will then be analyzed by gas chromatography/mass spectrometry select ion monitoring (GC/MS/SIM) following EPA SW-846 Method 8270.

## Osprey Egg

Egg samples will be analyzed by the NWRC for PBDEs using the method NWRC-MET-ORG RES-BFR-ver. 4 (Letcher, 2008) (Appendix B). The egg samples will be extracted using 50% dichloromethane/hexane, and then analyzed by gas chromatography/high-resolution mass spectrometry (GC/HRMS).

Egg sample analysis at MEL will follow the same procedures as for fish tissue.

# Quality Control Procedures

## Field

No field quality control (QC) samples will be analyzed in conjunction with fish tissue sampling. Field variability associated with fish tissue is being addressed by analyzing composite samples.

Field variability pertaining to egg sampling will be judged by contaminant concentration differences between eggs obtained from each specific river reach. Due to increased risk of nest failure, only one osprey egg can be collected from each nest.

## Laboratory

Standard QC procedures routinely followed by MEL and NWRC for the analyses requested will be satisfactory for the project. Table 8 lists the types of QC samples and the frequency at which they will be analyzed.

Table 8. Laboratory Quality Control Procedures.

Matrix	Analysis	LCS	Method Blanks	Surrogate Spike	Analytical Duplicate*	MS/MSD	Lab
Tissue <sup>^</sup>	PBDEs	1/batch	1/batch	every sample	3/project	2/batch~	MEL
	Lipids						
Osprey Egg <sup>^</sup>	PBDEs			every sample	1/batch		NWRC
	Lipids						

\* 2 fish, 1 egg submitted blind to the laboratory.

<sup>^</sup> Fish and egg.

~ Egg = 1/batch.

<sup>^</sup> A detailed description of QC is available in Appendix B, section 11.

LCS = Laboratory Control Sample.

MS/MSD = Matrix spike/matrix spike duplicate.

Surrogate spikes at MEL will consist of 100 ng of decachlorobiphenyl (DCB) spiked prior to extraction. Osprey egg samples at NWRC will be spiked with a 100 µL internal standard solution of BDE-30, BDE-156, and <sup>13</sup>C<sub>12</sub>-BDE-209 prior to extraction. Surrogate spikes provide an estimate of recovery of target compounds. NWRC concentrations of PBDEs in osprey eggs will be recovery corrected as an internal standard method of quantification and used to compensate for recovery differences between samples.

Laboratory control samples (LCS) will be analyzed once per batch for both fish tissue and eggs to assess analytical precision and bias. The MEL LCS consists of blank water spiked with all target compounds. NWRC LCSs for osprey eggs will consist of an in-house standard reference material (SRM) of double-crested cormorant (*Phalacrocorax auritus*) egg homogenate.

Method blanks will be analyzed once per batch for tissue and egg samples to identify contamination originating from the laboratory environment. Two fish tissue duplicates and one egg duplicate will be submitted blind to MEL to assess analytical precision. The fish tissue

duplicates will be chosen to represent anticipated low and high concentrations. NWRC will perform a duplicate analysis of selected egg extracts every three samples.

A matrix spike and matrix spike duplicate will be included with every MEL tissue batch to assess bias. Matrix spikes will consist of approximately 25 ng/Kg of all target compounds.

## Budget

Total costs for the project are estimated at \$83,352 (Table 9). The USGS will cover approximately \$38,337 of the project costs with Ecology contributing the remaining \$45,015. Ecology will contribute \$35,015 to the USGS in the form of an inter-agency agreement. Costs include a 50% discount for Manchester Lab.

Table 9. Laboratory Cost Estimates.

MEL PBDE Analysis	\$10,000
Contribution to USGS	\$35,015
Ecology Total	\$45,015
USGS Total	\$38,337
Grand Total	\$83,352

## Data Management Procedures

Field data will be recorded on waterproof paper and checked for legibility and completeness. All field data will be stored with the project manager. Field notes and observations will be transferred to Microsoft Excel spreadsheets.

Analytical data from MEL and NWRC staff will be provided in an electronic format. MEL staff will verify their data before sending case narratives to the project manager. Data generated by NWRC will be verified by USGS staff. Reviewed analytical data will be entered into Ecology's EIM database. EIM data entry is conducted following formal Ecology guidelines. Data entered into EIM are reviewed by the project manager, data entry staff, and an independent reviewer.

## **Audits and Reports**

NWRC and MEL laboratories participate in routine audits of their laboratory facilities, capabilities, and analytical performance. Results of audits are available upon request.

Two technical reports will be prepared from data collected for the project. Ecology will report on the findings related to the fish tissue collections. A draft technical report will be prepared for review in September 2009. A final Ecology report is anticipated to be completed by December 2009. See *Organization and Schedule* within this Quality Assurance (QA) Project Plan for a complete project timeline.

The USGS will report on findings related to the osprey egg collections. A final USGS report is expected in February 2010. See Work and Reporting Schedule in Appendix A for a complete timeline related to reporting of osprey data.

Finalized project data for all fish and egg results will be entered into EIM by December 2009.

## **Data Verification**

MEL will review all analytical data pertaining to fish tissue analysis. MEL will verify that all laboratory procedures outlined in the QA Project Plan were followed and provide their findings to the project manager in a case narrative. Parameters reviewed by MEL include, but are not limited to, acceptability of holding times, instrument calibration, procedural blanks, spiked samples, surrogate spikes, precision data, laboratory control samples, and assigned data qualifiers.

The project manager and MEL staff will examine the complete data record and determine whether results are acceptable as specified in the QA Project Plan.

The results of field and laboratory QC samples will be reviewed in order to determine if MQOs were met. Estimates of accuracy and precision will be based on laboratory QC. Data will be accepted, accepted with qualifiers, or rejected at the discretion of the project manager.

## **Data Quality (Usability) Assessment**

The quality of the data will be determined based on whether project objectives can be met using the verified data. The entire data package will be assessed by the project manager to determine the usability of the data for the analysis of Spokane River PBDE levels. The final report will provide detail on data quality and usability.

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# Appendices

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## **Appendix A. Possible sources and effects of Polybrominated Diphenyl Ethers (PBDEs) on Ospreys nesting along the Spokane River.**

This appendix describes the role of USGS in the overall project.

Source: U.S. Geological Survey Forest and Rangeland Ecosystem Science Center,  
January 29, 2009.

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## Possible sources and effects of Polybrominated Diphenyl Ethers (PBDEs) on Ospreys nesting along the Spokane River

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Prepared for:

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Washington State Department of Ecology  
Environmental Assessment Program

November 12, 2008

(revised January 29, 2009)

## Background/Introduction

Several formulations of PBDEs have been used as fire-retarding additives to paint, plastics, textiles, and electronics for about three decades. There is increasing experimental evidence that PBDE exposure may be detrimental to wildlife health including effects on sex and thyroid hormones as well as effects on the modulation of liver enzyme activity, immunotoxicity and neurological development (Kierkegaard et al. 2006, Darnerud et al. 2001, Birnbaum and Staskal 2004). PBDEs are distributed globally, environmentally persistent, and bioaccumulative, and in contrast to the organochlorines (including DDE and other pesticides, and PCBs) their levels have been increasing in biota since the 1970s (de Wit 2002, Norstrom et al. 2000, Law et al. 2006). In North America, several studies have shown PBDE concentrations to be increasing at an exponential rate in fish, bird and mammal species, e.g., the Arctic (Ikonou et al. 2002), St. Lawrence Estuary (Lebeuf et al. 2004), Great Lakes ecosystem (Norstrom et al. 2002) and Columbia River (Rayne et al. 2003). PBDE levels are greatest in predator fish and fish-eating wildlife in aquatic food webs (Alaee and Wenning, 2002). Reports of PBDEs in the tissues of avian species, and particularly fish-eating birds, remain very limited.

All 109 Osprey eggs analyzed from Oregon and Washington between 2002 and 2007 contained PBDEs (Henny et al. In press). In fact, the PBDE concentrations along the Upper Willamette River in 2006 (geo. mean 898 ng/g ww) were significantly higher ( $P=0.02$ ) than recorded along the Lower Columbia River in 2007 (geo. mean 570 ng/g). In addition, PBDEs in Osprey eggs from the lower segment of the Columbia River increased 41% from 2004 (geo. mean 403 ng/g) to 2007 (geo. mean 570 ng/g). Evidence of reduced reproductive success associated with PBDE concentrations was also documented at Osprey nests along the Upper Willamette in 2006 (USGS unpublished data). Controlled laboratory studies with several avian species are underway by other researchers to further evaluate reproductive effects. The Upper Willamette River Osprey eggs had the highest PBDE concentrations in the Pacific Northwest with expectations of higher concentrations in recent collections made in 2008. Washington Department of Ecology (WDOE) recently reported the highest PBDE concentrations in Spokane River fish from twenty rivers and lakes sampled throughout Washington in 2005 (WDOE 2006) and there is concern about the presence and effects of this emerging contaminant on nesting Ospreys and other species along the River. In view of the limited data that has been collected and the potential adverse effects of PBDEs on wildlife that rely on the Spokane River food web, this study will provide baseline data that could be used in the future to evaluate the effectiveness of the Washington State PBDE Chemical Action Plan (Ecology and DOH, 2006) and other efforts to reduce PBDE inputs to the environment.

This study will use the Osprey (*Pandion haliaetus*) to gather PBDE contaminant information using a sample egg and nestling blood sample from select nests along the Spokane River and at a reference area south of Spokane. The Osprey is a large (ca. 1.6 kg) fish-eating bird of prey, which has a nearly world-wide breeding distribution. Because of its position at the top of the aquatic food web as an obligate piscivore, its ability to accumulate lipophilic contaminants and sensitivity

to many contaminants, the U.S. Geological Survey, U.S. Fish and Wildlife Service, Canadian Wildlife Service and scientists from other organizations have often selected the osprey for contaminant studies (Grove et al. 2009). Several Osprey life history traits make it a species of choice for contaminant monitoring and research, justifying its use as a sentinel species.

These characteristics include:

1. An aquatic diet consisting almost exclusively (99+ %) of fish.
2. Localized feeding habits, within relatively short distance of the nest. Fish species captured can be identified based on prey remains at nest sites, direct observations and/or photographs.
3. A long-lived species, living up to 25 years, with strong nest site fidelity, returning year after year to the same or a nearby nest.
4. Ospreys have readily observable nests, constructed of large sticks at exposed locations that are easily detected during both aerial and ground/boat surveys. Ospreys commonly nest on artificial structures, generally facilitating access for egg and blood/tissue collection.
5. Adapts to human landscapes including industrial and municipal sites where contamination may be most severe, and readily habituates to human activity.
6. Tolerates short term nest disturbance for egg/blood collection, resulting in little or no effect on nest success.
7. Removal of a "sample egg" from the usual 3-egg clutch for contaminant analyses has limited effect on productivity at the nest, i.e., loss of 0.28 young fledged for each egg collected on the Columbia River in 1997-98 (Henny et al. 2004, 2008).
8. Nests spatially distributed at regular intervals along waterways as opposed to clumped at a limited number of regional colonies. This distribution permits random egg and tissue collections along river segments or strategic collections related to potential contaminant sources.

### **Objectives:**

1. Determine if reproductive success of Ospreys nesting on the Spokane River is negatively affected by PBDE exposure.
2. Determine PBDE concentrations in plasma of Osprey nestlings and measure various blood parameters to evaluate possible effects on the general health of the birds.
3. Determine prey preferences of Ospreys nesting on the Spokane River and Reference Site.

### **Methods:**

1. Determine the distribution and abundance of nesting pairs of Ospreys along a 65 mile segment of the Spokane River from Stateline to Little Falls Dam and at a reference site south of Spokane near Rock Lake in 2009. (Note: 27 occupied nests were present along the Spokane River study area in 2008, based on brief nest survey via car on 7/8/08). The reference area for this study is

Rock Lake and two adjacent lakes located approximately 25 miles southwest of Spokane, Washington. Fish fillets analyzed from Rock Lake in 2005 had very low Total PBDE concentrations (WDOE 2006). Nests will be located and nest activity (unoccupied, occupied or active) determined by conducting a helicopter survey when most birds are incubating. A relatively small percentage of the nests will need to be visited from the ground to verify if the pair is in incubation position (active nest) or if the pair did not lay eggs (occupied nest, usually about 5-10% of the nests with birds present). Nest locations will be plotted on aerial photographs, noting nest activity, nest structure type and possible access points. Private landowners will be approached for permission to monitor nests which occur on private lands and collect prey remain, egg and blood plasma samples. Permission to enter private property will be obtained in writing. Methods of contact will include a combination of phone calls, letters, and personal contact.

2. Determine the reproductive success (number of young fledged) at each nest. Osprey nests will be monitored throughout the nesting season at about 2 week intervals to determine nesting status (active, failed). The number of young produced at each nest will be determined by a second aerial survey or by ground survey when nestlings are about 40-45 days old (age of first flight usually 48-59 days).
3. Strategically select 20 accessible nests for sample egg collection (Blus et al. 1974), with 10 nests located above and 10 below the City of Spokane Waste Water Treatment Plant at river mile (RM) 67.5. Optimally, an egg will be collected for PBDE analysis from three nests within each of the six reaches along the Spokane River where Ecology sampled and analyzed fish for 12 PBDE congeners in 2005. This approach will answer two questions (a) are the cities and perhaps associated wastewater treatment plants the major PBDE source, i.e., are concentrations higher in eggs collected below the cities?, and (b) what is the reproductive "effect level" of PBDEs in Osprey eggs--a greater range of PBDE concentrations will be present if the first hypothesis is true, which should enhance the ability to evaluate reproductive effects at the nests with a sample egg collected. Up to ten eggs also will be collected from the reference area for analysis. Egg and blood plasma samples will be collected from nest sites built on non-energized platforms using bucket trucks and crew provided by local utility companies (Avista Utility and Inland Power and Light Company).
4. Collect blood plasma from advanced-age nestlings at 7 Spokane River nests and 7 reference nests where an egg was collected for PBDE residue analysis and to determine T4 (thyroid hormones) and a series of other blood parameters. Hematological measurements used in this study are known indicators of contaminant toxicity in birds, or are known to reflect organ damage and related physiological disturbances. Hematology will be conducted at the University of Miami-School of Medicine, Cooperative Pathology Laboratory. Eggs and blood plasma, as in the past, will be analyzed by National Wildlife Research Centre in Ottawa for PBDE/herbicide residues following procedures described by Letcher (2008). Findings will be compared to values determined from a reference Osprey population and to similar Osprey data from other locations in the Pacific Northwest.

5. Determine general foraging locations and identify species and size of prey fish delivered to each nest during pre-egg laying period and nestling period by conducting foraging and prey delivery observations and by examining prey remains collected from feeding perches at each nest site where egg or plasma sample collected. Constructed prey basket devices deployed at the same six river segments used by Ecology for sampling fish for PBDE analysis in 2005 (WDOE 2006), as well as a similar number of devices deployed at the Osprey reference area south of Spokane, will supplement the Osprey prey-fish data collected at nest sites. Some captured prey fish will be photographed at the nest sites and identified to species by noting body and caudal fin shape and color as well as other distinguishing characteristics. Determination of prey fish from prey remains collected at feeding perches, etc. will follow methods described by Johnson et al. (2008).

6. Evaluate reproductive success and egg residue concentrations for the Spokane River and compare the data with findings from the reference area and to PBDE concentrations for other Osprey populations. The Jonckhere-Terpstra Test (Hollander and Wolfe 1973) will be used to evaluate reproductive success at the nests with an egg collected in relation to PBDE concentrations in the sample egg from that nest.

### **Work and Reporting Schedule**

- November-December 2008: Draft/finalize study plan and work plan, prepare State & Federal collection permit applications, establish cooperative agreement with local power companies for accessing Osprey nest sites built on, or adjacent to power poles for sample collection.
- March-August 2009: Collect field data (landowner contacts, determine distribution and abundance of nesting pairs, identify feeding perches and collect prey remains from prior years, install prey baskets, conduct foraging and prey-delivery observations (Ecology staff may be available to assist with diet observations, April 1 – May 15), collect egg and plasma samples, determine reproductive success.
- September-October 2009: Egg and plasma residue, hormones and blood chemistry analysis.
- November-December 2009: Data Analysis
- January-February 2010: Prepare/Submit Final USGS peer-reviewed Report
- March-April 2010: Prepare/Submit peer-reviewed journal article

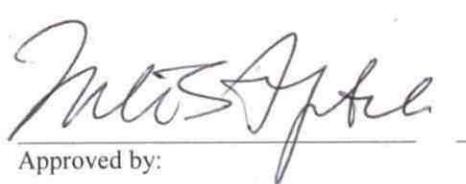
## Special Provisions

All pertinent scientific collection permits and animal care and use authorizations will be obtained prior to sample collection and will be in the possession of biologists during collection of authorized biological samples.

The requirement of the Biological Resources Division (BRD) of the U.S. Geological Survey for employees of our agency to obtain written permission from landowners before entering private property to conduct wildlife studies will be met.

Following completion of the study, field notebooks, data sheets, and electronic files originals will be stored at the Forest and Rangeland Ecosystem Science Center, Corvallis, OR.

Metadata and data archival requirements for the data collected in this study will be met. The metadata record will be created by the USGS FRESC Metadata Coordinator based on scientist responses to a metadata interview for the data set.

	01/29/09
Submitted by: _____	_____ Date
	2/4/09
Approved by: _____	_____ Date

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## Budget

Item		FY2009
<i>Washington Department of Ecology (Contribution)</i>		
Fish Tissue PBDE Analysis <sup>a</sup>	\$	8,000
USGS Wildlife Biologist GS-11 (0.20 FTE) <sup>b</sup>	\$	16,074
Travel (Lodging, Per Diem, Fuel 8/14 weeks) <sup>c</sup>	\$	8,960
<b>Subtotal</b>	<b>\$</b>	<b>25,034</b>
USGS Overhead (39.868%)	\$	9,981
<b>Ecology Net Compensation to USGS</b>	<b>\$</b>	<b>35,015</b>
Ecology Total	\$	43,015
<i>USGS (Contribution)</i>		
USGS Wildlife Biologist GS-11 (0.10 FTE) <sup>d</sup>	\$	8,037
Osprey Egg PBDE Analysis <sup>e</sup>	\$	9,000
Osprey PBDE-Plasma Residue Analysis <sup>f</sup>	\$	5,320
Travel (Lodging, Per Diem, Fuel) <sup>g</sup>	\$	10,080
Consumable Field Supplies	\$	400
Aerial Survey (2 flights)	\$	5,500
USGS Total	\$	38,337
Grand Total	\$	81,282

<sup>a</sup> Includes Laboratory QC and estimated re-analysis costs. Price reflects a 50% MEL discount and is not subject to USGS overhead.

<sup>b</sup> Salary for period March 3 – May 23, 2009 (10 weeks of fieldwork).

<sup>c</sup> Fieldwork related (WDOE: 8/14 weeks; Mar 15 – May 9). Lodging/Per diem: \$115/day, Fuel: \$45/day.

<sup>d</sup> Salary for study planning, data analysis, report writing and 4 weeks of fieldwork (excluding plasma sampling)

<sup>e</sup> Includes 20 Spokane River and 10 Reference Area egg samples at \$300/sample.

<sup>f</sup> Includes 7 Spokane River and 7 Reference Area blood/plasma samples at \$300/sample, plus University of Miami Advanced Well-Bird Exam (CBC, Advanced Avian Chemistry, EPH) and T4 test at \$80/sample.

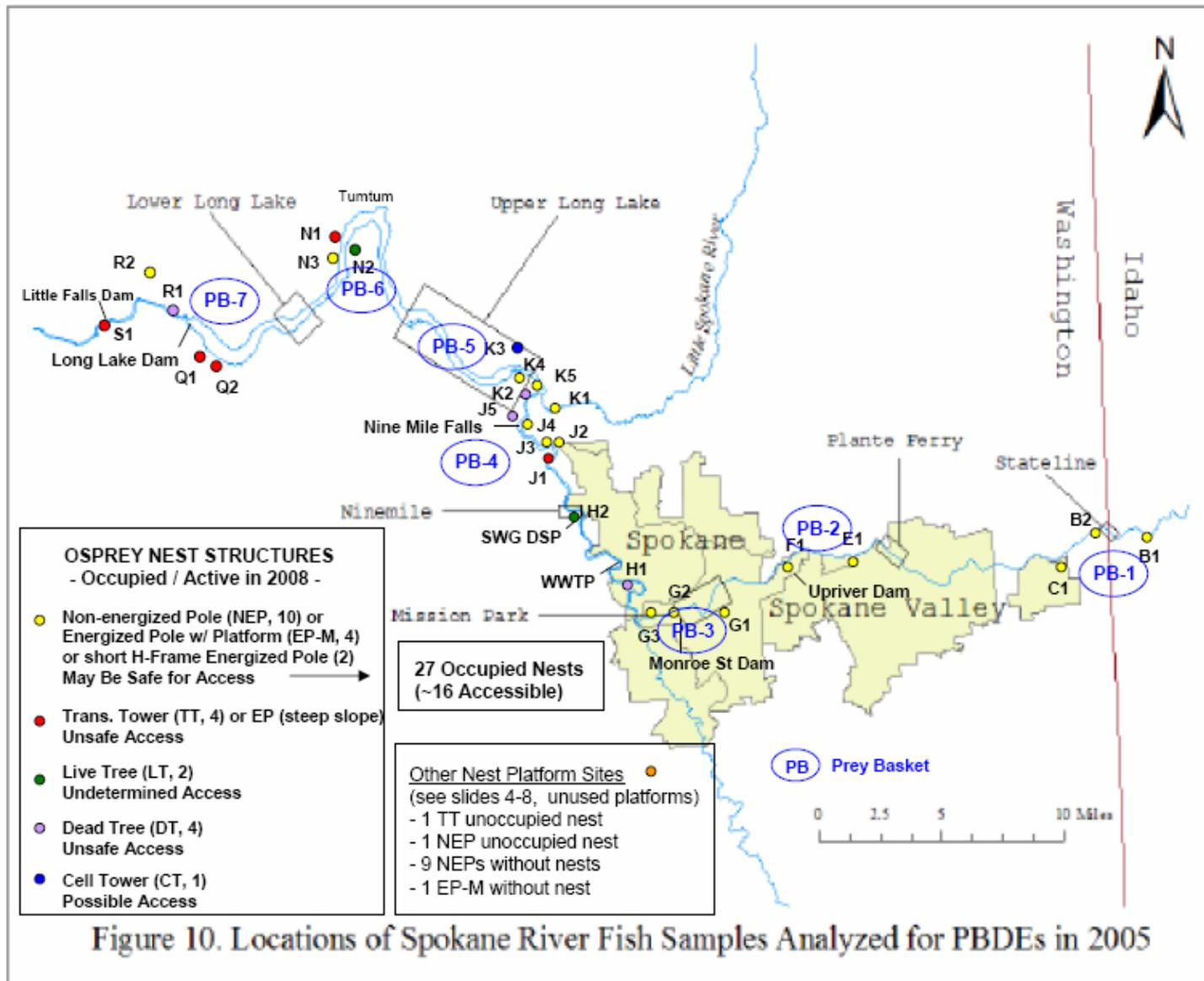
Performance of this task is dependent upon availability of USGS funding, as yet undetermined.

<sup>g</sup> Fieldwork related (USGS: 9/14 weeks (including 3 weeks for work related to plasma sampling).

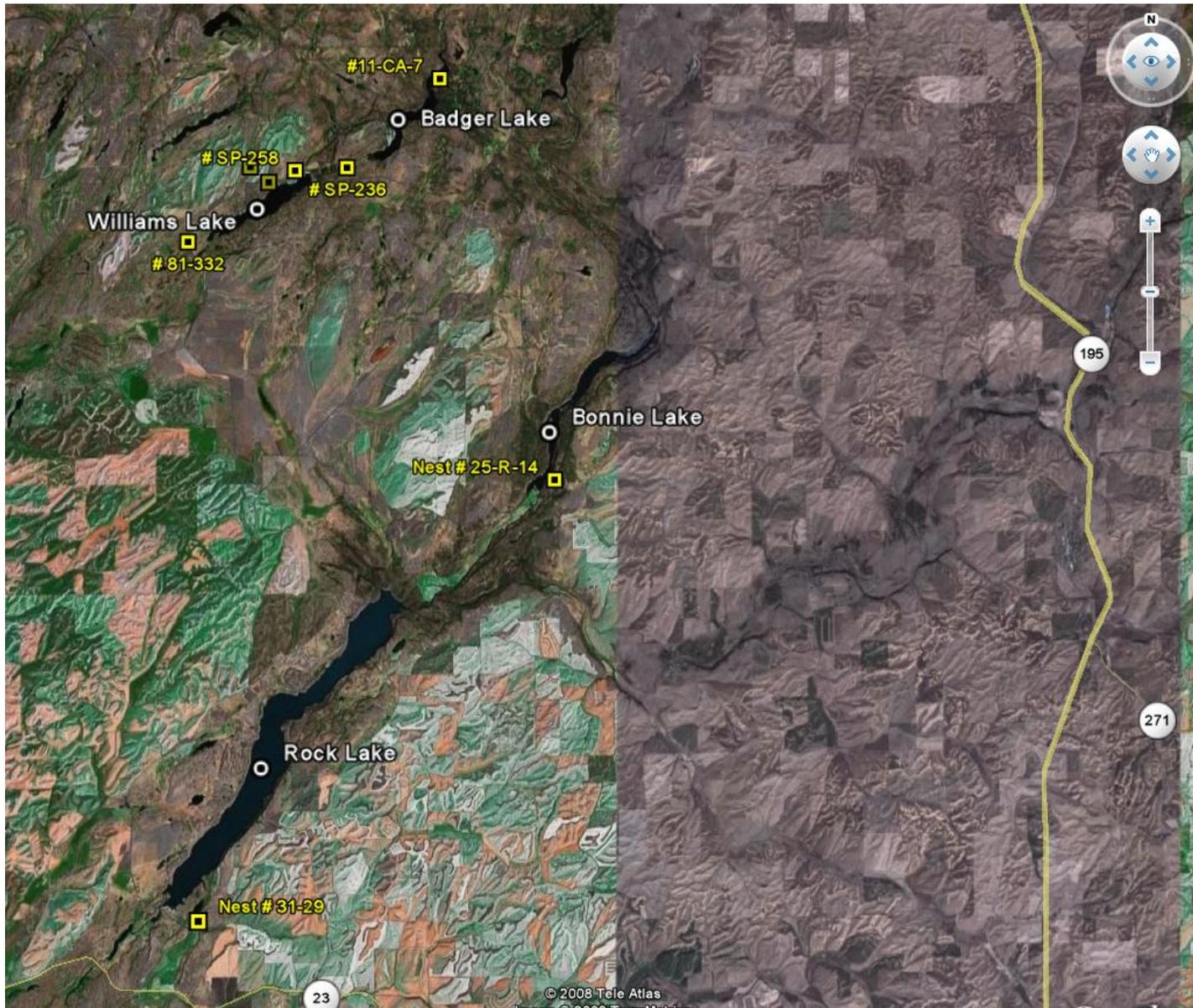
Note: Technical Assistance provided by Avista Utilities and Inland Power and Light Company (i.e., bucket truck and crew to access nests during sample collection).



**Overview map of Spokane River study area and reference area.**



Study area. Locations of occupied Osprey nests and structure types in 2008.  
(base map courtesy of Washington Department of Ecology, 2006)



Reference area. Locations of Osprey nests on pole-platform structures.

## **Appendix B. Method for the Determination of Multiple Brominated Flame Retardants (BFRs) and Isomers of the Chlorinated Flame Retardant, Dechlorane Plus, in Biological Matrices of Wildlife Species.**

This appendix describes analytical procedures used by NWRC for osprey egg analysis.

Source: National Wildlife Research Centre: Environmental Chemistry/Organic Research Group, November 2008.

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Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	1 of 51

## Method for the Determination of Multiple Brominated Flame Retardants (BFRs) and Isomers of the Chlorinated Flame Retardant, Dechlorane Plus, in Biological Matrices of Wildlife Species

### 1. SCOPE AND FIELD OF APPLICATION

This is an analytical method for the measurement of 42 polybrominated diphenyl ether (PBDE) congeners and 26 non-PBDE BFRs including their isomers, plus *syn*- and *anti*-isomers of the chlorinated flame retardant, Dechlorane Plus (DP), in various wildlife tissues matrices such as fat, liver and muscle as well as plasma and in eggs. The method is based on several published methods as listed in the references below.

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Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	2 of 51

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<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>3 of 51</b>

## 2. PRINCIPLES AND DEFINITIONS

This method incorporates the extraction of PBDEs, non-PBDE BFRs and degradation/metabolic products from wildlife tissues, plasma and eggs. With the exception of plasma, samples are extracted by accelerated solvent extraction (ASE) using dichloromethane(DCM)/hexane. In the case of plasma, protein denaturing occurs first followed by DCM/hexane extraction. Except for plasma and low lipid tissues (e.g. muscle and liver), bulk lipid removal is accomplished using gel permeation chromatography (GPC). In the case of plasma, muscle and liver extracts, a small amount of residual lipids are removed during the solid phase cartridge (SPE) chromatography step. The target BFRs in the final, isolated chemical fractions are determined via high resolution gas chromatography-mass spectrometry (GC-MS). Isomer-specific determination of hexabromocyclododecanes is described in a separate SOP, MET-ORGRES-HBCD isomers-01.

## 3. REAGENTS, SOLUTIONS AND STANDARDS

### SAFETY PRECAUTIONS

- ⇒ The toxicity or carcinogenicity of each reagent and standard used in this method has not been precisely defined. Each chemical must be treated as a potential health hazard and exposure should be reduced to the lowest possible level.
- ⇒ Operations with organic solvents such as DCM, hexane, methanol and acetone should be performed in a fume hood and dermal contact with solvents should be avoided.
- ⇒ Standards should always be opened and used in a fume hood. Handling of these compounds must be done only by qualified technical staff.
- ⇒ General safety rules and waste disposal procedures that apply to the Trace Organic Chemistry Laboratory must be followed (ref. Safety Manual).
- ⇒ Material Safety Data Sheets (MSDSs) for the products used in the assay must be read.

### 3.1. Reagents

- 3.1.1.** Acetone, Caledon Laboratories<sup>®</sup>, Distilled 1201-2-40,
- 3.1.2.** Hexane, Caledon Laboratories<sup>®</sup>, 5502-2-40
- 3.1.3.** Dichloromethane, Caledon Laboratories<sup>®</sup>, 3601-2-40
- 3.1.4.** 2,2,4-Trimethylpentane (Iso-octane or TMP), Omnisolv<sup>®</sup>, EM Science TX1389-1
- 3.1.5.** Nitrogen, compressed gas, MEGS Specialty Gases Inc., N<sub>2</sub> Ultra High Purity, 106605

<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>4 of 51</b>

- 3.1.6.** Formic acid (98-100 %) AnalaR® B100115
- 3.1.7.** Methanol HPLC grade, Fisher, A452-4
- 3.1.8.** De-ionized water from the Milli-Q system, MILLIPORE
- 3.1.9.** Sulfuric acid (98%)

### **3.2. Adsorbents for Sample Cleanup**

- 3.2.1.** Sodium sulphate, anhydrous granular (Na<sub>2</sub>SO<sub>4</sub>), Caledon Laboratories, 8221-1
  - 3.2.1.1.** Wash about 600 g of Na<sub>2</sub>SO<sub>4</sub> in a large glass column with approximately 600 mL of 1:1 DCM:hexane (v/v).
  - 3.2.1.2.** Air dry in an open dish under the fume hood overnight.
  - 3.2.1.3.** Heat for eight hours at 600°C in a muffle furnace.
  - 3.2.1.4.** Cool and transfer to a tightly capped glass bottle.
  - 3.2.1.5.** If, after heating, the Na<sub>2</sub>SO<sub>4</sub> develops a grayish cast (due to the presence of carbon in the crystal matrix), discard that batch.
- 3.2.2.** LC-Si SPE cartridge (500 mg X 6 mL; 6 gram; J.T.Baker, USA)
  - 3.2.2.1.** SPE conditioned by successive washes of 6 mL of 10% methanol in DCM followed by 8 mL 5% DCM in hexane.
  - 3.2.2.2.** The sample is loaded onto the SPE and eluted with 8 mL 5% DCM/hexane (v/v).
- 3.2.3.** Vanillin reagent, 6 g/L
  - 3.2.3.1.** Dissolve 6.0 g of vanillin in water in a 1 litre volumetric flask and dilute to volume. Transfer to an amber bottle and store at room temperature. The shelf life is two months.
- 3.2.4.** Phospho-vanillin reagent
 

Add 350 mL of vanillin reagent from section 4.3.2 and 50 mL of water to a 2 litre beaker. Add 600 mL of concentrated o-phosphoric acid, with constant stirring. Transfer to an amber bottle and store at room temperature. The shelf life is two months.
- 3.2.5.** Olive Oil Stock solution
 

Add approximately 0.14g of olive oil to a 10 mL volumetric flask. Dilute to volume with absolute ethanol and mix well. Store solution at 4-7°C when not in use. Prepare a series of standard solution of 12, 10, 8, 6, 4, 2 mg/ml using the stock solution (13.93mg/mL).

<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>5 of 51</b>

### 3.3. Standards

Refer to SOP-CHEM-PROC-05C for details concerning the preparation and storage of standard solutions. PBDE, other BFR and DP isomer standards should be stored in amber glass vessels to protect from photocatalytically-mediated degradation. All PBDE, non-PBDE BFR and DP standard solutions described below are mostly obtained from Wellington Laboratories, Inc. (Guelph, ON) Sigma-Aldrich or AccuStandard.

**Table 1. Target PBDE Standards (42 congeners)**

(see Table 4 for bromine atom substitution of all theoretical PBDE congeners)

<b>PBDE CONGENERS</b>	<b>BROMINE SUBSTITUTION</b>	<b>CONC. (UG/ML)</b>	<b>COMMERCIAL SOURCE</b>
BDE17	2,2',4-TRIBDE	50	WELLINGTON LABS
BDE25	2,3',4- TRIBDE	50	WELLINGTON LABS
BDE28	2,4,4'-TRIBDE	50	WELLINGTON LABS
BDE33	2',3,4-TRIBDE	50	WELLINGTON LABS
BDE47	2,2',4,4'-TETRABDE	50	WELLINGTON LABS
BDE49	2,2',4,5'-TETRABDE	50	WELLINGTON LABS
BDE66	2,3',4,4'-TETRABDE	50	WELLINGTON LABS
BDE71	2,3',4',6-TETRABDE	50	WELLINGTON LABS
BDE75	2,4,4',6-TETRABDE	50	WELLINGTON LABS
BDE77	3,3'4,4'-TETRABDE	50	WELLINGTON LABS
BDE85	2,2',3,4,4'-PENTABDE	50	WELLINGTON LABS
BDE99	2,2',4,4',5-PENTABDE	50	WELLINGTON LABS
BDE100	2,2',4,4',6-PENTABDE	50	WELLINGTON LABS
BDE116	2,3,4,5,6-PENTABDE	50	WELLINGTON LABS
BDE119	2,3',4,4',6-PENTABDE	50	WELLINGTON LABS
BDE138	2,2',3,4,4',5'-HEXABDE	50	WELLINGTON LABS
BDE139	2,2',3,4,4',6-HEXABDE	50	WELLINGTON LABS
BDE140	2,2',3,4,4',6'-HEXABDE	50	WELLINGTON LABS
BDE153	2,2',4,4',5,5'-HEXABDE	50	WELLINGTON LABS
BDE154	2,2',4,4',5,6'-HEXABDE	50	WELLINGTON LABS
BDE155	2,2',4,4',6,6'-HEXABDE	50	WELLINGTON LABS

Unit		Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC		Method Number Authorization : Date: Page:	NWRC-MET-ORG-RES-BFR-ver. 4 Dr. R.J. Letcher Revision #4, November 2008 6 of 51
BDE170	2,2',3,3',4,4',5-HEPTABDE	50	WELLINGTON LABS
BDE171	2,2',3,3',4,4',6-HEPTABDE	50	WELLINGTON LABS
BDE179	2,2',3,3',5,6,6'-HEPTABDE	50	WELLINGTON LABS
BDE180	2,2',3,4,4',5,5'-HEPTABDE	50	WELLINGTON LABS
BDE181	2,2',3,4,4',5,6-HEPTABDE	50	WELLINGTON LABS
BDE183	2,2',3,4,4',5',6-HEPTABDE	50	WELLINGTON LABS
BDE184	2,2',3,4,4',6,6'-HEPTABDE	50	WELLINGTON LABS
BDE190	2,3,3',4,4',5,6-HEPTABDE	50	WELLINGTON LABS
BDE191	2,3,3',4,4',5',6-HEPTABDE	50	WELLINGTON LABS
BDE194	2,2',3,3',4,4',5,5'-OCTABDE	50	WELLINGTON LABS
BDE195	2,2',3,3',4,4',5,6-OCTABDE	50	WELLINGTON LABS
BDE196	2,2',3,3',4,4',5',6-OCTABDE	50	WELLINGTON LABS

<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>7 of 51</b>

**Table 1 (cont'd). Target PBDE Standards (42 congeners)**  
(see Table 4 for bromine atom substitution of all theoretical PBDE congeners)

BDE197	2,2',3,3',4,4',6,6'-OCTABDE	50	WELLINGTON LABS
BDE201	2,2',3,3',4,5',6,6'-OCTABDE	50	WELLINGTON LABS
BDE202	2,2',3,3',5,5',6,6'-OCTABDE	50	WELLINGTON LABS
BDE203	2,2',3,4,4',5,5',6-OCTABDE	50	WELLINGTON LABS
BDE206	2,2',3,3',4,4',5,5',6-NONABDE	50	WELLINGTON LABS
BDE205	2,3,3',4,4',5,5',6-OCTABDE	50	WELLINGTON LABS
BDE207	2,2',3,3',4,4',5,6,6'-NONABDE	50	WELLINGTON LABS
BDE208	2,2',3,3',4,5,5',6,6'-NONABDE	50	WELLINGTON LABS
BDE209	2,2',3,3',4,4',5,5',6,6'-DECABDE	50	WELLINGTON LABS

**Table 2. Internal PBDE/BFR/DP Standards**  
(see Table 4 for bromine atom substitution of all theoretical PBDE congeners)

<b>PBDE CONGENERS</b>	<b>BROMINE SUBSTITUTION</b>	<b>CONC. (UG/ML)</b>	<b>COMMERCIAL SOURCE</b>
BDE30	2,4,6-TRIBDE	50	WELLINGTON LABS
BDE156	2,3,3',4,4',5-HEXABDE	50	WELLINGTON LABS
<sup>13</sup> C <sub>12</sub> -BDE209	<sup>13</sup> C <sub>12</sub> -2,2',3,3',4,4',5,5',6,6'-DECABDE	25	WELLINGTON LABS

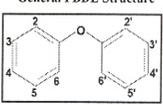
Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	8 of 51

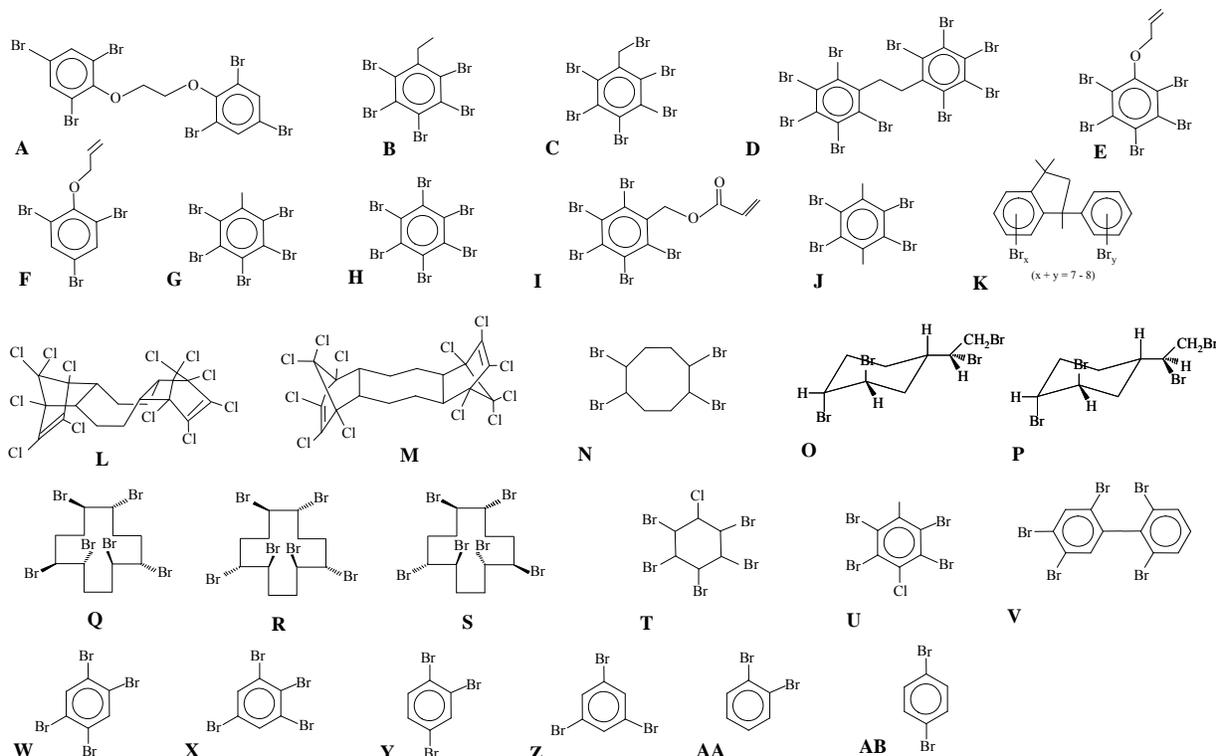
**Table 3. Target non-PBDE BFR and DP Isomer Standards (28 compounds/isomers)**  
(see Figure 1 for the chemical structures of the non-PBDE BFRs and DP isomers)

ABBREVIATION	FULL CHEMICAL NAME	CONC. (OR SOLID)	COMMERCIAL SOURCE
BTBPE	1,2,-Bis-(2,4,6-TriBromoPhenoxy)Ethane	50 µG/ML	WELLINGTON LABS
PBEB	PentaBromoEthyl Benzene	50 µG/ML	WELLINGTON LABS
PBBB	PentaBromoBenzyl Bromide	~2 G (SOLID)	SIGMA-ALDRICH
DBDPE	DecaBromoDiphenyl Ethane	25 µG/ML	WELLINGTON LABS
TBPAE	2,4,6-TriBromoPhenyl Allyl Ether	~100 G (SOLID)	SIGMA-ALDRICH
PBPAE	PentaBromoPhenyl Allyl Ether	~100 G (SOLID)	SIGMA-ALDRICH
HBB	HexaBromoBenzene	50 PG/ML	WELLINGTON LABS
PBB	PENTABROMOBENZENE	100 MG	SIGMA-ALDRICH
1,2,4,5-tetraBB	1,2,4,5-tetrabromobenzene	~100 G (SOLID)	SIGMA-ALDRICH
1,2,3,5-tetraBB	1,2,3,5 tetrabromobenzene	~100 G (SOLID)	SIGMA-ALDRICH
1,2,4-triBB	1,2,4-tribromobenzene	~100 G (SOLID)	SIGMA-ALDRICH
1,3,5-triBB	1,3,5-tribromobenzene	~100 G (SOLID)	SIGMA-ALDRICH
1,2-diBB	1,2-dibromobenzene	~100 G (SOLID)	SIGMA-ALDRICH
1,4-diBB	1,4-dibromobenzene	~100 G (SOLID)	SIGMA-ALDRICH
PBBA	PentaBromoBenzyl Acrylate	~2 G (SOLID)	SIGMA-ALDRICH
PTBX	TetraBromo-p-Xylene	50 µG/ML G/ML	WELLINGTON LABS
OBTMI	OctaBromo-1,3,3-TriMethyl-1-phenyl	50 µG/ML	BERGMAN/MARSH
<i>syn</i> -DP	<i>syn</i> -Dechlorane Plus	100 µG/ML	CIL
<i>anti</i> -DP	<i>anti</i> -Dechlorane Plus	100 µG/ML	CIL
TBCO	1,2,5,6-TetraBromoCycloOctane	57.5 µG/ML	SIGMA-ALDRICH
PBT*	PentaBromoToluene	1000 PG/µL	SIGMA-ALDRICH
PBCH	PentaBromoChlorocycloHexane	100 µG/ML	ACCUSTANDARD
TBCT	TetraBromoChloroToluene	100 µG/ML	ACCUSTANDARD
α-TBECH	1,2-dibromo-4-(1,2-dibromoethyl)-	100 µG/ML	SIGMA-ALDRICH
β-TBECH	1,2-dibromo-4-(1,2-dibromoethyl)-	100 µG/ML	SIGMA-ALDRICH
α-HBCD	α-HexaBromoCycloDodecane	50 µG/ML	WELLINGTON LABS
BB101	2,2',4,5,5'-pentaBromoBiphenyl	50 µG/ML	WELLINGTON LABS

\* An impurity peak is present in corresponding GC chromatograms.

Table 4. Bromine atom substitution of all theoretical PBDE congeners  
(adapted from Ballschmiter *et al.* 1992)

Monochlorobiphenyls or Monobromodiphenyl ethers		Trichlorobiphenyls or Tribromodiphenyl ethers		Tetrachlorobiphenyls or Tetrabromodiphenyl ethers		Pentachlorobiphenyls or Pentabromodiphenyl ethers		Hexachlorobiphenyls or Hexabromodiphenyl ethers		Heptachlorobiphenyls or Heptabromodiphenyl ethers	
1	2-	37	3,4,4'-	75	2,4,4',6-	113	2,3,3',5',6-	151	2,2',3,5,5',6-	189	2,3,3',4,4',5,5'-
2	3-	38	3,4,5-	76	2',3,4,5-	114	2,3,4,4',5-	152	2,2',3,5,6,6'-	190	2,3,3',4,4',5,6-
3	4-	39	3,4',5-	77	3,3',4,4'-	115	2,3,4,4',6-	153	2,2',4,4',5,5'-	191	2,3,3',4,4',5',6-
Dichlorobiphenyls or Dibromodiphenyl ethers		Tetrachlorobiphenyls or Tetrabromodiphenyl ethers		78	3,3',4,5-	116	2,3,4,5,6-	154	2,2',4,4',5,6'-	192	2,3,3',4,5,5',6-
				79	3,3',4,5'-	117	2,3,4',5,6-	155	2,2',4,4',6,6'-	193	2,3,3',4',5,5',6-
4	2,2'-	40	2,2',3,3'-	80	3,3',5,5'-	118	2,3',4,4',5-	156	2,3,3',4,4',5-	Octachlorobiphenyls or Octabromodiphenyl ethers	
5	2,3-	41	2,2',3,4-	81	3,4,4',5-	119	2,3',4,4',6-	157	2,3,3',4,4',5'-		
6	2,3'-	42	2,2',3,4'-	Pentachlorobiphenyls or Pentabromodiphenyl ethers		120	2,3',4,5,5'-	158	2,3,3',4,4',6-	194	2,2',3,3',4,4',5,5'-
7	2,4-	43	2,2',3,5-			121	2,3',4,5',6-	159	2,3,3',4,5,5'-	195	2,2',3,3',4,4',5,6-
8	2,4'-	44	2,2',3,5'-	82	2,2',3,3',4-	122	2',3,3',4,5-	160	2,3,3',4,5,6-	196	2,2',3,3',4,4',5',6-
9	2,5-	45	2,2',3,6-	83	2,2',3,3',5-	123	2',3,4,4',5-	161	2,3,3',4,5',6-	197	2,2',3,3',4,4',6,6'-
10	2,6-	46	2,2',3,6'-	84	2,2',3,3',6-	124	2',3,4,5,5'-	162	2,3,3',4',5,5'-	198	2,2',3,3',4,5,5',6-
11	3,3'-	47	2,2',4,4'-	85	2,2',3,4,4'-	125	2',3,4,5,6'-	163	2,3,3',4',5,6-	199	2,2',3,3',4,5,5',6'-
12	3,4-	48	2,2',4,5-	86	2,2',3,4,5-	126	3,3',4,4',5-	164	2,3,3',4',5',6-	200	2,2',3,3',4,5,6,6'-
13	3,4'-	49	2,2',4,5'-	87	2,2',3,4,5'-	127	3,3',4,5,5'-	165	2,3,3',5,5',6-	201	2,2',3,3',4,5',6,6'-
14	3,5-	50	2,2',4,6-	88	2,2',3,4,6-	Hexachlorobiphenyls or Hexabromodiphenyl ethers		166	2,3,4,4',5,6-	202	2,2',3,3',5,5',6,6'-
15	4,4'-	51	2,2',4,6'-	89	2,2',3,4,6'-			167	2,3',4,4',5,5'-	203	2,2',3,3',4,4',5,5',6-
Trichlorobiphenyls or Tribromodiphenyl ethers		52	2,2',5,5'-	90	2,2',3,4',5-	128	2,2',3,3',4,4'-	168	2,3',4,4',5',6-	204	2,2',3,3',4,4',5,6,6'-
		53	2,2',5,6'-	91	2,2',3,4',6-	129	2,2',3,3',4,5-	169	3,3',4,4',5,5'-	205	2,3,3',4,4',5,5',6-
16	2,2',3-	54	2,2',6,6'-	92	2,2',3,5,5'-	130	2,2',3,3',4,5'-	Heptachlorobiphenyls or Heptabromodiphenyl ethers		Nonachlorobiphenyls or Nonabromodiphenyl ethers	
17	2,2',4-	55	2,3,3',4-	93	2,2',3,5,6-	131	2,2',3,3',4,6-				
18	2,2',5-	56	2,3,3',4'-	94	2,2',3,5,6'-	132	2,2',3,3',4,6'-	170	2,2',3,3',4,4',5-	206	2,2',3,3',4,4',5,5',6-
19	2,2',6-	57	2,3,3',5-	95	2,2',3,5',6-	133	2,2',3,3',5,5'-	171	2,2',3,3',4,4',6-	207	2,2',3,3',4,4',5,6,6'-
20	2,3,3'-	58	2,3,3',5'-	96	2,2',3,6,6'-	134	2,2',3,3',5,6-	172	2,2',3,3',4,5,5'-	208	2,2',3,3',4,5,5',6,6'-
21	2,3,4-	59	2,3,3',6-	97	2,2',3',4,5-	135	2,2',3,3',5,6'-	173	2,2',3,3',4,5,6-	Decachlorobiphenyls or Decabromodiphenyl ether	
22	2,3,4'-	60	2,3,4,4'-	98	2,2',3',4,6-	136	2,2',3,3',6,6'-	174	2,2',3,3',4,5,6'-		
23	2,3,5-	61	2,3,4,5-	99	2,2',4,4',5-	137	2,2',3,4,4',5-	175	2,2',3,3',4,5',6-	209	2,2',3,3',4,4',5,5',6,6'-
24	2,3,6-	62	2,3,4,6-	100	2,2',4,4',6-	138	2,2',3,4,4',5'-	176	2,2',3,3',4,6,6'-	General PCB Structure 	
25	2,3',4-	63	2,3,4',5-	101	2,2',4,5,5'-	139	2,2',3,4,4',6-	177	2,2',3,3',4',5,6-		
26	2,3',5-	64	2,3,4',6-	102	2,2',4,5,6'-	140	2,2',3,4,4',6'-	178	2,2',3,3',5,5',6-	General PBDE Structure 	
27	2,3',6-	65	2,3,5,6-	103	2,2',4,5',6-	141	2,2',3,4,5,5'-	179	2,2',3,3',5,6,6'-		
28	2,4,4'-	66	2,3',4,4'-	104	2,2',4,6,6'-	142	2,2',3,4,5,6-	180	2,2',3,4,4',5,5'-		
29	2,4,5-	67	2,3',4,5-	105	2,3,3',4,4'-	143	2,2',3,4,5,6'-	181	2,2',3,4,4',5,6-		
30	2,4,6-	68	2,3',4,5'-	106	2,3,3',4,5-	144	2,2',3,4,5',6-	182	2,2',3,4,4',5,6'-		
31	2,4',5-	69	2,3',4,6-	107	2,3,3',4',5-	145	2,2',3,4,6,6'-	183	2,2',3,4,4',5',6-		
32	2,4',6-	70	2,3',4',5-	108	2,3,3',4,5'-	146	2,2',3,4',5,5'-	184	2,2',3,4,4',6,6'-		
33	2',3,4-	71	2,3',4',6-	109	2,3,3',4,6-	147	2,2',3,4',5,6-	185	2,2',3,4,5,5',6-		
34	2',3,5-	72	2,3',5,5'-	110	2,3,3',4',6-	148	2,2',3,4',5,6'-	186	2,2',3,4,5,6,6'-		
35	3,3',4-	73	2,3',5',6-	111	2,3,3',5,5'-	149	2,2',3,4',5',6-	187	2,2',3,4',5,5',6-		
36	3,3',5-	74	2,4,4',5-	112	2,3,3',5,6-	150	2,2',3,4',5,6'-	188	2,2',3,4',5,6,6'-		



**Figure 1.** Chemical structures of non-PBDE flame retardants currently under investigation: **A**, 1,2-bis-(2,4,6-tribromophenoxy) ethane (BTBPE); **B**, pentabromoethylbenzene (PBEB); **C**, pentabromobenzyl bromide (PBBB); **D**, decabromodiphenyl ethane (DBDPE); **E**, pentabromo allyl ether (PBAE); **F**, 2,4,6-tribromo allyl ether (TBAE); **G**, pentabromotoluene (PBT); **H**, hexabromobenzene (HBB); **I**, pentabromobenzyl acrylate (PBBA); **J**, tetrabromo-p-xylene (pTBX); **K**, octabromo-1,3,3-trimethyl-1-phenyl indane (OBTMI); **L**, *syn*-Dechlorane Plus (*syn*-DP); **M**, *anti*-Dechlorane Plus (*anti*-DP); **N**, 1,2,5,6-tetrabromocyclooctane (TBCO); **O/P**, alpha and beta-1,2-dibromo-4-(1,2-dibromoethyl)-cyclohexane ( $\alpha$ - and  $\beta$ -TBECH) respectively; **Q/R/S**, alpha-, beta- and gamma-hexabromocyclododecane ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCD) respectively; **T**, pentabromochlorocyclohexane; **U**, tetrabromochlorotoluene; **V**, 1,1',3,4,6'-pentabromobiphenyl (BB-101); **W**, 1,2,4,5-tetrabromobenzene (1,2,4,5-tetraBB); **X**, 1,2,3,5 tetrabromobenzene (1,2,3,5-tetraBB); **Y**, 1,2,4-tribromobenzene (1,2,4-triBB); **Z**, 1,3,5-tribromobenzene (1,3,5-triBB); **AA**, 1,2-dibromobenzene (1,2-diBB); **AB**, 1,4-dibromobenzene (1,4-diBB). The hydrogen atoms have been omitted for simplicity.

Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	11 of 51

**Table 5. List of Abbreviations**  
(In addition to those listed in Tables 1, 2 and 3)

DCM	dichloromethane
ECNI	electron capture negative ionization
EI	electron impact
GC	gas chromatograph
GPC	gel permeation chromatography
HP	Hewlett-Packard
HPC	halogenated phenolic compound
HPLC	high performance liquid chromatography
I.D.	inner diameter
MSD	mass selective detector
MS	mass spectrometer
OPR	ongoing precision and recovery
PFTBA	perfluorotributylamine
PFDTD	perfluorodimethyltrioxado-decane
QA/QC	quality assurance/quality control
%R	percent recovery
RRF	relative response factor
SIM	selected ion monitoring
SPE	solid phase extraction
SRM	standard reference material
TMP	2,2,4-trimethyl pentane

### 3.4. Standard Solutions

**3.4.1. PBDE, BFR, DP stock solutions, many at 50 ppm conc. in nonane** (see Tables 1, 2 and 3 for exact stock conc.) *Note:* The original solutions provided by Wellington Labs, Sigma-Aldrich, etc. were packaged in amber glass ampoules. The ampoules were broken and the solutions were transferred in chemically cleaned 1.5 mL Certan Vials (CE015). The weight of the vial was recorded without the cap and it must be recorded before and after every future use. Store the amber Certan vial at 4°C (in the dark) until further use.

**3.4.2. Calibration solution #1 of mixed PBDEs/BFRs/DPs (1000 pg/μL each in TMP)**

For the BFRs listed in Table 6, the necessary volume of each of original stock solutions are transferred into a 10 mL Certan vial. Also, necessary volumes of

the internal standard stock solutions of BDE30, BDE156 and <sup>13</sup>C<sub>12</sub>-BDE209 are added. Dilute with TMP to 2.5 mL to achieve individual BFR standard concentrations of around 1000 pg/μL. This solution is mixed well at room temperature and away from light and heat. Store the amber Certan vial at 4°C (in the dark) until further use.

**Table 6: Composition of PBDE, BFR and DP Calibration Standard Solution # 1**

Congener	Stock Conc. (pg/μL)	Stock Vol. Added (μL)	Calibration Solution Concentration (pg/μL)
BDE30*	50000	50	1000
BDE54	50000	50	1000
BDE77	50000	50	1000
BDE139	50000	50	1000
BDE140	50000	50	1000
BDE156*	50000	50	1000
BDE170	50000	50	1000
BDE171	50000	50	1000
BDE179	50000	50	1000
BDE180	50000	50	1000
BDE184	50000	50	1000
BDE194	50000	50	1000
BDE195	50000	50	1000
BDE196	50000	50	1000
BDE197	50000	50	1000
BDE201	50000	50	1000
BDE202	50000	50	1000
BDE205	50000	50	1000
BDE207	50000	50	1000
BDE208	50000	50	1000
BDE209	50000	50	1000
<sup>13</sup> C <sub>12</sub> -BDE209*	50000	50	1000
Total Volume (mL)		1.1	2.5

\* Denotes Internal Standard

**3.4.3. Calibration solution #2 of mixed PBDEs (1000 pg/μL each in TMP)**

For the BFRs listed in Table 7, the necessary volume of each of original stock solutions are transferred into a 10 mL Certan vial. Also, necessary volumes of the internal standard stock solutions of BDE30, BDE156, <sup>13</sup>C<sub>12</sub>-BDE209 are added. Dilute with TMP to 2.5 mL to achieve individual BFR standard concentrations of approximately 1000 pg/μL. This solution is mixed well at room temperature and away from light and heat. Store the amber Certan vial at 4°C (in the dark) until further use.

**Table 7: Composition of PBDE and BFR Calibration Standard Solution # 2**

Congener	Stock Conc. (pg/μL)	Stock Vol. Added (μL)	Calibration Solution Conc. (pg/μL)
BDE17	50000	50	1000
BDE25	50000	50	1000
BDE28	50000	50	1000
BDE30*	50000	50	1000
BDE47	50000	50	1000
BDE49	50000	50	1000
BDE66	50000	50	1000
BDE71	50000	50	1000
BDE75	50000	50	1000
BDE85	50000	50	1000
BDE99	50000	50	1000
BDE100	50000	50	1000
BDE116	50000	50	1000
BDE119	50000	50	1000
BDE138	50000	50	1000
BDE153	50000	50	1000
BDE154	50000	50	1000
BDE155	50000	50	1000
BDE156*	50000	50	1000
BDE181	50000	50	1000
BDE183	50000	50	1000
BDE190	50000	50	1000
BDE191	50000	50	1000
BDE203	50000	50	1000

BDE206	50000	50	1000
<sup>13</sup> C <sub>12</sub> -BDE209*	50000	50	1000
Total Volume (mL)		1.3	2.5

\* Denotes Internal Standard

### 3.4.4. Calibration solution #3 of mixed non-PBDE BFRs and DPs (1000 pg/μL each in TMP)

For the BFRs listed in Table 8, the necessary volume of each of original stock solutions are transferred into a 10 mL Certan vial. Also, necessary volumes of the internal standard stock solutions of BDE30, BDE156, <sup>13</sup>C<sub>12</sub>-BDE209 are added. Dilute with TMP to 2.5 mL to achieve individual BFR standard concentrations of around 1000 pg/μL. This solution is mixed well at room temperature and away from light and heat. Store the amber Certan vial at 4°C (in the dark) until further use.

**Table 8: Composition of PBDE and BFR Calibration Standard Solution # 3**

Congener	Stock Conc. (pg/μL)	Stock Vol. Added (μL)	Calibration Solution Concentration (pg/μL)
BTBPE	50 000	50	1000
PBEB	50 000	50	1000
PBBB	50 000	50	1000
DBDPE	25 000	400	4000
TBPAAE	61 500	50	1230
PBPAAE	70 000	50	1400
HBB	50 000	50	1000
PBB	100 000	50	2000
PBBA	50 000	50	1000
PTBX	50 000	50	1000
OBTMI	50 000	50	1000
<i>syn</i> -DP	50 000	50	1000
<i>anti</i> -DP	50 000	50	1000
TBCO	50 000	50	1000
PBT*	50 000	50	1000
PBCCH	100 000	50	2000
TBCT	100 000	50	2000

α-TBECH	50 000	50	1000
β-TBECH	50 000	50	1000
α-HBCD	50 000	50	1000
BB101	50 000	50	1000
BB153	50 000	50	1000
BDE-30	50 000	50	1000
BDE-156	50 000	50	1000
BDE-205	50 000	50	1000
<sup>13</sup> C <sub>12</sub> BDE-209	50 000	50	1000
Total Volume (mL)		1.65	2.5

**3.4.5. Working/single point calibration solution #1 of mixed PBDEs/BFRs/DPs (250 pg/μL each in TMP)**

A volume of 125 μL of calibration mixed solution #1 is transferred to a 1 mL GC vial, and 375 μL of TMP is added. This solution is mixed well at room temperature and away from light and heat. A 500 μL working/single point calibration solution results where each of the standard and internal standard concentrations are 250 pg/μL, with the exception of TBECH which is 500 pg/μL. Store the amber Certan vial at 4°C (in the dark) until further use.

**3.4.6. Working/single point calibration solution #2 of mixed PBDEs (250 pg/μL each in TMP)**

A volume of 125 μL of calibration mixed solution #2 is transferred to a 1 mL GC vial, and 375 μL of TMP is added. This solution is mixed well at room temperature and away from light and heat. A 500 μL working/single point calibration solution results where each of the standard and internal standard concentrations are 250 pg/μL. Store the amber Certan vial at 4°C (in the dark) until further use.

**3.4.7. Working/single point calibration solution #3 of mixed non-PBDE BFRs and DP (250 pg/μL each in TMP)**

A volume of 125 μL of calibration mixed solution #2 is transferred to a 1 mL GC vial, and 375 μL of TMP is added. This solution is mixed well at room temperature and away from light and heat. A 500 μL working/single point calibration solution results where each of the standard and internal standard concentrations are 250 pg/μL. Store the amber Certan vial at 4°C (in the dark) until further use.

Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	16 of 51

#### 3.4.8. Intermediate mixed internal standard solution (1000 pg/μL in TMP)

A volume of 50 μL each of the 50000 pg/μL stock solutions of the internal standards BDE30, BDE156 and <sup>13</sup>C<sub>12</sub>-BDE209 are transferred into a 10 mL Certan vial. The volume in the Certan vial is adjusted to 2.5 mL with TMP to give a final conc. of 1000 pg/μL for each of the three internal standards. Mix thoroughly by vortexing. Store the amber Certan vial at 4°C (in the dark) until further use.

#### 3.4.9. Working internal standard spiking solution (250 pg/μL in TMP)

A volume of 1500 μL of the intermediate internal standard solution of BDE30, BDE156 and <sup>13</sup>C<sub>12</sub>-BDE209 is transferred into a 10 mL Certan vial. The volume in the Certan vial is adjusted to 6 mL with TMP to give a final conc. of 250 pg/μL for each of the three internal standards. Mix thoroughly by vortexing. If 100 μL sample spikes are used, there is sufficient volume for 60 spikes. Store the amber Certan vial at 4°C (in the dark) until further use.

#### 3.4.10. Standard solutions for the calibration curve

The calibration curves (for 3 mixtures) are composed of 8 concentration levels (e.g., 1000, 500, 250, 125, 50, 25, 10 and 2 pg/μL) of target compounds that span the range of the anticipated analyte concentrations in the samples. Each standard contains a fixed concentration of 250 pg/μL of each of the three internal standards. A calibration curve is run periodically with a sample batch to confirm that the use of a single point calibration is analyte quantification.

***NOTE: The external standard calibration solutions made be prepared first, and then the same amount and final concentration of internal standards spiked after, so that the concentration of all internal standards are the same in all calibration solutions.***

#### 3.4.10. Mass Spectrometer Calibration Standards

Perfluorodimethyltrioxadodecane (PFDTD) For GC-MSD 5973 with CI (chemical ionization) Source. Catalogue #: 8500-8130 from Agilent.

### 3.5. QA Reference Material

**3.5.1.** For egg samples, to assess reproducibility (precision) of analyses, an in-house standard reference material (SRM) of double-crested cormorant (*Phalacrocorax auritus*) (DCCO) egg homogenate is used, or alternately a 10 time diluted herring gull egg homogenate (SRM 8929). In 2003, CWS collected 160 individual eggs from double-crested cormorant (DCCO) nests at

<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>17 of 51</b>

the Scotch Bonnet Island colony (Lake Ontario), combined and homogenized them to generate a very large sample pool. The DCCO SRM (SRM K03-67609-00) was chosen for the present study since it is reflective of recent, naturally-contaminated PBDEs and other BFRs in a top predator avian species. Along with PCBs and several organochlorine pesticides, 14 PBDE congeners (BDE-17, -28, -47, -49, -66, -85, -99, -100, -138, -153, -154, -183 and -190) and including BDE209, were recently assessed in the DCCO SRM.

- 3.5.2.** NWRC Polar Bear Plasma Pool. Non-certified. Validated as an in-house SRM for precision only, as it has not been rigorously assessed by outside labs for proper QA/QC, for any ultratrace analyte. Presently used as precision SRM for PCBs, OC pesticides, MeSO<sub>2</sub>-PCBs, PBDEs and all halogenated phenolic compounds (HPCs).
- 3.5.3.** NIST SRM 1945 (pilot whale blubber). Certified for major PBDEs (e.g., BDE-47, -99, -100 and -153).

### **3.6. Method Blank**

Sodium sulphate, spiked with the internal standard, and processed through the entire method is used as a blank.

## **4. AUXILIARY EQUIPMENT**

### **4.1. Glassware and Labware**

- 4.1.1.** Aluminum foil
- 4.1.2.** Amber glass bottle - 1 L and 4 L with Teflon lined screwed cap
- 4.1.3.** Autosampler vials – 1.5 mL, amber with silver, aluminum, red/orange crimp caps (Chromatographic Specialties, Catalogue # C781110LM (vials), C221150 (caps))
- 4.1.4.** Column glass - 3 cm ID x 50 cm long with Teflon™ stopcock and reservoir (for preparing the Na<sub>2</sub>SO<sub>4</sub>)
- 4.1.5.** Flasks, flat bottom - 125, 250 and 500 mL all with 24/40 outer joint
- 4.1.6.** Fluted funnels, glass - 65 mm I.D., 65 mm Stem length
- 4.1.7.** Glass wool (Canadawide Scientific 54100-11), pre-washed with DCM/hexane (1:1) and air dried
- 4.1.8.** GPC glass column - 3 cm I.D. x 70 cm long - Envirosep-ABC column assay (ABC Laboratories Inc., Columbia, MO, USA), packing of 46 cm.
- 4.1.9.** GPC tubes, 16 mm x 125 mm (Fisherbrand, Catalogue No. 14-961-30)
- 4.1.10.** GPC sample loop, size 4 mL.

<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>18 of 51</b>

- 4.1.11. Graduated cylinders, glass - 10, 50, 100 and 500 mL, and 1 L
- 4.1.12. Hand crimper, 8 mm - for crimping aluminum seals to autosampler vials (Chromatographic Specialties)
- 4.1.13. Mortars and pestles, glass
- 4.1.14. Pasteur pipets
- 4.1.15. Pipets, Eppendorf - 5-100 µL (with tips), 100-1000 µL (with tips), 1-5 mL (with tips)
- 4.1.16. Pipets, glass, disposable - 0.5, 1, 5 and 10 mL
- 4.1.17. Spatulas
- 4.1.18. Syringe, glass - 5 mL Hamilton™
- 4.1.19. Syringes, Hamilton™ - 10, 50, 100 and 250 µL
- 4.1.20. Volumetric flasks, glass - 10, 50 and 100 mL, and 1 L
- 4.1.21. Weighing aluminum dishes – disposable
- 4.1.22. 10 mL CERTAN™ vial (brown amber glass)
- 4.1.23. 22 mL stainless steel extraction cells, Dionex™
- 4.1.24. Cellulose filters for 22mL cells for Dionex ASE 200™
- 4.1.25. O-rings, Teflon for Dionex ASE 200™ cell cap
- 4.1.26. 40 mL amber collection vials for ASE Dionex ASE 200™

## 4.2. Equipment

- 4.2.1. Analytical balance (Sartorius BP210D) and top-loading balance (Mettler PB3002)
- 4.2.2. Vortex mixer, Fisher Scientific™
- 4.2.3. Rotary evaporator with water bath at ca 30°C (Büchi Rotavapor-R, Brinkman Instruments)
- 4.2.4. Refrigerated circulating bath at a minimum of ca -4°C (Lauda RM20)
- 4.2.5. Drying oven (Fisher Scientific, Model 516 G)
- 4.2.6. Muffle furnace (Blue M Electric Company, Blue Island, IL, USA)
- 4.2.7. Centrifuge (VWR™ Clinical 200)
- 4.2.8. Visiprep solid phase extraction vacuum manifold (Supelco 57030)
- 4.2.9. Water bath at 100°C (Blue M) - for plasma lipids
- 4.2.10. Water bath at 37°C (Precision Instruments) - for plasma lipids
- 4.2.11. Roller culture apparatus (Wheaton Instruments)

## 4.3. Instrumentation

- 4.3.1. GPC Autoprep™ 2000 (AP 2000), with automatic sample loading and sample collection unit (from O.I. Analytical) containing a collection tray capacity of sixteen (16) 250-mL round bottom flasks sample tray capacity of sixty (60)

<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>19 of 51</b>

16 x 100 mm (15 mL) tubes, a 10 mL syringe pump and sample loop of 5 mL.

- 4.3.2.** ASE™ 200 Accelerated Solvent Extraction System. Sample tray capacity of twenty-four (24 ). Sample stainless steel cell sizes can be 1, 5, 11, 22 and 33 mL. Collection bottles can be 40 or 60 mL in size. Operating pressures can range from 500 to 3000 psi.
- 4.3.3.** GC/MSD, Hewlett-Packard (HP) gas chromatograph (GC) 5890 Series II equipped with an autosampler (7673A), and linked to a Hewlett-Packard 5973 mass selective detector (MSD) equipped with Electron Capture Negative Ionization sources controlled with MS ChemStation (HP G1034C, Rev. C.02.00); GC column: 15 m DB-5 (J&W) fused silica column, 0.25 mm ID, 0.25 µm film thickness (Chromatographic Specialties, J1225711).
- 4.3.4.** Spectrophotometer (Hewlett-Packard Diode Array, Model 8452A) for total lipid determination in plasma samples.

## **5. SPECIMEN OR SAMPLE HANDLING REQUIREMENTS**

Samples provided to the Organics Research Lab are prepared as described in the Tissue Preparation Unit’s standard operating procedure SOP-TP-PROC-07. These tissues were usually collected and preserved as recommended in the document “Protocol for Field Collection and Storage of Wild Birds for Biomarker Studies” (S. Trudeau, Biomarker Laboratory, NWRC, 1992).

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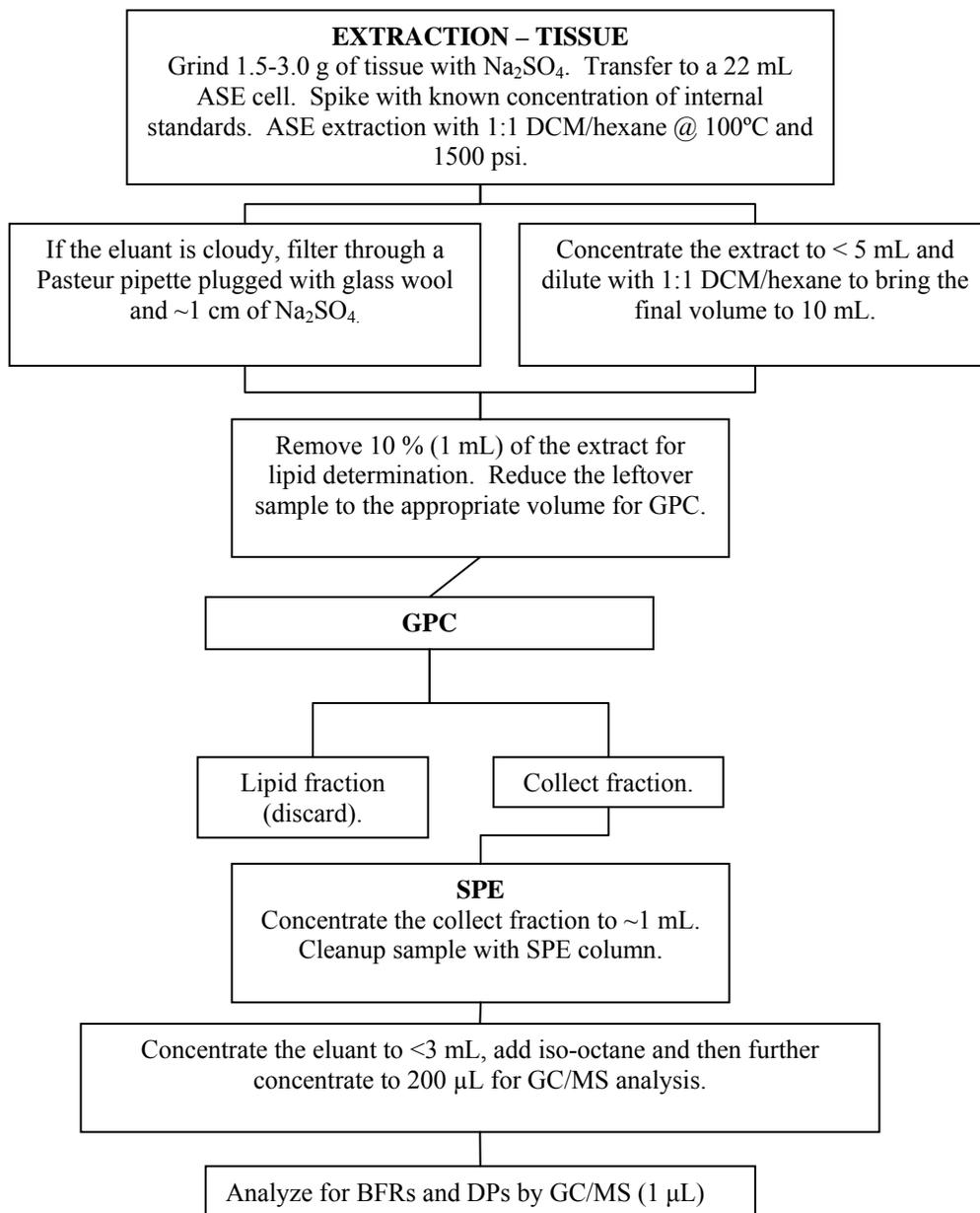
<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>20 of 51</b>

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## **6. PROCEDURE**

A summary flow chart for BFR/DP sample extraction, cleanup and analysis is shown in Figure 2. Subsequent sub-section describes the procedures in more detail.

**Figure 2: Summary Flow Diagram of Extraction, Cleanup and Analysis of BFRs/DPs from Tissue and Egg Samples**



### 6.1. Sample and Column Preparation

<p>Unit</p> <p>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</p>	<p>Environmental Chemistry/Organic Research Group (NWRC)</p> <p>Method Number : NWRC-MET-ORG-RES-BFR-ver. 4 Authorization : Dr. R.J. Letcher Date: Revision #4, November 2008 Page: 22 of 51</p>
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### 6.1.1. GPC Column

Place 70 g Envirobeads™ S-X3 in a 500 mL beaker. Cover the beads with DCM/hexane (1:1) and allow to swell overnight (a minimum of 12 h). Pack a GPC column (Section 5.1.11) with the pre-swelled beads. This material generally makes 43 to 45 cm in column length.

6.1.2 Remove tissue, plasma or egg samples and QC samples from the freezer and leave at room temperature to thaw. Thawing can also be done in a refrigerator overnight.

6.1.3 While the samples are thawing, record the USOX number of each sample and other pertinent information on the worksheet. (see Appendix E)

6.1.4 Assign the laboratory number as follows: For tissue and plasma samples : (BFR-Y-T-#### for BFR/DP compound, year of analysis, sample type and sample # 1-999; for example for liver “BFR-07-L-001”). For egg samples : (PFC-Y-E-#### for BFR/DP compound, year of analysis, egg sample and sample #1-999; for example “BFR-07-E-001”).

6.1.5 With a permanent marker, identify each of the plastic tubes with the sample lab number.

## 6.2. Sample Extraction

### 6.2.1. Eggs and All Other Tissue Samples Except Plasma and Brain

6.2.1.1. Ensure material is thawed and grind between 1.5 g to 3.0 g of the homogenized sample with ca 25 g of the treated anhydrous Na<sub>2</sub>SO<sub>4</sub> in a glass mortar and pestle until a free-flowing mixture is obtained. (*Note:* A sample size of 3 g is preferred). 25 grams of Na<sub>2</sub>SO<sub>4</sub> is used because this is the capacity of the 22 mL ASE cell (washed with acetone, then hexane). Include at least one standard reference material, an spiked method blank sample with each batch of samples (a typical batch contains 10 samples). The sample is then transferred to a 22 mL stainless steel extraction cell (after insertion of a cellulose filter), and the cell is then loaded onto the Automated Solvent Extractor System (ASE) 200 (Dionex Corp.).

6.2.1.2 Spike the contents of the ASE cell with a known volume and concentration of internal standards. Record the volume and concentration, since this will be used in calculating the concentration of your BFRs of interest.

6.2.1.3 Cap the cell tightly and then load it onto the ASE 200. High purity

Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	23 of 51

nitrogen and compressed air are the gases used to pressurize the contents of the cell. Pressure used is 1500 psi, temperature setting is at 100 °C. The extractions cycles can be set from 1 to 3 cycles, but 1 cycle is sufficient in the case of the present BFR extraction. The eluant is eluted into a tightly capped 40 mL amber glass vial (note: ensure quality of septa). Septa should be replaced after ~3 uses, when it begins to look ragged.

#### Operation Parameters of the ASE 200

Preheat time	1 minute
Heating time	5 minutes
Static period	5 minutes
Flush percent	60 % volume
Purge time	60 seconds
Extraction Cycles	1 cycle
Extraction Temperature	100°C
Extraction Pressure	1500 psi

- 6.2.1.4** If the extract is clear, then skip to section 6.2.1.5, if it has a cloudy appearance, the sample should be dried by a sodium sulphate dry column. The dry column can be a Pasteur pipette (5mL) plugged with glass wool and packed with 2-3 cm of sodium sulphate. After the extract elute through the dry column, the column is then eluted with 5 mL more of 1:1 DCM/hexane. Collect the extract into a 125 mL round bottom flask and then do section 6.2.1.6.
- 6.2.1.5** Transfer the extract (~30 mL) from the collection vial to a 125 mL round bottom flask. Rinse the collection vial 3 times with 1:1 DCM/hexane, adding the rinses to the round bottom flask. Alternately, with a large sample set, while still in 40 mL amber glass vials, solvent can be reduced using the N-Evap. From here, the extract can be quantitatively transferred to a 15 mL graduated centrifuge tube, rinsing 3 times with 1:1 DCM:HEX and vortexing in between. If moisture is observed (this likelihood increases with increasing sample size, add a little sodium sulfate and vortex with each wash.
- 6.2.1.6** Concentrate the eluant to less than 5 mL on a rotary evaporator with the water bath temperature at 30°C. Quantitatively transfer the concentrate to a 15 mL graduated conical tube. Rinse the flask 3

<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>24 of 51</b>

times with 1:1 DCM:hexanes, and add the rinsing into the tube

- 6.2.1.7.** If the final volume of the sample is greater than 10 mL, then concentrate the sample to exactly 10 mL using a nitrogen evaporator. If the volume of the sample is less than 10 mL, rinse the round bottom flask with 1:1 DCM/hexane and transfer enough of it to the Pasteur pipette and elute into the conical vial until the total volume is 10 mL.
- 6.2.1.8.** Transfer 1 mL (10 % of the total volume) from the graduated centrifuge tube and into a pre-weighed aluminum dish for gravimetric lipid determination (8.5.1).
- 6.2.1.9.** If the results of the gravimetric determination show that the sample contains greater than 2 % lipid, then the sample must be cleaned up using GPC. Otherwise the sample can be cleaned up with SPE directly (6.4)
- 6.2.1.10.** Reduce the remaining 90 % of the sample solution to 2.0 mL under a gentle stream of nitrogen and transfer this to a GPC tube. Rinse the sample tube with two successive 1.0 mL washes of DCM, and make the total sample volume to 4.0 mL with 1:1 DCM:HEX. Follow the section for sample GPC cleanup procedure (6.3).
- 6.2.1.11.** After GPC cleanup, rotary-evaporate the sample to 0.5 to 1.0 mL

## **6.2.2. Plasma Samples**

- 6.2.2.1.** Accurately weigh about 3 g aliquot of the thawed plasma into a 50 mL screw-top centrifuge tube. Spike with a known volume and concentration of internal standards. Mix the spiked plasma gently with a vortex mixer, and let it stand for 30 minutes to equilibrate.
- 6.2.2.2.** Add 1 mL of 6M HCl and vortex for 30 seconds. Add 3 mL of 2-propanol and vortex for another 30 seconds.
- 6.2.2.3.** Add 6 mL of MtBE: Hexane (50:50), replace cap, vortex for 1 minute and then sonicate in an ultrasonic bath for 20 minutes. Vortex for 30 seconds (to wash down sides) and then centrifuge at

<b>Unit</b>  <b>Methods Manual</b> <b>Wildlife Toxicology &amp; Disease Program</b> <b>NWRC</b>	<b>Environmental</b> <b>Chemistry/Organic</b> <b>Research Group (NWRC)</b>  <b>Method Number</b> <b>NWRC-MET-ORG-RES-BFR-ver. 4</b> <b>Authorization :</b> <b>Dr. R.J. Letcher</b> <b>Date:</b> <b>Revision #4, November 2008</b> <b>Page:</b> <b>25 of 51</b>
---	---

1000 rpm for 10 minutes. Transfer the organic phase into a 60 mL separatory funnels. Repeat these steps 2 more times and combine the organic extract in the funnel.

- 6.2.2.4.** Add 6 mL of 1% KCl (wt./vol.) to the separatory funnel and shake for 1 minute. Drain off aqueous layer (discard) and collect the organic phase in a 125 mL round bottom flask. Rotary-evaporate the sample to ~ 1.0 mL and transfer it into a 60 mL separatory funnel (rinse with hexane).

**\*\*NOTE\*\*** At this work-up stage, it is possible to fractionate the neutral and phenolic contaminant containing fraction. To do this, add 6 mL of 1.0 M KOH (5.611 g in 100 mL) in 50:50 ethanol (95%) and water (Milli-Q water or hexane washed deionized water). Mix for 1 minute. Rinse down sides and stopper with hexane. Transfer aqueous phase into a 50 mL screw-top centrifuge tube. Repeat this entire procedure two more times. Use the organic phase to analyze for the target compounds.

### **6.2.3. Brain samples**

- 6.2.3.1.** Homogenize approximately 1 g of brain tissue with 8-10 grams of treated anhydrous Na<sub>2</sub>SO<sub>4</sub> (Section 3.2.1.) in a glass mortar and pestle until a free-flowing mixture is obtained.
- 6.2.3.2.** Transfer the sample into a pre-cleaned extraction thimble, which already contains 5 gram of anhydrous Na<sub>2</sub>SO<sub>4</sub>. Another 5 grams of anhydrous Na<sub>2</sub>SO<sub>4</sub> is added on top of the sample.
- 6.2.3.3.** Spike with internal standard solution into the thimble.
- 6.2.3.4.** Sample is extracted (Soxhlet) for 8 hours with 150 ml hexanes-acetone (1-1), and extract is concentrated by rotary evaporation to app. 10 ml, and transferred to a 30ml centrifuge tube.
- 6.2.3.5** 10% of the extract is transferred to a cleaned aluminum dish for gravimetric lipid determination (6.2.1.9), remaining extract concentrated to ~2 mL.
- 6.2.3.6.** Extract the hexanes solution twice with 2 ml conc. H<sub>2</sub>SO<sub>4</sub> (30 sec

<p>Unit</p> <p>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</p>	<p>Environmental Chemistry/Organic Research Group (NWRC)</p> <p>Method Number : NWRC-MET-ORG-RES-BFR-ver. 4 Authorization : Dr. R.J. Letcher Date: Revision #4, November 2008 Page: 26 of 51</p>
--	--

vortex), the acid phase is separated by centrifugation 4000 rpm, 30 min.

- 6.2.3.7.** Acid phase is backwashed 3 times with 4 ml hexanes, separated by centrifugation at 4000 rpm, 30 min. All hexanes fraction are combined. Continue the process as with other tissues from 6.2.1.4 to 6.2.1.11.

### 6.3. Sample Cleanup by GPC

- 6.3.1.** Calibrate the GPC system when the column is changed, when channeling occurs, when column drying has occurred or when recoveries are not acceptable. Refer to the GPC operating manual for specific instructions.
- 6.3.2.** Place the GPC tubes onto the tube rack and collection flasks on the instrument tray.
- 6.3.3.** Set GPC flow-rate to 5 mL/min of DCM/hexane (1:1), the “dump” time to 28 mins, the “collect” time to 40 mins and the wash time to 3 mins. Initiate the operation of the GPC. *Note:* It is possible to load and run as many as 16 samples simultaneously (typically 12-16 are run). The sequence can be run overnight.
- 6.3.4.** Evaporate the collected eluant from 6.3.3 to ~ 0.5 to 1.0 mL on a rotary evaporator. The sample is now ready for solid phase extraction (SPE) cleanup.

### 6.4. Sample Cleanup by SPE

- 6.4.1.** Sample extract cleaned is accomplished using a LC-Si SPE cartridge (500 mg X 6 mL; 6 gram; J.T.Baker, USA).
- 6.4.2.** After conditioning the column with 1) 6 mL of 10 % methanol in DCM and then 2) 8 mL of 5 % DCM in hexane, the sample is loaded on the cartridge, the tube is rinsed with 5% DCM/hexane (v/v) (and the rinsings added to the cartridge, and then the cartridge is eluted with 8 mL of 5 % DCM/hexane (v/v) into a 16 × 100 mm disposable test tube.
- 6.4.3.** The eluant is concentrated under a gentle stream of nitrogen and solvent exchanged with isoctane to a final volume of approximately 200 µL (if necessary confirm by exact mass) for GC/MS determination.

Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	27 of 51

## 6.5 GC Operating Conditions

### 6.5.1. Instruments and Columns

**6.5.1.1.** Agilent gas chromatograph (GC) 6890 equipped with a 5973 quadrupole mass spectrometer (MS) detector

**6.5.1.2.** The GC column is a was a 15 m × 0.25 mm×0.10 μm DB-5HT (J&W) fused-silica capillary column.

### 6.5.2. Injection Information

♦ Injection port temperature	280 °C
♦ Location	Front
♦ Sample washes	3
♦ Sample pumps	3
♦ Sample volume	1 μL
♦ Viscosity delay	1 s
♦ Solvent washes	5A, 5B
♦ Splitless injection	YES (pulsed)
♦ On column	NO
♦ Injection pulse	25.0 psi until 0.50 min.
♦ Purge A on	1.5 min

### 6.5.3. Oven Temperature Program

**6.5.3.1** 100°C, hold 2 min.; 25°C/min to 260°C; 1.5°C/min to 280°C; 25°C/min to 325°C hold for a final 7 min; total run time 30.50 min

### 6.5.4. Carrier gas (He)

Head pressure	0.85 psi (with variance due to carrier gas flow rate)
Carrier Gas Flow Rate	0.9 mL/min
Purge Gas Flow Rate	96.4 mL/min (to 2 min.)
Total Gas Flow Rate	100 mL/min

## 6.6. MSD Operating Conditions



## 6.9. GC-MSD Parameters

**Table 9: Retention Time and Target Ions for PBDEs and Internal Standards**

BROMINATED FLAME RETARDANTS <sup>A</sup>	COMPOUND NAME	RT (MINS)	TARGET <sup>b</sup> (M/Z)
BDE17	2,2',4-TRIBDE	6.744	79+81
BDE25	2,3',4- TRIBDE	6.850	79+81
BDE28 <sup>C</sup>	2,4,4'-TRIBDE	6.967	79+81
BDE30 (I.S.)	2,4,6-TRIBDE	6.467	79+81
BDE33 <sup>C</sup>	2',3,4-TRIBDE	6.967	79+81
BDE47	2,2',4,4'-TETRABDE	7.833	79+81
BDE49	2,2',4,5'-TETRABDE	7.693	79+81
BDE66	2,3',4,4'-TETRABDE	7.728	79+81
BDE71	2,3',4',6-TETRABDE	7.657	79+81
BDE75	2,4,4',6-TETRABDE	8.134	79+81
BDE77	3,3'4,4'-TETRABDE	9.222	79+81
BDE85	2,2',3,4,4'-PENTABDE	8.738	79+81
BDE99	2,2',4,4',5-PENTABDE	8.508	79+81
BDE100	2,2',4,4',6-PENTABDE	8.840	79+81
BDE116	2,3,4,5,6-PENTABDE	8.582	79+81
BDE119	2,3',4,4',6-PENTABDE	10.810	79+81
BDE138	2,2',3,4,4',5'-HEXABDE	10.26	79+81
BDE139	2,2',3,4,4',6-HEXABDE	10.408	79+81
BDE140	2,2',3,4,4',6'-HEXABDE	10.034	79+81
BDE153	2,2',4,4',5,5'-HEXABDE	9.542	79+81
BDE154	2,2',4,4',5,6'-HEXABDE	9.542	79+81
BB153	2,2',4,4',5,5'- TETRABB	9.542	79+81
BDE155	2,2',4,4',6,6'-HEXABDE	9.32	79+81
BDE156 (I.S.)	2,3,3',4,4',5-HEXABDE	11.177	79+81

Unit		Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC		Method Number Authorization : Date: Page:	NWRC-MET-ORG-RES-BFR-ver. 4 Dr. R.J. Letcher Revision #4, November 2008 30 of 51
BDE170	2,2',3,3',4,4',5-HEPTABDE	11.774	79+81
BDE171	2,2',3,3',4,4',6-HEPTABDE	13.124	79+81
BDE179	2,2',3,3',5,6,6'-HEPTABDE	13.467	79+81
BDE180	2,2',3,4,4',5,5'-HEPTABDE	12.027	79+81
BDE181	2,2',3,4,4',5,6-HEPTABDE	11.633	79+81
BDE183	2,2',3,4,4',5',6-HEPTABDE	13.678	79+81
BDE184	2,2',3,4,4',6,6'-HEPTABDE	12.710	79+81
BDE190	2,3,3',4,4',5,6-HEPTABDE	15.458	79+81
BDE191	2,3,3',4,4',5',6-HEPTABDE	15.213	79+81
BDE194	2,2',3,3',4,4',5,5'-OCTABDE	14.914	79+81
BDE195	2,2',3,3',4,4',5,6-OCTABDE	15.774	79+81
BDE196	2,2',3,3',4,4',5',6-OCTABDE	16.240	79+81
BDE197	2,2',3,3',4,4',6,6'-OCTABDE	17.552	409+407
BDE201	2,2',3,3',4,5',6,6'-OCTABDE	17.319	409+407
BDE202	2,2',3,3',5,5',6,6'-OCTABDE	17.210	409+407
BDE203	2,2',3,4,4',5,5',6-OCTABDE	18.974	79+81
BDE205	2,3,3',4,4',5,5',6-OCTABDE	18.974	79+81
BDE206	2,2',3,3',4,4',5,5',6-NONABDE		79+81
BDE207	2,2',3,3',4,4',5,6,6'-NONABDE		484+486
BDE208	2,2',3,3',4,5,5',6,6'-NONABDE		484+486
BDE209	2,2'3,3',4,4',5,5',6,6'-DECABDE		484+486
<sup>13</sup> C <sub>12</sub> BDE209	<sup>13</sup> C <sub>12</sub> -2,2'3,3',4,4',5,5',6,6'-DECABDE		494+496
BTBPE	1,2,-Bis-(2,4,6-TriBromoPhenoxy)Ethane		79+81
PBEB	PentaBromoEthyl Benzene		79+81
PBBB	PentaBromoBenzyl Bromide		79+81
DBDPE	DecaBromoDiphenyl Ethane		79+81
TBPAE	2,4,6-TriBromoPhenyl Allyl Ether		79+81
PBPAE	PentaBromoPhenyl Allyl Ether		79+81
HBB	HexaBromoBenzene		79+81

Unit		Environmental Chemistry/Organic Research Group (NWRC)
Methods Manual Wildlife Toxicology & Disease Program NWRC		Method Number : Authorization : Date: Page:
		NWRC-MET-ORG-RES-BFR-ver. 4 Dr. R.J. Letcher Revision #4, November 2008 31 of 51
PBB	PENTABROMOBENZENE	79+81
PBBA	PentaBromoBenzyl Acrylate	79+81
PTBX	TetraBromo-p-Xylene	79+81
OBTMI	OctaBromo-1,3,3-TriMethyl-1-phenyl Indane	79+81
<i>syn</i> -DP	<i>syn</i> -Dechlorane Plus	652+654
<i>anti</i> -DP	<i>anti</i> -Dechlorane Plus	652+654
TBCO	1,2,5,6-TetraBromoCycloOctane	79+81
PBT*	PentaBromoToluene	79+81
PBCH	PentaBromoChlorocycloHexane	79+81
TBCT	TetraBromoChloroToluene	79+81
$\alpha$ -TBECH	1,2-dibromo-4-(1,2-dibromoethyl)-cyclohexane	79+81
$\beta$ -TBECH	1,2-dibromo-4-(1,2-dibromoethyl)-cyclohexane	79+81
$\alpha$ -HBCD	$\alpha$ -HexaBromoCycloDodecane	79+81
BB101	2,2',4,5,5'-pentaBromoBiphenyl	79+81
BB153	2,2',4,5,5'-hexaBromoBiphenyl	79+81

*a* Target ions – mass-to-charge ratio (*m/z*) of the quantifier and quantifier ion, respectively. Quantification based on the TIC-ECNI response of these two anions and ion the case of BDE207, BDE208 and BDE209 *m/z* 484 and 486 (bromophenoxy isotope anions).

*b* BDE-28 and BDE-33 coelute and thus their peak areas are combined.

## 7. MS Tuning

For detailed instructions on the operation of the instruments, consult the equipment operator manuals

**7.1.** Tune the mass spectrometer prior to sample acquisition with a PFDTD calibration standard for GC-MSD-NCI. Detailed procedure and acceptance criteria are described in SOP-CHEM-PROC-12.

**7.2.** Print hard copies of the tuning data. An example is given in **Figure 3**

## 8. GC/MS Sequence Set Up

### 8.1. Analysis of Standards and Samples

**8.2.1.** In an autosampler vial, combine a volume of the internal standard solution spike used in the procedure as well as a volume of the PBDE/PBFR calibration solution(s). Cap the vial and vortex to ensure mixing. Record information for

Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	32 of 51

sample run in BFR/DP analysis worksheet (Appendix F). *Note:* The combined solution should be prepared just prior to injection - used for PBDE/BFR/DP quantitation.

- 8.2.2.** Establish the operating conditions given in Sections 6.5-6.6.
- 8.2.3.** Prepare sequence injection table as described in SOP-CHEM-PROC-10C. A typical sequence would consist of the following chemical fraction solutions:

Blank solvent  
 PBDE/BFR/DP quantification standard (3.4)  
 Blank solvent  
 QA/QC SRM  
 Method blank  
 Samples (a series of 5)  
 Duplicate injection  
 Blank solvent \_\_\_\_\_

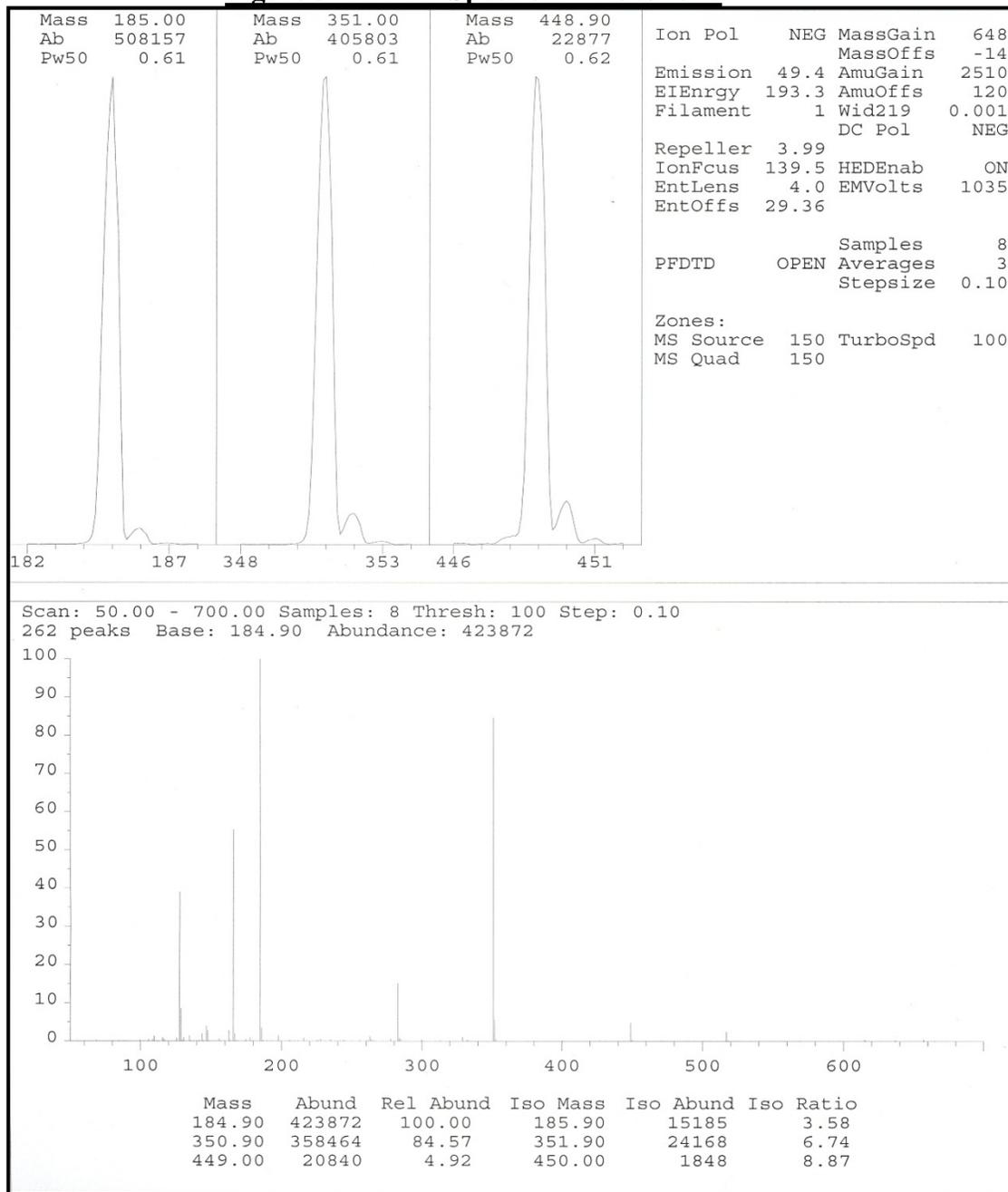
Unit

Environmental  
Chemistry/Organic  
Research Group (NWRC)

Methods Manual  
Wildlife Toxicology & Disease Program  
NWRC

Method Number      NWRC-MET-ORG-RES-BFR-ver. 4  
Authorization :      Dr. R.J. Letcher  
Date:                    Revision #4, November 2008  
Page:                    33 of 51

Figure 3: PFDTD Spectrum – AutoTune



Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	34 of 51

**8.2.4** Start the injection sequence.

**8.2.5.** Once the sequence is completed, calculate and print the results as described in SOP-CHEM-DOC-06.

## **8.2. Initial Calibration Curve**

**8.2.1.** To calibrate the analytical system and determine linearity, inject sequentially, 1 µL of the 8 calibration standard in increasing order of concentration for each of the standard mixtures #1 and #2, using the instrument conditions.

**8.2.2.** Repeat these initial calibrations whenever new calibration standard solutions are prepared or if the analytical acceptance criteria for the daily, single point, calibration verification standard have not been met.

## **8.3. Moisture Determination**

**8.3.1.** Accurately weigh ca 0.5 g of sample (tissue homogenate) into a pre-weighed aluminum dish and record the weight to 5 decimal places.

**8.3.2.** Place the dish in a drying oven at 105°C for about two hours, until constant weight is obtained.

**8.3.3.** The calculation of the moisture content is as follows:

$$\% \text{ moisture} = 100 - (W_d/W_w) \times 100$$

where:  $W_d$  = weight of dry sample  
 $W_w$  = weight of wet sample

## **8.4. Olive Oil Standard**

Used for plasma lipid determination. Add about 150 mg of olive oil to a 10 mL volumetric flask and dilute to volume with ethyl alcohol. Mix well and store at 4°C. Shelf life: one month. For each set of assays, prepare 4 calibration standards to have concentration ranging from 30 to 150 mg/10 mL by diluting the olive oil stock solution in ethyl alcohol.

## **8.5. Lipid Determination**

Lipid levels are determined to allow calculations of contaminants based on lipid content instead of wet-weight. For plasma samples, see Section 8.5.2.2.

Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	35 of 51

### 8.5.1. Egg and tissue samples

- 8.5.1.1.** Allow solvent in the aluminum dish (prepared in Section 7.2.1.7) to evaporate to dryness in the fume hood.
- 8.5.1.2.** Heat dish in oven at 105°C for 10 min or overnight.
- 8.5.1.3.** Take dish out of the oven, allow to cool, then reweigh the dish. Record the difference in weight as the weight of lipids in the sample. The calculation of the lipid content is as follows:

$$\% \text{ lipids} = [(W_1 \times V_1) / (W_{te} \times V_{tl})] \times 100$$

where:  $W_1$  = weight of lipids (8.5.1.3)

$W_{te}$  = weight of sample extracted

$V_{tl}$  = total volume of extract

$V_1$  = volume of extract used for lipid determination (7.2.1.7)

### 8.5.2. Plasma samples

- 8.5.2.1.** Add 20  $\mu$ L of water (blank), standard olive oil solution of different concentrations (Section 4.8.2) or plasma (unknown) to 15 mL glass centrifuge tubes. Add 0.20 mL of concentrated sulfuric acid to each tube, stopper and mix the contents well on a Vortex mixer.
- 8.5.2.2.** Place all tubes in boiling water bath (Section 5.2.9) for 10 min ( $\pm 1$  min), then cool them in cold water for ca 5 min.
- 8.5.2.3.** Add 10 mL of the phospho-vanillin reagent (Section 4.3.3) to each tube, stopper and mix well on a Vortex mixer. Incubate in a water bath adjusted to 37°C ( $\pm 2^\circ$ C) for 15 min.
- 8.5.2.4.** Cool the tubes for ca 5 min and then, within 30 min, measure the absorbance at 540 nm using the following operating parameters (Turn the spectrophotometer's lamp on one hour prior to the assay):

**8.1.2.4.1.** Computer file path : C:\HP8452

**8.5.2.4.2** Mode : F2 – Quantitation

**8.5.2.4.3** Wavelength : F1 - Single Wavelength (540,0)

**8.5.2.4.4** Press F8 to blank the instrument with the tube containing water.

Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	36 of 51

**8.5.2.5.** Press F5 (Calibration) to access the “standard information table”. Measure the absorbance of each of the standard olive oil solutions to create a calibration curve, specifying the concentration of each one. When readings are done, press F7 to evaluate the curve and F10 to exit.

**8.5.2.6.** Press F7 (Analysis) to access the “analysis results table”. Measure the absorbance of the unknown plasma samples. The results of the absorbance and concentration will be automatically recorded based on the calibration curve. Print a hard-copy of the results by pressing F9.

## 9. EXPRESSION OF RESULTS

The concentration of each PBDE/BFR/DP is expressed as ng/g (ppb) wet weight (ww) or ng/g (ppb) lipid weight (lw).

### 9.1 Calculation by Internal Standard Quantification

A relative response factor (RRF) is first generated for each analyte relative to an appropriate internal standard (IS) in an external standard solution. Multiple ISs may be used, and in the ideal case a <sup>13</sup>C-labeled IS surrogate would be available and used for every analyte that is to be determined. However, this is not necessary as the IS (or minimum number of ISs) simply needs to be representative of a class or sub-class of analytes in a sample.

A range of external standard solutions are prepared where the concentrations of the IS is held constant. Because there is generally relatively stable linear response (as represented by the RRF) over approximately the 10 to 1000 pg range (by GC-MS (ECNI)), a single point calibration can suffice for analyte determination in a sample. Although periodically full calibration curves are generated to confirm use of the single point calibration. The external solution for RRF determination is generally run every 5 samples. The replicate RRF determinations over the entire duration of the GC run of a sample set are generally very reproducible (within 5%), and thus the average RRFs of all external standard runs can be used for analyte quantifications.

**RRF Calculation for an Analyte.** A RRF is the ratio of analyte response factor to the response factor of a representative internal standard surrogate. A mixture of native and internal standards is analyzed prior to sample analysis (every n=12

<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>37 of 51</b>

injected samples) and RRF values are determined as follows:

$$RRF_{analyte X} = \frac{A_{analyte X - ext. standard}}{C_{analyte X - ext. standard}} \times \frac{C_{IS - ext. standard}}{A_{IS - ext. standard}}$$

Where:

- RRF<sub>analyte X</sub> = Relative response factor of an analyte to the IS in the external standard solution
- A<sub>analyteX-ext. standard</sub> = Area of the ECNI *m/z* response for the analyte in the external standard solution
- A<sub>IS-ext. standard</sub> = Area of the ECNI *m/z* response for the internal standard in the external standard solution
- C<sub>analyteX-ext. standard</sub> = Concentration (pg/uL) of the analyte in the external standard solution
- C<sub>IS-ext. standard</sub> = Concentration (pg/uL) of the internal standard in the external standard solution

**Determination of the Concentration of an Analyte Using an RRF.** Using the IS responses from the sample run, and the RRF values, recovery-corrected concentrations of analytes (pg/g wet weight) are calculated directly. A Microsoft Excel spreadsheet is used to automatically calculate these values following sample acquisition on the GC-MS (ECNI). The calculation is done as follows:

$$C_{analyteX-sample} = \frac{A_{analyteX-sample}}{A_{IS-sample}} \times C_{IS-sample spike} \times V_{IS spike} \times 1 / RRF_{analyte X} \div W_{sample}$$

where:

- C<sub>analyte X-sample</sub> = Analyte concentration in sample (pg/g wet weight)
- A<sub>analyteX-sample</sub> = Area of the ECNI *m/z* response for the analyte in the sample extract
- A<sub>IS-sample</sub> = Area of the ECNI *m/z* response for the internal standard in the sample extract
- C<sub>IS-sample spike</sub> = Concentration (pg/uL) of the internal standard solution spiked to the initial sample matrix
- V<sub>IS spike</sub> = Volume (uL) of the internal standard solution spiked to the initial sample matrix

Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	38 of 51

$RRF_{\text{analyte } X}$	= Relative response factor of the analyte to IS in the external standard solution
$W_{\text{sample}}$	= Wet weight of the sample (g wet weight)

## 9.2 Calculation of the Recovery of An Analyte Surrogate Standard in a Sample

The recovery is automatically corrected when using an internal standard method for the determination of the concentration of an analyte in a sample. However, often the IS spike (if there is only one representative IS) can be used as a surrogate to also find out the actual analyte recovery. Generally, it is more analytically acceptable that an additional, separate surrogate is used to determine analyte recovery. Recoveries (%R) are calculated and reported, as these values indicate the overall quality of the residue data. Even with the IS determination approach, where the recovery is automatically corrected, if the actual %R is too low, then the sample analysis should be rejected, and a new sample aliquot re-analyzed. General rules vary considerably with respect to %R, although >75% appears to be the standard level for analysis of organohalogen contaminants in biological matrices. However, as long as the IS (or ISs) maintain their representation of the analytes in question, and that the detector sensitivity remains well above the detection limit, analytical precision and accuracy of the analyte concentration is likely maintained.

The following is a formula for comparing the areas in two separate GC injections, i.e., one the original recovery surrogate (or IS) spike alone, and the other of the recovery surrogate (or IS) in the sample fraction. When using an internal standard method of quantification for an analyte, a normalization standard is not necessary as the calculation based on the absolute amount of RS (or IS) added rather than the final volume of the extract for GC injection.

$$\%R = \frac{A_{RS(orIS)-sample}}{A_{RS(orIS)-original\ spike}} \times 100\%$$

where:

%R	= Percent recovery of recovery surrogate (or IS spike) in the sample extract
$A_{RS(orIS)-sample}$	= Area of the ECNI $m/z$ response for the recovery surrogate (RS) (or IS) in the sample extract
$A_{IS-ext. standard}$	= Area of the ECNI $m/z$ response for the recovery surrogate (RS) (or IS) in the original spiking solution

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<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>39 of 51</b>

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## **10. REPRESENTATIVE DOCUMENTS**

**10.1 Chromatogram of PBDE, BFR and DP standards (Figure 4)**

**10.2 Chromatogram of DCCO SRM (Figure 5)**

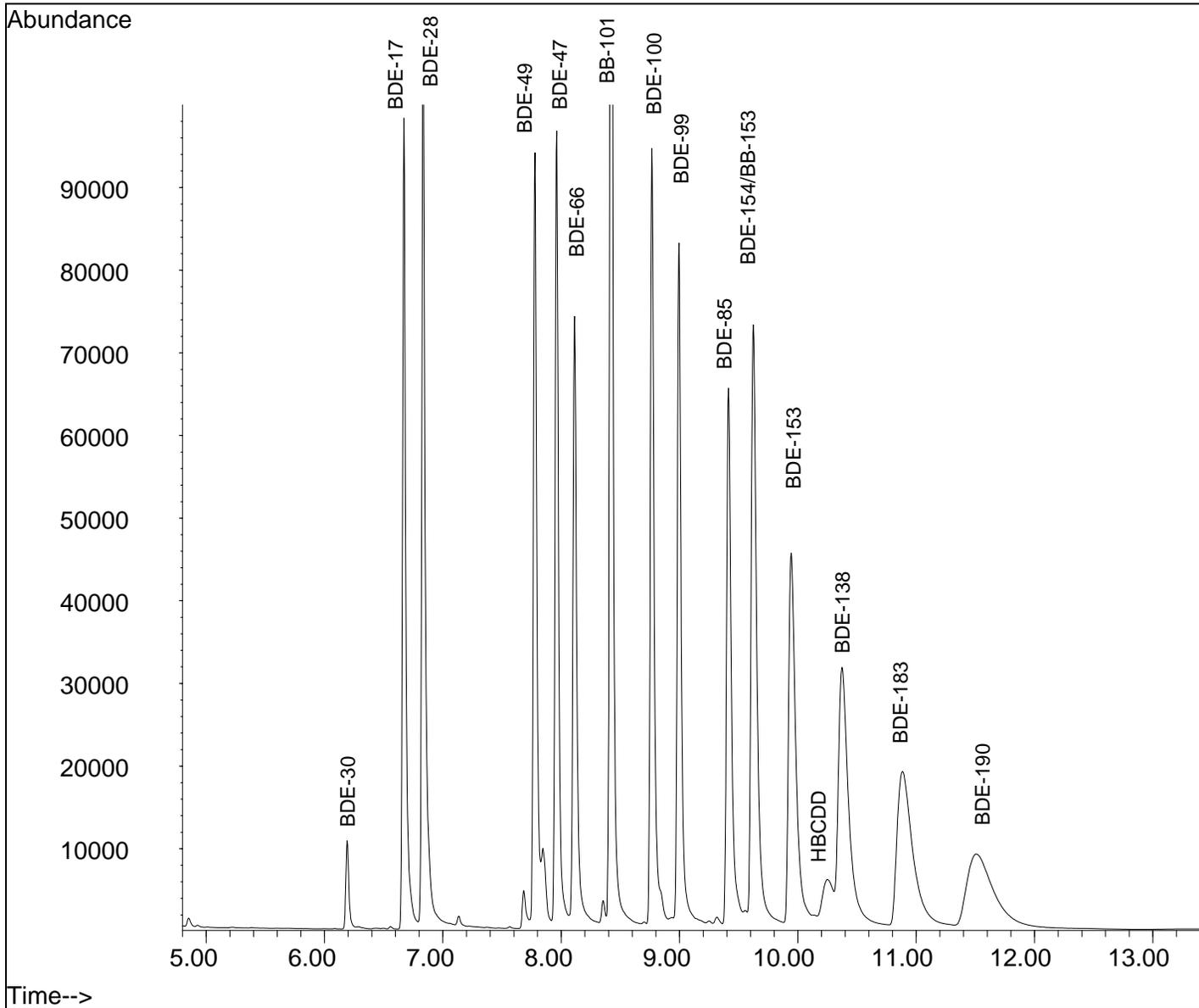
Unit

Environmental  
Chemistry/Organic  
Research Group (NWRC)

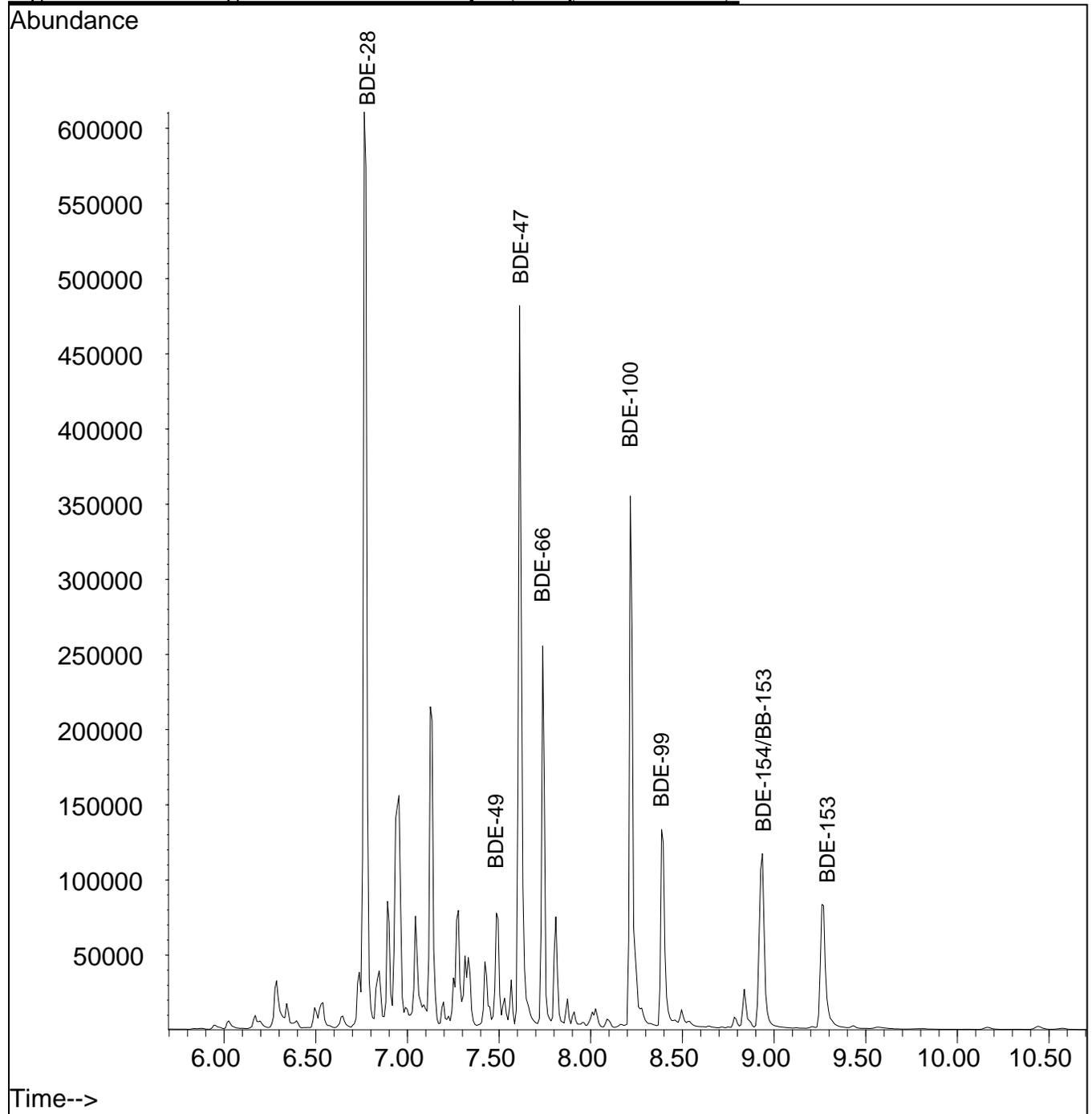
Methods Manual  
Wildlife Toxicology & Disease Program  
NWRC

Method Number: NWRC-MET-ORG-RES-BFR-ver. 4  
Authorization: Dr. R.J. Letcher  
Date: Revision #4, November 2008  
Page: 40 of 51

**Figure 4: Chromatogram of BFR Standards.**



**Figure 5: Chromatogram of a DCCO Sample (Analyzed for BFRs)**



Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	42 of 51

## 11. QUALITY CONTROL

### 11.1. MSD Tuning

The MSDs are tuned weekly with the PFTBA or PFDTD calibration standard using the AutoTune program, and weekly with the QuickTune program (Figure 2). The tuning of the instrument must meet the criteria for conformance outlined in SOP-CHEM-PROC-12 before sample analysis. Tune files are archived in a logbook.

### 11.2. Calibration Verification of Response Linearity

A 8 point initial calibration standard curve is made with the PBDEs and BFRs standard mixtures to cover the range of interest. This established calibration curve is verified periodically, by analyzing a calibration verification standard (quantification standard) having a mid-point concentration. The calculated concentration of each compound must be within 20% of its actual known value. The final concentration of any reportable compounds must be within the demonstrated linearity of the detector. If necessary, samples are diluted with iso-octane to meet the calibration range.

The linearity of the detector response is evaluated for all the target compounds by examining the correlation coefficient of the linear regression of the calibration curve. The criteria for an acceptable linearity of response is when the coefficient of determination ( $r^2$ ) is  $>0.98$ .

### 11.3. Detection Limits and Reporting Limits

In this multi-residue method, it is possible but not practical to list the detection limits for each compound of interest. The background noise is affected by several factors, such as tissue size and tissue type, and instrument effects such as column bleed and cleanliness of the source on the day of the analysis.

According to the U.S. EPA, the MDL value is measured by performing replicate analyses ( $n=8$ ) of matrix samples (in this case 1 g of pork liver) spiked with analytes at a concentration of 3-5 times the estimated detection limit, and calculating the standard deviation:

$$\underline{\underline{MDL = t \times S}}$$

<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>43 of 51</b>

Where  $t$  = Student's value for a 99 % confidence interval and a standard deviation estimate with  $n-1$  degree of freedom, and  $S$  = standard deviation of the replicate control. In this experiment  $t_{(n-1, 0.99)} = 2.998$ .

If the concentration of the target compound is below the method detection limit (MDL), the concentration should be reported as "<MDL". In this method, the method limit of quantification (MLOQ) was defined as equal to method detection limit (MDL).

The limit of detection (LOD) was estimated based on a ratio, peak to peak, of 3 between the signal of the analyte and the baseline noise ( $S/N \text{ PtP} = 3$ ). If the  $S/N$  ratio is below this value, it means the peak is not detected, and the concentration is reported as "ND".

<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>44 of 51</b>

As a general rule, a detection limit of at least 0.5 ppb is achievable for all compounds. For the purposes of reporting data, no results less than this concentration are reported and a result of ND (not detected) appears in the Laboratory Services Section analytical test report. . If a computed result falls in the range 0.1 and 0.5 ppb, the compound is defined as being detected but the result would be too variable to be reliable so a designation of TR (trace) is listed beside the compound in the final report.

#### **11.4. Quality Control Check Sample**

A Quality Control Check Sample is a SRM containing the analytes of interest in known concentrations in a sample matrix similar to the matrix under test. An aliquot of the QA Reference Material (Herring gull eggs, Section 4.9 and DCCO eggs) is analyzed along with each batch of about fifteen samples. The concentration of the major compounds (BDE47, BDE99, BDE100, BDE153, BDE138, BDE183, BDE209,  $\alpha$ -HBCDD and BB-101) is determined and the results are compared to the previously established acceptance limits (i.e.,  $\pm 2$  SD of the long-term mean plotted in a Shewart chart - ref. SOP-CHEM-DOC-02).

##### **11.4.1. FOR PBDE/BFRs**

To determine the degree of analyte loss during sample cleanup, each sample (including the PBDEs and BFRs standard mixture 7.11.1) is spiked with internal/recovery standard mixture (4.6.1). It is assumed that during sample cleanup, native PBDEs and BFRs behave the same way as the labeled compounds. Analysis is accepted when the % surrogate standard recoveries for most PBDEs and BFRs are between 80% and 110%.

#### **11.5. Replicates**

Performing duplicate extractions of samples is required to reveal any variability in workup between samples and sample batches, when sample abundance allows. In addition, duplicate injections of the same sample vial allows for verification of instrument's accuracy and precision.

#### **11.6. Method Blank**

The method blank consists of spiked sodium sulphate (~25 grams) carried through the entire extraction, concentration and analysis procedure. This is done with each batch

Unit	Environmental Chemistry/Organic Research Group (NWRC)	
Methods Manual Wildlife Toxicology & Disease Program NWRC	Method Number	NWRC-MET-ORG-RES-BFR-ver. 4
	Authorization :	Dr. R.J. Letcher
	Date:	Revision #4, November 2008
	Page:	45 of 51

of about fifteen samples to demonstrate freedom from cross-contamination and contaminants that would interfere with analysis and to determine the levels of contamination associated with the processing and analysis of samples. The aliquot of sodium sulphate is treated exactly as the sample including exposure to all glassware, equipment, solvents, reagents, internal standards and surrogates that are used with samples. The method blank is used to determine if analytes or interferences are present in the laboratory environment, the reagents, or the apparatus. The concentrations of (choose one BDE) are charted on a control chart. If problems with the blank exist, associated data are carefully evaluated and appropriate corrective actions are applied. Blank values are subtracted from reportable values. A compound found in a blank and also in an associated sample is flagged in the analytical test report when present at a ratio of at least 5/1, sample to blank.

### 11.7. Ongoing Precision and Recovery Blank

A method blank is spiked with known quantities of unlabelled analytes and is analyzed exactly like a sample. Its purpose is to assure that the results produced remain within the limits specified in the method for precision and recovery.

The concentrations are compared with the limits for the Ongoing Precision and Recovery (OPR) control chart. If the concentration is in the range specified, the extraction, evaporation, and weighing processes are in control and analysis of blanks and samples may proceed. If, however, the concentration is not in the specified range, the analytical process is not in control. In this event, correct the problem, re-extract the analytical batch, and repeat the ongoing precision and recovery test.

The OPR control charts are updated with all acquired OPR data to form a graphic representation of continued laboratory performance.

### 11.8. Target Compound Identification

Peak GC retention time must be within their pre-defined retention time windows. All ions present in the standard mass spectrum at a relative intensity greater than 10% must be present in the sample spectrum.

The relative intensities of these ions must agree within  $\pm 20\%$  between the standard and sample spectra (e.g., for an ion with an abundance of 50% in the standard spectrum, the corresponding sample ion abundance must be between 30% and 70%).

<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
Methods Manual Wildlife Toxicology & Disease Program NWRC	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	46 of 51

## 11.9. Standard Operating Procedures

SOPs relevant to this analytical method:

- 11.9.1.** SOP-CHEM-DOC-01B: For the use of log-books
- 11.9.2.** SOP-CHEM-DOC-04B: For the archiving of gas chromatography data files
- 11.9.3.** SOP-CHEM-DOC-07B: For the archiving of analytical test reports
- 11.9.4.** SOP-CHEM-PROC-01D: For pipette calibration
- 11.9.5.** SOP-CHEM-PROC-03D: For balance calibration
- 11.9.6.** SOP-CHEM-PROC-05C: For the preparation and storage of standard solutions
- 11.9.7.** SOP-CHEM-PROC-06C: For the monitoring of refrigerator's temperatures
- 11.9.8.** SOP-CHEM-PROC-09C: For glassware cleaning
- 11.9.9.** SOP-CHEM-PROC-10C: For running a sequence with the GC/MSD
- 11.9.10.** SOP-CHEM-PROC-12: For the tuning and calibration of the MSD
- 11.9.11.** SOP-CHEM-PROC-13: For verification of standard with a second source standard
- 11.9.12.** SOP-CHEM-MAIN-04: For the maintenance of the GC/MSD

### 11.10 Sample accountability

Possible errors due to contamination of solvent, material or other are recorded and reported in the final results report.

### 11.11 Randomisation of samples

To avoid any biased results, samples are analyzed at random, not taking into account the sample's collection date or site.

### 11.12 Data validation and reporting

Data validation is ensured by filling out FORM-ECT-01 "Data validation and reporting for PBDE/BFR analysis - Checklist" (see appendix E). The report writing and distribution status is insured by filling out FORM-ECT-02 (see appendix E). The original data about sampling and other details should be recorded on the worksheet (see appendix ?).

## 12. CRITICAL CONTROL POINTS

A number of critical points are identified at various steps in Section ?. The following are repeated for emphasis:

<b>Unit</b>	<b>Environmental Chemistry/Organic Research Group (NWRC)</b>	
<b>Methods Manual Wildlife Toxicology &amp; Disease Program NWRC</b>	<b>Method Number</b>	<b>NWRC-MET-ORG-RES-BFR-ver. 4</b>
	<b>Authorization :</b>	<b>Dr. R.J. Letcher</b>
	<b>Date:</b>	<b>Revision #4, November 2008</b>
	<b>Page:</b>	<b>47 of 51</b>

- 
- 12.1** The original stock standard solutions are kept in an amber Certan vial sealed with parafilm and kept in the refrigerator when not in use. The weight of the vial is recorded before and after every use. The weight is adjusted by adding trimethylpentane if the weight is lower than the last recorded weight.
  - 12.2** The working standards should be kept in the refrigerator when not in use.
  - 12.3** Always include a blank (trimethylpentane) after injecting the last standard of the calibration curve and in between each sample.
  - 12.4** Run a set of calibration standards before each set of 5 samples.
  - 12.5** Run a standard from the calibration curve after 5 samples.
  - 12.6** Trace contaminant levels are determined by this method and the elimination of interferences is essential. They could occur through sample handling, reagents, solvents, instruments or labware.

**13. REVISION HISTORY**

NWRC-MET-ORG-RES-BFR-version 4 (November 2008): This method is based on MET-CHEM-06A.

## APPENDIX E: FORM-ECT-01 Report writing and distribution status

FORM-ECT-01 / Apr. 2008

### BFR ANALYSIS – DATA VALIDATION CHECKLIST

Report no.: \_\_\_\_\_ Project leader: \_\_\_\_\_ Project no.: \_\_\_\_\_

√	Worksheet – should include the following information:
	Laboratory No. (BFR-YR-TISSUE-NO.), USOX no., ID no., GC-MS file, any appropriate comments
	Tissue weight (g):
	A QC sample or a spiked sample was analysed with every batch (10 samples)
	When a sample was re-extracted, the letter “R” was added to the original lab number
	If a sample was re-extracted, a new lab number was issued (BFR-YR-TISSUE-NO.)
	The worksheet was initialized by the GC-MS analyst after completion
√	Quantification
	The correlation coefficient of the linear regression of the calibration curve was >0.98 for all compounds
	The sample area counts were within the range of the calibration curve
	The appropriate factors were used to transform the detector’s response in ppb (ng/g)
	A blank correction was applied if appropriate
	The peak threshold was adjusted if the MS sensitivity changed
√	The Chemstation Sample List – should include the following information:
	File name (identified by date of analysis and sequence of analysis)
	File Text includes standard concentration or lab no. (BFR-YR-TISSUE-NO.)
	Sample ID (USOX#)
	GC Method and MS Method
	Vial number and volume injected
	Type of sample (standard; blank or analyte)
	Concentration of standard in ppb (ng/mL)
	Appropriate multiplier and divisor
	Project number or submitter
	The sample list was initialized by the GC-MS analyst after verification
√	The Chemstation Sample Summary report – should contain the following information:
	File name, Date and Time of analysis, Identification and Description are included I the sample header
	The compound name, ions, retention time, response, amount and factors (divisor/multiplier)
	The concentration of the target compounds in the tissue sample
	The GC-MS analyst initializes the data once it is verified and the values are accepted
	If the data is rejected, the analyst includes a comment beside the sample data
	The report is saved with the date of the analysis (ex. 31072007 for July 31, 2007)
√	Test Report
	Method modifications are detailed
	Appropriate significant figures are used
	Test data were verified to insure that theres was no transcription error
	Non-conformity situations were reported on FORM-QAP-06 / May 2007
	Final test report was signed by the GC-MS analyst
	Final test report was authorized for release by the Laboratory Services Section Head

## APPENDIX E: FORM-ECT-02 Report writing and distribution status

FORM-ECT-02 / Apr. 2008

### REPORT WRITING AND DISTRIBUTION STATUS

Report no.: \_\_\_\_\_      Project leader: \_\_\_\_\_      Project no.: \_\_\_\_\_

*Information needed*

	Project file (includes project title and project information)	
	Date of shipment and condition of samples	
	Analysis dates and analyst name	
	Photocopy of laboratory notebooks	
	Method used for sample preparation	
	Modification to the method	
	Sample list	
	Calibration Report	
	Sample and Compound Summary Report	
	Experiment Parameters (for GC-MS)	
	GC method (Inlet File) (*.M)	

*Steps*

	General information	
	Sample preparation and method of analysis	
	Quality assurance	
	Results / e.g. of chromatograms	
	Discussion	
	Report approval	

*Report distribution*

	Project Leader (hard and electronic copy)	
	Head, Laboratory Services (hard and electronic copy)	
	Unit Project file	
	Database Manager (hard and electronic copy)	
	Record of distribution	

*Documentation and others*

	Archive electronic copy of report on R:/protected drive	
	Archive results in specific analysis box	
	Identify or discard draft copies	
	Verify storage location of samples	

### Appendix F: BFR Analysis – Worksheet

Analyst:	Date of extraction:	Date of analysis:
Method:	Species:	Tissue:

Project no.:	Notebook no.:
Project Leader:	Pages:

Extraction done on unhomogenized tissue: <input type="checkbox"/>
Extraction done on homogenized tissue: <input type="checkbox"/>

INTERNAL STANDARD			
Compound:	Concentration (pg/μL):	Spiked volume (μL):	Amount spiked (ng):
Compound:	Concentration (pg/μL):	Spiked volume (μL):	Amount spiked (ng):
Compound:	Concentration (pg/μL):	Spiked volume (μL):	Amount spiked (ng):
Compound:	Concentration (pg/μL):	Spiked volume (μL):	Amount spiked (ng):



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## **Appendix C. Protocol for Bird Egg Collection, Measurement, Preparation, and Shipment for Contamination Residue Analysis.**

This appendix describes protocol for collection, measurement, preparation, and shipment of osprey egg samples.

Source: U.S. Fish and Wildlife Service: Oregon Office, May 2, 2008.

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Oregon Fish and Wildlife SOP-F003

Date Prepared: December 14, 1993

Date Revised: May 2, 2008

**Protocol for Bird Egg Collection, Measurement, Preparation,  
and Shipment for Contaminant Residue Analysis**

U.S. Fish and Wildlife Service  
Oregon Fish and Wildlife Office  
2600 SE 98<sup>th</sup> Ave, Suite 100  
Portland, Oregon 97206

Revisions made by Jeremy Buck  
Environmental Contaminant Specialist  
Oregon Fish and Wildlife Office

Date Prepared: December 14, 1993

Date Revised: May 2, 2008

### **Background and Objectives**

Environmental contaminants can be transferred into eggs from adult female birds at concentrations that can be detrimental to the developing embryo. Contaminants can directly impact the embryo, or cause eggshell thinning which can dehydrate the embryo or lead to egg breakage during incubation. Collection of bird eggs is useful in wildlife toxicology to evaluate contaminant concentrations in egg contents, and measure the degree for eggshell thinning, to monitor trends over time or evaluate risk from contaminant exposure to the species. The objectives of this Standard Operating Procedure (SOP) are to 1) provide a consistent method for egg collection; 2) ensure consistent measurements of the egg and provide standard methods to measure eggshell thickness; and 3) ensure accurate analysis of contaminants in eggs by providing standard methods for harvesting and transferring egg contents into a clean container without introducing contamination. It is important to standardize the process for collecting eggs, harvesting egg contents, and measuring eggshell thickness to improve data comparisons among contaminant investigations. Collection of various measurements during egg processing is necessary for interpretation of analytical results.

### **Materials**

For field collection: Appropriate State and Federal permits; waterproof pen or pencil; specimen jar labels; egg collection cans or boxes (padded coffee can, hard-sided container such as plastic kitchen ware, or tackle box with foam padding); aluminum foil; small plastic bags with zip closure.

For egg processing in laboratory: Data sheets; writing utensils; dull pencil; safety glasses; powder-free latex gloves; laboratory paper wipes such as Kimwipes®; distilled, deionized (DDI) water or equivalently pure water; balance (to 0.01 g); vernier calipers (to 0.01 mm); immersion chamber with beaker and wire loops (Figure 1) or similar vessel to measure egg volume; chemically-clean jar (one per egg); chemically-clean stainless steel scalpel blades (No. 21 or No. 22 with No. 4 handles or similar size); chemically-clean aluminum foil sheets (approximately 30 x 30 cm square), 1 per egg; ball-tip micrometer (to 0.01 mm).

### **Collection**

The procedure for accessing the nest and determining which egg to collect, and how many eggs, will vary depending on the species and study objectives. Typically, one egg per nest is randomly selected and removed.

1. Remove egg(s) from nest. Wrap egg in chemically-clean aluminum foil - dull side in - if needed (aluminum foil keeps the eggshell together and the contents inside should the egg be cracked in transit), and/or place egg in zip-closure plastic bag. Clearly label egg in pencil or place label (written in pen) in plastic bag with egg. Include on label the date, collector, nest identification, and location. Place protective material around egg (e.g., gently wrap egg in bubble wrap, place in foam rubber in which egg holes have been cut, or place in an egg carton).
2. Place each egg in container (a clean 1-gallon or quart paint can, cardboard box, Tupperware container, etc.) and fill spaces with soft packing material. Seal container with tape.

3. Pack the egg container inside ice chest with blue ice (to maintain 4EC temperature) and cushion with appropriate amount of packaging material.
4. If eggs cannot be processed immediately after collection, store eggs in a refrigerator (4EC). **Do not freeze** whole eggs since this will crack the shell.

### **Egg Processing**

#### **Whole Egg Measurements**

1. If possible, candle the egg to determine if cracks are present in the shell. Any cracked egg should not be rinsed or immersed in water as this may contaminate the sample.
2. If the egg is not cracked and is dirty, clean gently with a soft towel and distilled or deionized water that is at or near the temperature of the egg. Dry the egg.
3. Write the sample identification number on both ends of the eggshell with a dull pencil.
4. Record on data sheet any distinguishing characteristics of the egg (e.g. cracked, dented, discolorations, etc.).
5. Measure and record the length (mm) [caliper jaws parallel to the longitudinal axis] and the breadth (mm) [caliper jaws perpendicular to the longitudinal axis] of the egg with calipers at their greatest dimensions.
6. Measure and record the mass (g) of the egg on data sheet.
7. Measure and record the egg volume (cm<sup>3</sup>) by following instructions given below for intact versus cracked shells. NOTE: Egg volume is important for estimating lipid and moisture loss of an egg, and is used along with other eggshell metrics to convert analytical results from wet weight to fresh weight (which incorporates lipid and moisture loss). If egg is cracked, do not immerse it in water; rather, see Cracked Egg Technique below.

#### ***Intact Shell-Water Displacement Technique***

1. Place a water receptacle adjacent and underneath the egg immersion volumeter side arm (see Figure 1).
2. Place the wire egg holder in the volumeter.
3. Fill the volumeter with distilled or deionized water until it flows freely from the volumeter side arm (*Note: the temperature of the water should be as close to the temperature of the egg as possible*).
4. When the water stops flowing, the receptacle should be emptied, weighed, and returned to its position adjacent to the volumeter.
5. Gently raise the wire egg holder and place the egg on it. Gently lower the egg into the volumeter until it is completely submerged.
6. Weigh the water receptacle and its contents. Subtract the mass of the water receptacle alone. The mass of the displaced water is the approximate egg volume, assuming that egg density is similar to water (1gm = 1 ml). For example, 40 gm displaced water = 40 ml of water, and 40 ml egg volume.

7. Repeat the procedure 3 times for each egg and report the average value. Dry the egg.
8. Calculate the fresh weight conversion factor and record value on data sheet. Use the equation:

$$\text{Conversion factor} = \frac{\text{egg contents mass (g)}}{\text{displaced water mass (g)}}$$

Alternative method for measuring volume:

1. Place egg in a graduated cylinder with 10 ml graduations.
2. Adjust water level to a given graduation, then immerse the egg with a wire egg holder.
3. Determine the first volume of water displaced in complete 10 ml units. Then, use a 10 ml syringe to collect the water in the graduated cylinder down to the nearest 10 ml graduation. Measure the difference in water volume as determined on the graduated cylinder.
4. Add the amount of water in the 10 ml syringe (measured in 0.1 ml increments) e.g., 50 ml + 5.2 ml = volume of egg, 55.2 ml.

### ***Cracked Egg Technique***

Do not measure volume using egg immersion. Instead, estimate egg volume based on length and breadth measurements, depending on species, as described in Stickel *et al.* (1973):

### **Embryo Harvest**

Wear surgical gloves and safety glasses when performing embryo harvest. **CAUTION: EGGS MAY EXPLODE UPON OPENING IF CONTENTS HAVE DECOMPOSED!**

1. If eggs have a strong odor (indicating advanced decomposition) or are suspected to be addled, it is advisable to vent before attempting to open to avoid possible explosions. To vent, don safety glasses and gently insert a chemically-clean needle into the blunt end of the egg. Use gentle, steady pressure to pierce the shell.
2. Place a labeled, chemically-clean glass jar and its lid on a scale, tare, and remove lid. Jars should be labeled with the following information; sample number, species, date, location, and collector. An additional label containing the sample number and date should be placed on the lid of each jar.
3. Hold the egg lengthwise over the jar. Using a sharp scalpel, gently score the egg around its equator. Apply gentle, steady pressure while making several rotations around the egg. Once the scalpel pops through the shell, insert the tip of the scalpel blade to cut the membrane and separate the two halves. Cut 1/2 to 2/3 the distance around the egg. Invert the egg while pulling apart the shell halves and pour the contents into the opened jar. Use a chemically clean stainless steel or teflon spatula to scrape any contents remaining on the shell into the jar (be careful not to tear the shell membrane).
4. Weigh (g) the jar (including lid) containing the egg contents. Record the mass (g) of the egg contents by subtracting the mass of the jar and lid alone from the jar and lid containing the egg contents.

5. Visually inspect the egg contents. Record the presence or absence of an embryo, estimated stage of development as early, mid, or late. Note any abnormalities.
6. Rinse the interior of the shell halves with tap water being careful not to tear the membrane, or erase the sample identifications. After the shells have dried, use an indelible ink pen to remark the shells with their sample IDs. Store the shells in a cool dry place for at least 30 days at which time they should have attained a constant mass. Store egg shells in egg cartons.
7. Store embryos in freezer at -13EC.

### **Shell Thickness Measurement**

1. Determine the eggshell mass or weight (to the nearest 0.001 g) of dried shells.
2. Measure the eggshell thickness using a dial micrometer with rounded contacts. Take thickness measurements of each shell-half along the equator in at least three places. Report the average of all measurements as the final thickness measurement. If the membrane has separated from the shell, take measurements without the membrane but be sure to make note of this on the data sheet.
3. If determining the Ratcliff thickness Index (Ratcliffe 1967), calculate using the following formula:

$$\text{Thickness Index} = \frac{\text{eggshell mass (mg)}}{\text{egg length (mm)} \times \text{egg width (mm)}}$$

### **Sample Shipment**

1. Wrap sample jars with bubble wrap and secure with tape or parafilm.
2. Place samples in cooler with a sufficient supply of dry ice (1 gm dry ice: 1 gm sample) to last at least 24 hours.
3. Cushion samples in cooler with packing material such as foam rubber, bubble wrap, or peanuts.
4. Enclose the working catalog, a pre-addressed return label, and chain of custody forms in a sealed plastic bag inside cooler.
5. Wrap cooler with mailing tape.
6. If needed, inform the shipper that the cooler contains dry ice by labeling two opposite sides and the top of the cooler with a dry ice label containing the amount of dry-ice inside.
7. Call the designated laboratory contact to inform him/her that samples have been shipped.

### **References**

- Ratcliffe, D.A. 1967. Decrease in eggshell weight in certain birds of prey. *Nature* 215(5097):208-210.
- Stickel, L.F., S.N. Wiemeyer, and L.J. Blus. 1973. Pesticide residues in eggs of wild birds: adjustment for loss of moisture and lipid. *Bull. Environ. Contam. Toxicol.* 9:193-196.

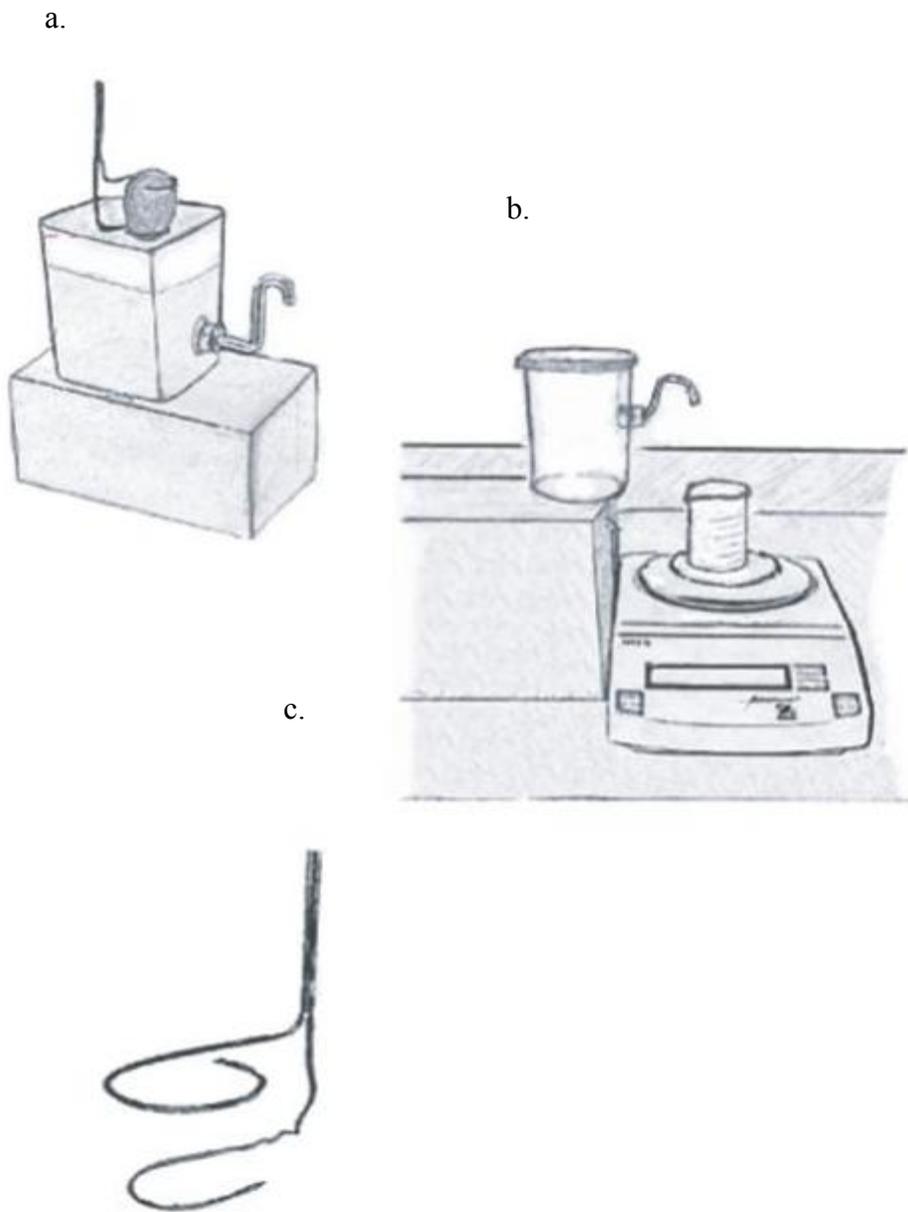


Figure 1. Measuring total egg volume. a. Egg immersion chamber (can be round or square shaped). The top bend of the spigot is high enough so that an egg can be completely immersed below it. b. Immersion chamber set up to drain into beaker on balance. c. Wire loops used to hold the egg.

## Appendix D. Acronyms and Abbreviations

BDE	Brominated Diphenyl Ether
BFR	Brominated Flame Retardants
DOH	Washington State Department of Health
EA	Environmental Assessment (Program)
Ecology	Washington State Department of Ecology
EIM	Environmental Information Management
EPA	U.S. Environmental Protection Agency
HBCDD	Hexabromocyclododecane
LCS	Laboratory Control Sample
MEL	Manchester Environmental Laboratory
MQO	Measurement Quality Objective
NWRC	National Wildlife Research Centre
PBDE	Polybrominated Diphenyl Ethers
QA	Quality Assurance
QC	Quality Control
USGS	U.S. Geological Survey