Published in final edited form as:

Aquat Toxicol. 2010 April 1; 97(1): 42–50. doi:10.1016/j.aquatox.2009.11.013.

Flow cytometric analysis of BDE 47 mediated injury to rainbow trout gill epithelial cells

Jing Shao[†], Michael J. Dabrowski[†], Collin C. White, Terrance J. Kavanagh, and Evan P. Gallagher^{*}

Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA 98105 USA

Abstract

The polybrominated diphenyl ethers (PBDEs) are ubiquitous environmental contaminants whose residues are increasing in fish, wildlife and human tissues. However, relatively little is known regarding the mechanisms of cell injury caused by PBDE congeners in fish. In the present study, we employed flow cytometry-based analyses to understand the onset and mechanisms of cell injury in rainbow trout gill cells (RTgill-W1 cells) exposed to 2,2',4,4'-tetrabromodiphenyl ether (BDE 47). Substantial optimization and validation for flow cytometry protocols were required during assay development for the trout gill cell line. Exposure to micromolar concentrations of BDE 47 elicited a significant loss in RTgill-W1 cell viability that was accompanied by a decrease in NAD(P)H autofluorescence, a marker associated with disruption of cellular redox status. This loss in NAD(P)H content was accompanied by a decrease in nonylacridine orange fluorescence, indicating mitochondrial membrane lipid peroxidation. Furthermore, low doses of BDE 47 altered cellular forward angle light scatter (FS, a measure of cell diameter or size) and side light scatter properties (SS, a measure of cellular internal complexity), consistent with the early stages of apoptosis. These changes were more pronounced at higher BDE 47 concentrations, which lead to an increase in the percentage of cells undergoing frank apoptosis as evidenced by sub-G1 DNA content. Apoptosis was also observed at a relatively low dose (3.2 μ M) of BDE 47 if cells were exposed for an extended period of time (24 hr). Collectively, the results of these studies indicate that exposure of rainbow trout gill cells to BDE47 is associated with the induction of apoptosis likely originating from disruption of cellular redox status and mitochondrial oxidative injury. The current report extends observations in other species demonstrating that oxidative stress is an important mechanism of BDE 47 mediated cellular toxicity, and supports the use of oxidative stress-associated biomarkers in assessing the sublethal effects of PBDEs and their replacements in fish. The application of flow cytometry endpoints using fish cell lines should facilitate study of the mechanisms of chemical injury in aquatic species.

Keywords

rainbow trout; polybrominated diphenyl ethers; oxidative stress; lipid peroxidation; flow cytometry

© 2009 Elsevier B.V. All rights reserved.

^{*}Address correspondence to: School of Public Health Department of Environmental and Occupational Health Sciences 4225 Roosevelt Way NE, Suite100 University of Washington Seattle, WA 98105-6099 Phone (206) 616-4739 Fax: (206) 543 8458 evang3@u.washington.edu

[†]The first two authors contributed equally to this work and are therefore listed as co-lead authors.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Polybrominated diphenyl ethers (PBDEs) are halogenated compounds used as flame retardants in textiles, plastics and electronics that represent a novel group of persistent environmental contaminants (McDonald, 2002). Because these compounds are not chemically bound into the polymers, they readily migrate into environmental media (McDonald, 2002; Sjodin *et al.*, 2003). The high lipid solubility, low vapor pressure and general resistance to degradation of these compounds has contributed to their environmental persistence and subsequent bioaccumulation throughout food chains (Birnbaum and Cohen Hubal, 2006; Darnerud, 2003; Gill *et al.*, 2004; Hites, 2004; McDonald, 2002). The fact that PBDEs are also commonly detected in ambient air in industrialized regions (Butt *et al.*, 2004), remote Arctic locations (de Wit *et al.*, 2006), as well as in indoor air (Schechter *et al.*, 2005; Stapleton *et al.*, 2005), underscores the widespread distribution and concerns associated with these emerging contaminants.

Of the various PBDE congeners, 2,2',4,4'-tetrabromodiphenyl ether (IUPAC, BDE 47) is of lower molecular weight and typically the major PBDE congener detected in highest concentrations in fish and human tissues (Darnerud, 2003; Mazdai *et al.*, 2003; Schechter *et al.*, 2007). Interestingly, while BDE 47 often predominates in the tissues of fish, it constitutes a minor contribution to global PBDE production and usage (Darnerud, 2003; Hale *et al.*, 2003). Collectively, PBDE congeners 47, 49, 99, and 100 account for the highest proportion of total PBDE concentrations in the aquatic environment and biota (Betts, 2002; Burreau *et al.*, 1997; Gustafsson *et al.*, 1999; Hites, 2004; Sjodin *et al.*, 2003; Stapleton and Baker, 2003). Furthermore, PBDEs in the edible portions of fish tissues may lead to dietary exposures and increase the PBDE body burdens in humans (McDonald, 2002; Sjodin *et al.*, 2003). Although the human and ecological health risks associated with PBDE exposures are unknown, studies in rodents have shown developmental, reproductive, neurological and endocrine toxicity (Birnbaum and Staskal, 2004; de Wit, 2002; Eriksson *et al.*, 2001; Zhou *et al.*, 2001; Zhou *et al.*, 2002).

Mechanisms of PBDE toxicity appear to be varied and include disruption of thyroid hormone homeostasis, dysregulation of genes involved in endocrine function (Birnbaum and Staskal, 2004; Darnerud *et al.*, 2001), and biotransformation reactions (Holm *et al.*, 1993; Tjarnlund *et al.*, 1998). Although less studied relative to their mammalian counterparts, these mechanisms also appear to be relevant in aquatic species. For example, chronic exposure of sticklebacks (*Gasterosteus aculeatus*) to a commercial penta-BDE mixture (Bromkal 70-5DE) reduces spawning success and causes mild hepatic lipid accumulation (Holm *et al.*, 1993), whereas chronic exposures of rainbow trout to tetra- and penta-BDE are associated with reductions in cytochrome P4501A and glutathione reductase activities, as well as perturbations in blood glucose and hematocrit (Tjarnlund *et al.*, 1998). In addition, embryonic exposure of zebrafish to BDE 47 has been shown to cause developmental toxicity, and impair cardiovascular function in zebrafish (Lema *et al.*, 2007). It is also apparent that oxidative stress is an important component of PBDE toxicity *in vivo* (Ferne *et al.*, 2005; Lam *et al.*, 2006; van Boxtel *et al.*, 2008), as well as *in vitro* in primary human fetal liver hematopoietic stem cells (Shao *et al.*, 2008b) and rat neuronal cells (Costa and Giordano, 2007; Giordano *et al.*, 2008). However, oxidative stress encompasses a broad spectrum of subcellular perturbations and these mechanisms of PBDE toxicity have not well characterized on a cellular level in aquatic species.

We have previously reported that transformed gill and liver cells from rainbow trout increase reactive oxygen species production in the presence of BDE 47 (Shao *et al.*, 2008a). In the current study, we have optimized several flow cytometry assays for use in RTgill-W1

gill epithelia cells to more closely investigate the mechanisms of cellular injury of a common PBDE congener found in fish. Fish gills receive continuous exposure to waterborne toxicants and comprise a first line of defense against the toxicity of aquatic chemicals (McKim *et al.*, 1985; Wood *et al.*, 2002). Gill epithelial cells in particular, perform important physiological functions such as gas exchange, ion transport, acid–base regulation, osmoregulation, and excretion of endogenous metabolic byproducts. Others have reported that gill epithelial cells are more sensitive than cultured hepatocytes to environmental toxicants (Zhou *et al.*, 2006). Although primary fish gill cells more closely approximate *in vivo* function, the use of immortalized gill cell lines such as RTgill-W1 cells avoids logistical issues associated with microbial contamination as well as the variability among cell preparations which can arise from the use of individual animals (Bols *et al.*, 1994). We focused on flow cytometry endpoints such as pyridine nucleotide redox status, mitochondrial cardiolipin content, mitochondrial membrane potential, and apoptosis to investigate the roles of mitochondrial injury and oxidative stress as mechanisms of BDE 47 toxicity.

Materials and methods

Chemicals and biochemicals

Leibovitz's L-15 media was purchased from Sigma-Aldrich (St. Louis, MO). Fetal Bovine Serum (FBS), heat-inactivated, was purchased from Atlanta Biologicals (Lawrenceville, GA). L-Glutamine and 0.05% Trypsin-EDTA were purchased from GibcoBRL (Life Technologies Inc., Gaithersburg, MD). Penicillin-Streptomycin was purchased from Fisher Scientific (Pittsburgh, PA). BDE 47 (2,2',4,4'-tetrabromodiphenyl ether, >99% purity) was purchased from Chem Service, Inc. (West Chester, PA). DMSO (dimethylsulfoxide) was purchased from Sigma-Aldrich. Acridine orange 10-nonyl bromide (nonyl acridine orange, NAO), JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolo-carbocyanineiodide), MitoTracker Red and propidium iodide (PI) were purchased from Invitrogen (Carlsbad, CA). Vented tissue culture flasks were purchased from Corning Inc. (Corning, NY). All other chemicals were purchased from either Sigma-Aldrich or Fisher Scientific.

Cells and Exposures

RTgill-W1 cells originated from histologically normal gill lamella of a rainbow trout *Oncorhynchus mykiss* (Bols *et al.*, 1994) were purchased from American Type Culture Collection (Manassas, VA). Gill cells were maintained without CO₂ incubation in free gas exchange with air at 19°C in Leibovitz's L-15 medium supplemented with 10% FBS (v/v), 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin according to the manufacturer's instructions. The medium was filtered through a Durapore 0.22 µm filter (Millipore, Billerica, MA).

All experimental treatments were carried out in serum-free L-15/ex solution (Lee *et al.*, 2008). Cells were plated into 25 cm² or 75 cm² flasks at a density of 1×10^7 or 2×10^7 cells, respectively, and allowed to attach and acclimate for 48 hours prior to chemical exposures. BDE 47 was dissolved in DMSO and prepared in L-15/ex solution for final concentrations of 0 µM (0.02% DMSO, throughout all experiments), 0.2 µM, 0.8 µM, 3.2 µM, 12.5 µM or 50 µM. These concentrations were based upon preliminary range-finding experiments establishing a dose response for cell injury and similar to other studies of PBDE toxicity in human, rodent and fish cells (He *et al.*, 2008; Hu *et al.*, 2007; Shao *et al.*, 2008b). In all experiments, 2 mM hydrogen peroxide (H₂O₂) was used as positive control for cell toxicity and validation of flow cytometry assays. Following the appropriate exposure times, the cells were trypsinized and transferred to sterile 15 ml conical tubes. The samples were then

centrifuged at 4°C for 5 min at $250 \times g$. The supernatant was removed and the pelleted cells were resuspended in L-15/ex solution for further analysis via flow cytometry.

Effect of BDE 47 on cell viability

A commonly used quantitative method for cell viability is propidium iodide (PI) staining. PI is a non-permeant dye which stains the DNA of dead or dying cells and thus viable cells with intact cell membranes exclude PI. We used PI in conjunction with flow cytometry and confirmed the effects of BDE 47 on cell viability using a biochemical assay based upon alamarBlue reduction (Shao *et al.*, 2008a). AlamarBlue is an indicator dye which measures the proliferative ability of cells based upon oxidation-reduction reflecting mitochondrial function. Following BDE 47 exposures, media from each treatment (including cells detached during the incubation) was transferred to 15 ml polypropylene tubes. Gill cells which remained attached were trypsinized and combined with their respective “floater” population. These samples represented total cell populations for each treatment and were centrifuged at $150 \times g$ for 5 min at 4°C prior to resuspension to a density of 1×10^6 cells/ml. The cells were then incubated with 1 μ g/ml PI and placed at 4°C. PI positive populations were quantified via flow cytometry using 488 nm excitation and fluorescence emissions were collected using a 645 nm long pass filter.

Flow cytometric analysis of cellular NADPH levels, and early and late stages of apoptosis

NAD(P)H redox status—Gill cells contain a variety of endogenous fluorescent molecules including aromatic amino acids, flavins and the reduced pyridine nucleotides NADH and NADPH. Excitation of NAD(P)H at ~360nm gives rise to fluorescent emission signal which peaks at 450 nm (blue) (del *et al.*, 2008; Laing *et al.*, 1980; Masters *et al.*, 1989). While NAD(P)H molecules are fluorescent, the oxidized forms of NAD(P)H (NAD^+ and NAD^+P) lose their fluorescence characteristics (Laing *et al.*, 1980). Accordingly, changes in NAD(P)H autofluorescence reflect alterations in cellular pyridine nucleotide redox status (Laing *et al.*, 1980; Masters *et al.*, 1989). NAD(P)H autofluorescence intensities were measured in conjunction with other indicators via flow cytometry on a Coulter EPICS Elite flow cytometer (Beckman Coulter, Miami, FL) using 355 nm (UV) excitation. The resulting blue fluorescence emission signals were collected using a 450/35 band-pass filter.

Effect of BDE 47 on forward angle and side light scatter properties—Cells undergoing apoptosis or necrosis display distinct physiological and morphological characteristics, which can be readily measured by flow cytometry (Darzynkiewicz *et al.*, 1992; Tomer *et al.*, 1988). One such technique involves analysis of the cell's light scattering properties. Forward angle light scatter (FS) is a measure of cell diameter or size while side angle, or 90°, light scatter (SS) is a measure of cellular internal complexity (Darzynkiewicz *et al.*, 1992). One morphological characteristic of early apoptosis is cellular shrinkage reflected by a decrease in the FS signal (Darzynkiewicz *et al.*, 1992). As membrane integrity is not compromised in early apoptosis, the SS signal either remains unchanged, or can increase as a result of chromatin condensation or mitochondrial swelling. In contrast to apoptotic cells, cells undergoing necrosis display an initial increase in FS as the SS signal decreases. Accordingly, the effect of BDE 47 on cell morphology was determined by monitoring the FS and SS properties of treated cells in relation to DMSO (vehicle control) treated cells via flow cytometry. Briefly, a gate was placed around the untreated, control population in a FS vs. SS bivariate plot. Shifts in the FS and SS of BDE47 treated cell populations were then assessed, relative to the gate established for control cell populations.

Effect of BDE 47 on sub-G1 DNA content by flow cytometry—The percentage of apoptotic cells was determined by propidium iodide (PI) staining of semi-permeabilized cells conducted as described previously with minor modifications (Nicoletti *et al.*, 1991). In

this assay, apoptotic cells were identified on the basis of hypodiploid DNA content that results from DNA fragmentation (Nicoletti *et al.*, 1991; Pozarowski *et al.*, 2004). Cells having DNA content less than those cells in the G₁ phase of the cell cycle (sub-G₁) were assumed to be apoptotic. In these experiments, approximately 1×10^6 gill cells from each treatment group were detached with Trypsin-EDTA for 5 min @ 37°C, transferred to 15 ml polypropylene tubes and centrifuged at $150 \times g$ for 5 min at 4°C. The cell pellet was resuspended in 200 μ l 1X phosphate buffered saline (PBS) at room temperature (Chemicon International, Temecula, CA) and cells were fixed by the addition of 2 ml of ice-cold 70% ethanol/30% PBS. The cells were incubated on ice for 30 min, centrifuged at $1000 \times g$ for 5 min, and resuspended in 800 μ l 1X PBS. The isolated nuclei were then incubated with 1 μ l RNase H (2 U/ μ l) and 40 μ l PI (1 mg/ μ l) in a 37°C water bath for 30 min, and filtered through a 70 micron pore filter prior to flow cytometry analysis. PI stained nuclei were excited at 488 nm and fluorescence emission was detected at >605 nm.

Flow Cytometry Analysis of Mitochondrial injury

Mitochondrial membrane potential—A decrease in mitochondrial membrane potential is often associated with mitochondrial injury and can serve as an early indicator of apoptosis. Two fluorescent dyes were used to determine the effects of BDE 47 on trout gill cell mitochondrial membrane potential. Specifically, JC-1 is a lipophilic, cationic dye, which selectively incorporates into mitochondrial membranes, exhibiting differential fluorescence characteristics dependent upon the mitochondrial membrane potential (Cossarizza *et al.*, 1993). In polarized membranes, JC-1 molecules form red fluorescent J-aggregates. Upon membrane depolarization, these J-aggregates increasingly shift to a monomeric form exhibiting green fluorescence (Cossarizza *et al.*, 1993). Briefly, approximately 1×10^6 gill cells from each treatment group were detached with Trypsin-EDTA for 5 min @ 37°C, transferred to 15 ml polypropylene tubes and centrifuged at $250 \times g$ for 5 min at 4°C. The cell pellet was resuspended in 800 μ l 1X PBS (Chemicon International, Temecula, CA). JC-1 was prepared in DMSO and added to the cell suspension to a final concentration of 1 μ g/ml and incubated for 45 min at room temperature. The fluorescence intensities were acquired on a Coulter Elite flow cytometer (Beckman Coulter, Miami, FL) using 488 nm excitation. Green (JC-1 monomer) and red (JC-1 aggregate) fluorescence emission were acquired with 525/40-nm band-pass and 590-nm long-pass filters, respectively.

Changes in mitochondrial membrane potential were further evaluated using MitoTracker Red (CMXRos), a mitochondrion-specific probe, which passively diffuses across the plasma membrane and is sequestered into functional mitochondria (Poot and Pierce, 1999). Mitochondria with a decreased membrane potential are less able to retain the probe and are therefore less fluorescent. Approximately 1×10^6 gill cells from each treatment group were detached with Trypsin-EDTA for 5 min @ 37°C, transferred to 15 ml polypropylene tubes and centrifuged at $250 \times g$ for 5 min at 4°C. The cell pellet was resuspended in 800 μ l 1X PBS (Chemicon International, Temecula, CA). MitoTracker Red was prepared in DMSO and added to the cell suspension at a final concentration of 125 μ g/ml and incubated for 15 min at 37°C. MitoTracker Red was excited with 488 nm, and fluorescence emission was collected using a 645 nm long pass filter.

Mitochondrial lipid peroxidation—Cardiolipin is an important component of the inner mitochondrial membrane, where it constitutes about 20% of the total lipid composition (Wright *et al.*, 2004). Peroxidation of cardiolipin leads to a collapse of mitochondrial membrane integrity and release of cytochrome c from mitochondria into the cytosol (Petit *et al.*, 1992; Wright *et al.*, 2004; McMillin and Dowhan, 2002). The fluorescent dye acridine orange 10-nonyl bromide (nonyl acridine orange, NAO) binds cardiolipin with a higher affinity for cardiolipin than other molecules, thus providing a quantitative measure of

mitochondrial lipid peroxidation and mitochondrial membrane integrity (Petit *et al.*, 1992; McMillin and Dowhan, 2002; Wright *et al.*, 2004). In this assay, approximately 1×10^6 gill cells from each treatment group were collected for NAO staining. NAO (Chemicon International, Temecula, CA) was prepared in DMSO and diluted 1:4 with culture medium to a concentration of 125 $\mu\text{g/ml}$ and 1.0 μl of NAO working stock was added to cells that were resuspended in 800 μl 1X PBS prior to incubation for 30 min at 37°C. NAO fluorescence was determined by flow cytometry using 488 nm excitation, and a 645 nm long pass filter to measure fluorescence emission.

Statistical analysis

Experimental values for all oxidative stress and viability indices represent the mean of a minimum of triplicate replications performed in three experiments. All statistical analyses were done using GraphPad Prism version 4.0c for Macintosh, (GraphPad Software, San Diego, California USA). Treatment-related effects on cell injury parameters were assessed using one-way Analysis of Variance (ANOVA) followed by Dunnett's post-hoc test. Treatment-related differences were considered statistically significant at $p \leq 0.05$.

Results

Effect of BDE 47 on viability and cellular redox status

Treatment of RTgill-W1 cells with low concentrations of BDE 47 ($<3.2 \mu\text{M}$) led to a substantial increase in the adherence of cells to culture dishes. At these dosages, collection of cells required the addition of trypsin over incubation, likely as a result of increases in the production of gill cell surface proteins that modulate cell-cell and cell-extracellular matrix interactions (Luo *et al.*, 2007). Presented in Figure 1 is a dose-response of the acute toxicity of BDE 47 in rainbow trout cells. As observed, BDE 47 caused a dose-dependent increase in PI staining relative to DMSO controls. However, the effects of BDE 47 were only statistically significant at the 50 μM exposure concentration. As shown in Figure 2, significant decreases in NAD(P)H autofluorescence were observed in live cells at concentrations of 12.5 and 50 μM BDE 47. Specifically, incubation of cells with 12.5 μM BDE 47 led to a 45% decrease in NAD(P)H autofluorescence, a concentration below those that resulted in acute cell death. Exposure to 50 μM BDE 47, a concentration that was associated with significant cell toxicity, resulted in a 68% decrease in NAD(P)H autofluorescence in live cells.

Effect of BDE 47 on the induction of apoptosis

As observed in Figure 3, exposure to 12.5 μM BDE 47, a concentration that was not associated with acute cell toxicity caused a moderate reduction in the FS signal and a concomitant increase in the SS signal, suggesting the presence of chromatin condensation and mitochondrial swelling. Treatment with the highest dose (50 μM) caused a more dramatic decrease in FS signal and a significant increase in SS signal, strongly suggesting stimulation of the early phase of apoptosis. To further examine whether BDE 47-induced cell death was mediated through apoptosis, we analyzed the DNA content of the RTgill cells by flow cytometry. As shown in Figure 4, at 6 hours post BDE 47 exposure, the sub-G1 cell populations were 10% and 22% for the 12.5 μM and 50 μM BDE 47 concentrations, respectively. At 24 hours post exposure, the observed apoptotic (sub-G1) populations were significantly increased at lower exposure levels (12% increase at 3.2 μM BDE 47) and apoptotic cells constituted more than 75% of total gill cell populations at both the 12.5 μM and 50 μM concentrations.

Analysis of mitochondrial injury

In contrast to our previous study in human cells, we were unable to acquire a reliable J-aggregate (red signal) using JC-1 (Shao *et al.*, 2008b). This may have been due to several factors, including; an intrinsically lower mitochondrial potential of gill cells relative to those from other tissues (O'Dowd *et al.*, 2006) which would not favor the formation of JC-1 aggregates, a low lipid composition of the gill cell membranes precluding the formation of J-aggregates, or quenching of red fluorescence of the aggregates when formed. However, we observed decreased membrane potential associated with an increase in JC-1 green fluorescence on exposure to BDE 47 and thus report the increased in JC-1 monomer (green signal) alone as reflective of mitochondrial depolarization. As shown in Figure 5A, exposure to BDE 47 at doses below 50 μ M did not change the percentage of cells exhibiting JC-1 green fluorescence, which in these cells is associated with an intact mitochondrial redox potential. These effects were most pronounced at the highest dose of BDE 47 where a 71% increase in JC-1 green fluorescence was observed. As shown in Figure 5B, a 56% decrease in MitoTracker Red staining was observed in cells exposed to 50 μ M BDE 47, further indicating a reduction in mitochondrial membrane potential and consistent with the results obtained using JC-1. The reduction in mitochondrial membrane potential at 50 μ M BDE 47 was associated with an increase in mitochondrial lipid peroxidation as evidenced by decreased NAO fluorescence (3% stained cells, Figure 6), compared to control cells.

Discussion

We have previously reported dose-response characteristics associated with BDE 47 toxicity in transformed rainbow trout gill and liver cells (Shao *et al.*, 2008a). The dose-response curves previously observed for BDE 47 were strikingly similar to other studies using mammalian primary and transformed cells exposed to either BDE 47 or to PBDE congener mixtures (Costa and Giordano, 2007; Giordano *et al.*, 2008; Shao *et al.*, 2008b; Yu *et al.*, 2008), suggesting that these agents may have similar mechanisms of toxicity in fish and mammalian cells. However, the biochemical events underlying the progression of BDE 47 cell injury in fish have not been well characterized. The application of flow cytometry approaches and use of fluorescently labeled probes that sensitively detect losses of cell function provides a valuable approach to test the hypothesis that the acute toxicity of BDE 47 in trout gill cells is associated with mitochondrial oxidative injury leading to apoptosis. To our knowledge, the flow cytometry probes used in the present study have not been applied to studies using fish cells, at least in a comprehensive approach to identify toxicants that disrupt cellular redox status.

In the current study, the use of the PI, a dye that binds to double-stranded DNA, enabled us to determine the effect of BDE 47 on the percentage of gill cells in the sub G1 phase of the cell cycle while concomitantly allowing for comparison of results obtained by flow cytometry and biochemically. We conducted the two viability assays (i.e., PI and alamarBlue) under the same conditions with similar results (Shao *et al.*, 2008a). The BDE 47-induced loss of NADPH autofluorescence was consistent with biochemical changes associated with the loss of mitochondrial energetics using the alamarBlue reduction assay that reported in our earlier studies (Shao *et al.*, 2008a; Shao *et al.*, 2008b). Because the alamarBlue assay measures the metabolic integrity of the mitochondria based upon the function of mitochondrial reductases (Ahmed *et al.*, 1994), the rate of alamarBlue reduction reflects oxidation-reduction activity of respiratory chain components in mitochondria, a major intracellular source of ROS (Lee and Wei, 2007). Therefore, our observations of decreased mitochondrial cardiolipin content and mitochondrial membrane potential on exposure to low concentrations of BDE 47 is consistent with the generation of oxidative stress within the mitochondria as a mechanism of cell injury, and indicates that mitochondria are a subcellular target of PBDEs in fish.

A close examination of the flow cytometry data reveals the nature of mitochondrial perturbations caused by BDE 47. For example, the BDE 47-induced reduction in the FS signal, and concomitant increase in the SS signal, reflects morphological characteristics associated with the onset of apoptosis. The results obtained by using the JC-1 fluorescent assay were consistent with those by MitoTracker Red indicating a loss of mitochondrial redox potential which is typically correlated with apoptosis. These data are consistent with analysis of sub G1 DNA content analysis by flow cytometry which indicated that exposure to BDE 47 increased the percentage of cells undergoing frank apoptosis. Endonucleases activated during apoptosis cleave sections of internucleosomal DNA, causing extensive DNA fragmentation, a primary indicator of apoptosis (Nagata, 2000). As a result of DNA fragmentation and chromatin condensation, apoptotic cells show reduced DNA staining with PI, reflecting lower quantitative DNA content relative to G1 phase nuclei (Gong *et al.*, 1994). The shedding of apoptotic bodies containing fragments of nuclear chromatin may further contribute to the loss of DNA from apoptotic cells (Pozarowski *et al.*, 2004). Accordingly, the emergence of sub-G1 peaks seen with increasing levels of BDE 47 treatment indicates that these cells were undergoing apoptosis. These results are consistent with other studies in primary human fetal liver hematopoietic stem cells (Shao *et al.*, 2008b) and primary mouse neurons and astrocytes (Giordano *et al.*, 2008) which also demonstrated that exposure to PBDEs lead to cellular injury through induction of apoptotic cell death.

A substantial body of work has shown that mitochondrial membrane depolarization plays an important role in regulating apoptosis (Green and Kroemer, 2004; Marchetti *et al.*, 1996). Chemical exposures can destabilize the mitochondrial membrane and cause a loss of mitochondrial integrity and cell survival (Loh *et al.*, 2006; Zorov *et al.*, 2006). In the current study, we observed a marked loss of mitochondrial membrane potential associated with the degradation of cardiolipin, a marker of mitochondrial membrane lipid oxidation, in gill cells treated with BDE 47, further supporting our hypothesis that BDE 47 targets the mitochondria and stimulates apoptosis via mitochondrial oxidative injury (Lugli *et al.*, 2005). Cardiolipin is a phospholipid located in the inner mitochondrial membrane, that when oxidized, plays a crucial role in cytochrome C release from mitochondria leading to apoptosis (Basova *et al.*, 2007). Normally, cytochrome C is localized to the outer surface of the inner mitochondrial membrane via electrostatic interactions with anionic lipids such as cardiolipin (Demel *et al.*, 1989). BDE 47 mediated cardiolipin oxidation likely disrupted the interaction between cytochrome C and cardiolipin, followed by its dissociation from the membrane and escape through the Bax-mediated permeabilized outer mitochondrial membrane (Nomura *et al.*, 2000; Ott *et al.*, 2002; Petrosillo *et al.*, 2001). It is also possible that BDE 47 triggered mitochondrial swelling and a rupture of the outer mitochondrial membrane (as reflected by light scattering measurements), which could have then caused cytochrome C release (Goldstein *et al.*, 2000), leading to the activation of caspase-dependent and independent mitochondrial death pathways (Antonsson, 2004).

It is important to note that all of the fluorescently-labeled commercial dyes used in this study required substantial optimization methodologies during assay validation. Furthermore, in addition to the battery of cell indicators discussed (i.e. JC-1, MitoTracker, NAO, PI staining, NADPH autofluorescence), we initially investigated other probes, including BODIPY FL C11, diphenyl-1-pyrenylphosphine (DPPP) and Annexin-V-Fluorescein (Annexin-V-FL). Specifically, BODIPY FL C11 is a lipophilic fluorophore that localizes to cell membranes and emits a fluorescence profile reflective of the oxidative state of the plasma membrane. Accordingly, as BODIPY FL C11 becomes oxidized, there is a concomitant shift from the red fluorescent excimer form to a green fluorescent monomeric form associated with peroxidation state of the membrane. By contrast, Annexin-V-FL is a phospholipid-binding protein with strong affinity for phosphatidylserine (PS). During the early stages of apoptosis, PS is translocated from the inner part of the plasma membrane to the external surface of the

cell. Thus, Annexin-V-FL binds exposed PS, providing a direct measure of apoptotic events. Although these aforementioned probes were used effectively in our studies addressing the mechanisms of BDE 47 injury to human fetal liver hematopoietic stem cells (Shao *et al.*, 2008b), they yielded inconsistent results in trout gill cells. The unsuccessful application of some of these flow cytometry probes may have been a result of the gill cell phospholipid content, which appears to be relatively low in trout gill cell membranes and can be further reduced during thermal acclimation (Hazel and Carpenter, 1985; Kraffe *et al.*, 2007). Furthermore, the fact that the trout gill cells were maintained at 19°C, a higher than normal physiological temperature for rainbow trout, may have further decreased the content of unsaturated phospholipids allowing for detection of lipid peroxidation by BODIPY FL C11 and DPPP. The lower mitochondrial potential of rainbow trout gill cells relative to other tissues may have contributed to the inability of JC-1 to form aggregates (O'Dowd *et al.*, 2006). Although we were not able to capture the red JC-1 signal by flow cytometry, the observed increase of the corresponding JC-1 green signal is similar to results observed using fetal liver hematopoietic stem cells, and in the current study using the MitoTracker Red dye.

As discussed, the dosing range of BDE 47 used in this study (3.2 µM -50 µM) allowed for comparison to other studies and were reasonable given that a only a single acute dose was employed, likely underestimating the environmental scenario involving chronic exposures. This range of exposures encompassed somewhat higher BDE 47 levels that caused cytotoxicity as well as lower BDE 47 levels that elicited subtle biochemical changes that allowed us to clearly evaluate with mechanisms of toxicity. The cellular effects observed with low micromolar concentrations of BDE 47 suggests a moderate sensitivity of trout gill cells to this, and potentially to other, PBDE congeners. BDE 47 toxicity to primary hepatocytes from rainbow trout show similar sensitivities which may be partially dependent upon the poor detoxification capabilities of the cells (Nakari and Pessala, 2005). Although the role of detoxification gene expression has not been clearly identified in detecting against PBDE toxicity, a number of genes critical to cellular protection are down-regulated in the liver of rainbow trout exposed to BDE 47 *in vivo* (Hook *et al.*, 2006). The sensitivity of trout gill epithelial cells to BDE 47 may be due to their poor ability to detoxify reactive oxygen species via cellular antioxidant pathways (Shao *et al.*, 2008a). However, we did not characterize the baseline expression of antioxidant enzymes in the gill cells, or determine if BDE 47 exposure modulates the expression of genes that protect against oxidative stress. These studies are ongoing in our laboratory.

In summary, BDE 47 is a predominant PBDE congener detected in fish that causes injury to cultured rainbow trout gill cells that is associated with increased cellular oxidative stress, mitochondrial injury, and apoptosis. Although the resulting cell death occurs at a level that is above PBDE concentrations encountered in the environment, we have not examined the effect of multiple or chronic exposures which may be more relevant to environmental exposures. Furthermore, the fact that BDE 47 bioaccumulates in fish relative to the other higher molecular weight congeners (Betts, 2002; Browne *et al.*, 2009; Sjodin *et al.*, 2003) is important in extrapolating our *in vitro* results to more relevant environmental scenarios. Collectively, our studies also indicate that oxidative stress-associated biomarkers may be useful in assessing the sublethal effects of PBDEs in fish, as well as in other species. To this end, the application of flow cytometry-based analyses and fluorescent probes that are sensitive markers of cell injury from aquatic species are of utility for broader use in the field of aquatic toxicology.

Acknowledgments

This work was supported by grants from the National Oceanic and Atmospheric Association (NOAA) Oceans and Human Health Initiative NA05NOS4781253, the National Institute of Environmental Health Sciences (NIEHS)

Superfund Basic Sciences Research Program P42-ES-04696, and the UW NIEHS sponsored Center for Ecogenetics and Environmental Health, P30ES07033.

References

- Ahmed SA, Gogal RM Jr, Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [³H]thymidine incorporation assay. *J. Immunol. Methods*. 1994; 170:211–24. [PubMed: 8157999]
- Antonsson B. Mitochondria and the Bcl-2 family proteins in apoptosis signaling pathways. *Mol. Cell Biochem*. 2004; 256-257:141–55. [PubMed: 14977177]
- Antonsson B, Montessuit S, Lauper S, Eskes R, Martinou JC. Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. *Biochem. J*. 2000; 345(Pt 2):271–8. [PubMed: 10620504]
- Basova LV, Kurnikov IV, Wang L, Ritov VB, Belikova NA, Vlasova II, Pacheco AA, Winnica DE, Peterson J, Bayir H, Waldeck DH, Kagan VE. Cardiolipin switch in mitochondria: shutting off the reduction of cytochrome c and turning on the peroxidase activity. *Biochemistry*. 2007; 46:3423–34. [PubMed: 17319652]
- Betts KS. Rapidly rising PBDE levels in North America. *Environ. Sci. Technol*. 2002; 36:50A–52A.
- Birnbaum LS, Cohen Hubal EA. Polybrominated diphenyl ethers: a case study for using biomonitoring data to address risk assessment questions. *Environ. Health Perspect*. 2006; 114:1770–5. [PubMed: 17107866]
- Birnbaum LS, Staskal DF. Brominated flame retardants: cause for concern? *Environ. Health Perspect*. 2004; 112:9–17. [PubMed: 14698924]
- Bols NC, Barlian A, Chirino-Trejo M, Caldwell SJ, Goegan P, Lee LEJ. Development of a cell line from primary cultures of rainbow trout, *Oncorhynchus mykiss* (Walbaum), gills. *Journal of Fish Diseases*. 1994; 17:601–611.
- Browne EP, Stapleton HM, Kelly SM, Tilton SC, Gallagher EP. In vitro hepatic metabolism of 2,2', 4,4',5-pentabromodiphenyl ether (BDE 99) in Chinook salmon (*Onchorhynchus tshawytscha*). *Aquat. Toxicol*. 2009; 92:281–7. [PubMed: 19346012]
- Burreau S, Axelman J, Broman D, Jakobsson E. Dietary uptake in pike (*Esox lucius*) of some polychlorinated biphenyls, polychlorinated naphthalenes and polybrominated diphenyl ethers administered in natural diet. *Environ. Toxicol. and Chem*. 1997; 16:2508–513.
- Butt CM, Diamond ML, Truong J, Ikononou MG, ter Schure AF. Spatial distribution of polybrominated diphenyl ethers in southern Ontario as measured in indoor and outdoor window organic films. *Environ. Sci. Technol*. 2004; 38:724–31. [PubMed: 14968856]
- Cai L, Kang YJ. Cell death and diabetic cardiomyopathy. *Cardiovasc. Toxicol*. 2003; 3:219–28. [PubMed: 14555788]
- Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C. A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). *Biochem. Biophys. Res. Commun*. 1993; 197:40–5. [PubMed: 8250945]
- Costa LG, Giordano G. Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. *Neurotoxicology*. 2007; 28:1047–67. [PubMed: 17904639]
- Darnerud PO. Toxic effects of brominated flame retardants in man and in wildlife. *Environ. Int*. 2003; 29:841–53. [PubMed: 12850100]
- Darnerud PO, Eriksen GS, Johannesson T, Larsen PB, Viluksela M. Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. *Environ. Health Perspect*. 2001; 109(Suppl 1):49–68. [PubMed: 11250805]
- Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P, Traganos F. Features of apoptotic cells measured by flow cytometry. *Cytometry*. 1992; 13:795–808. [PubMed: 1333943]
- de Wit CA. An overview of brominated flame retardants in the environment. *Chemosphere*. 2002; 46:583–624. [PubMed: 11999784]
- de Wit CA, Alaei M, Muir DC. Levels and trends of brominated flame retardants in the Arctic. *Chemosphere*. 2006; 64:209–33. [PubMed: 16458344]

- Dejean LM, Martinez-Caballero S, Manon S, Kinnally KW. Regulation of the mitochondrial apoptosis-induced channel, MAC, by BCL-2 family proteins. *Biochim. Biophys. Acta.* 2006; 1762:191–201. [PubMed: 16055309]
- del VCM, Reyes JM, Park CY, Gao X, Mori K, Chuck RS, Gehlbach PL. Demonstration by redox fluorometry that sulforaphane protects retinal pigment epithelial cells against oxidative stress. *Invest. Ophthalmol. Vis. Sci.* 2008; 49:2606–12. [PubMed: 18515589]
- Delhalle S, Duvoix A, Schnekenburger M, Morceau F, Dicato M, Diederich M. An introduction to the molecular mechanisms of apoptosis. *Ann. N. Y. Acad. Sci.* 2003; 1010:1–8. [PubMed: 15033687]
- Demel RA, Jordi W, Lambrechts H, van Damme H, Hovius R, de Kruijff B. Differential interactions of apo- and holocytochrome c with acidic membrane lipids in model systems and the implications for their import into mitochondria. *J. Biol. Chem.* 1989; 264:3988–97. [PubMed: 2537300]
- Eriksson P, Jakobsson E, Fredriksson A. Brominated flame retardants: a novel class of developmental neurotoxicants in our environment? *Environ. Health Perspect.* 2001; 109:903–8. [PubMed: 11673118]
- Fernie KJ, Shutt JL, Mayne G, Hoffman D, Letcher RJ, Drouillard KG, Ritchie IJ. Exposure to polybrominated diphenyl ethers (PBDEs): changes in thyroid, vitamin A, glutathione homeostasis, and oxidative stress in American kestrels (*Falco sparverius*). *Toxicol. Sci.* 2005; 88:375–83. [PubMed: 16120752]
- Gill U, Chu I, Ryan JJ, Feeley M. Polybrominated diphenyl ethers: human tissue levels and toxicology. *Rev. Environ. Contam. Toxicol.* 2004; 183:55–97. [PubMed: 15369322]
- Giordano G, Kavanagh TJ, Costa LG. Neurotoxicity of a polybrominated diphenyl ether mixture (DE-71) in mouse neurons and astrocytes is modulated by intracellular glutathione levels. *Toxicol. Appl. Pharmacol.* 2008; 232:161–8. [PubMed: 18656495]
- Goldstein JC, Waterhouse NJ, Juin P, Evan GI, Green DR. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat. Cell Biol.* 2000; 2:156–62. [PubMed: 10707086]
- Gong J, Traganos F, Darzynkiewicz Z. A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal. Biochem.* 1994; 218:314–9. [PubMed: 8074286]
- Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science.* 2004; 305:626–9. [PubMed: 15286356]
- Gustafsson K, Bjork M, Burreau S, Gilek M. Bioaccumulation kinetics of brominated flame retardants (polybrominated diphenyl ethers) in blue mussels (*Mytilus edulis*). *Environ. Toxicol. Chem.* 1999; 18:1218–1224.
- Hale RC, Alae M, Manchester-Neesvig JB, Stapleton HM, Ikononou MG. Polybrominated diphenyl ether flame retardants in the North American environment. *Environ. Int.* 2003; 29:771–9. [PubMed: 12850095]
- Hazel JR, Carpenter R. Rapid changes in the phospholipid composition of gill membranes during thermal acclimation of the rainbow trout, *Salmo gairdneri*. *J. Comp. Physiol. [B].* 1985; 155:597–602.
- He P, He W, Wang A, Xia T, Xu B, Zhang M, Chen X. PBDE-47-induced oxidative stress, DNA damage and apoptosis in primary cultured rat hippocampal neurons. *Neurotoxicology.* 2008; 29:124–9. [PubMed: 18054389]
- Hu XZ, Xu Y, Hu DC, Hui Y, Yang FX. Apoptosis induction on human hepatoma cells Hep G2 of decabrominated diphenyl ether (PBDE-209). *Toxicol Lett.* 2007; 171:19–28. [PubMed: 17509781]
- Hites RA. Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. *Environ. Sci. Technol.* 2004; 38:945–56. [PubMed: 14998004]
- Hites RA, Foran JA, Schwager SJ, Knuth BA, Hamilton MC, Carpenter DO. Global assessment of polybrominated diphenyl ethers in farmed and wild salmon. *Environ. Sci. Technol.* 2004; 38:4945–9. [PubMed: 15506184]
- Holm G, Norrgren L, Andersson T, Thuréa A. Effects of exposure to food contaminated with PBDE, PCN or PCB on reproduction, liver morphology and cytochrome P450 activity in the three-spined stickleback, *Gasterosteus aculeatus*. *Aquat. Toxicol.* 1993; 27:33–50.

- Hook SE, Skillman AD, Small JA, Schultz IR. Gene expression patterns in rainbow trout, *Oncorhynchus mykiss*, exposed to a suite of model toxicants. *Aquat. Toxicol.* 2006; 77:372–85. [PubMed: 16488489]
- Kraffe E, Marty Y, Guderley H. Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss*: roles of membrane proteins, phospholipids and their fatty acid compositions. *J. Exp. Biol.* 2007; 210:149–65. [PubMed: 17170158]
- Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol. Rev.* 2007; 87:99–163. [PubMed: 17237344]
- Laing RA, Fischbarg J, Chance B. Noninvasive measurements of pyridine nucleotide fluorescence from the cornea. *Invest. Ophthalmol. Vis. Sci.* 1980; 19:96–102. [PubMed: 7350140]
- Lam JC, Kajiwaru N, Ramu K, Tanabe S, Lam PK. Assessment of polybrominated diphenyl ethers in eggs of waterbirds from South China. *Environ. Pollut.* 2006; 148:258–67. [PubMed: 17150292]
- Lee LE, Dayeh VR, Schirmer K, Bols NC. Fish cell lines as rapid and inexpensive screening and supplemental tools for whole effluent testing. *Integr Environ Assess Manag.* 2008; 4:372–4. [PubMed: 18597561]
- Lee HC, Wei YH. Oxidative stress, mitochondrial DNA mutation, and apoptosis in aging. *Exp. Biol. Med.* (Maywood). 2007; 232:592–606. [PubMed: 17463155]
- Lema SC, Schultz IR, Scholz NL, Incardona JP, Swanson P. Neural defects and cardiac arrhythmia in fish larvae following embryonic exposure to 2,2',4,4'-tetrabromodiphenyl ether (PBDE 47). *Aquat. Toxicol.* 2007; 82:296–307. [PubMed: 17412433]
- Loh KP, Huang SH, De Silva R, Tan BK, Zhu YZ. Oxidative stress: apoptosis in neuronal injury. *Curr. Alzheimer Res.* 2006; 3:327–37. [PubMed: 17017863]
- Lugli E, Troiano L, Ferraresi R, Roat E, Prada N, Nasi M, Pinti M, Cooper EL, Cossarizza A. Characterization of cells with different mitochondrial membrane potential during apoptosis. *Cytometry A.* 2005; 68:28–35. [PubMed: 16184612]
- Luo BH, Carman CV, Springer TA. Structural basis of integrin regulation and signaling. *Annu. Rev. Immunol.* 2007; 25:619–47. [PubMed: 17201681]
- Maines MD, Costa LG, Reed DJ, Sassa S, Sipes IG, Morgan KS. The use of fish-derived cell lines. *Current Protocols in Toxicology.* 2001; (Supplement 15) 1.5.8.
- Marchetti P, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A, Haeflner A, Hirsch F, Geuskens M, Kroemer G. Mitochondrial permeability transition is a central coordinating event of apoptosis. *J. Exp. Med.* 1996; 184:1155–60. [PubMed: 9064332]
- Masters BR, Ghosh AK, Wilson J, Matschinsky FM. Pyridine nucleotides and phosphorylation potential of rabbit corneal epithelium and endothelium. *Invest. Ophthalmol. Vis. Sci.* 1989; 30:861–68. [PubMed: 2722442]
- Mazdai A, Dodder NG, Abernathy MP, Hites RA, Bigsby RM. Polybrominated diphenyl ethers in maternal and fetal blood samples. *Environ. Health Perspect.* 2003; 111:1249–52. [PubMed: 12842781]
- McDonald TA. A perspective on the potential health risks of PBDEs. *Chemosphere.* 2002; 46:745–55. [PubMed: 11999798]
- McKim J, Schmieder P, Veith G. Absorption dynamics of organic chemical transport across trout gills as related to octanol-water partition coefficient. *Toxicol. Appl. Pharmacol.* 1985; 77:1–10. [PubMed: 3966233]
- McMillin JB, Dowhan W. Cardiolipin and apoptosis. *Biochim Biophys Acta.* 2002; 1585:97–107. [PubMed: 12531542]
- Mikhailov V, Mikhailova M, Pulkrabek DJ, Dong Z, Venkatachalam MA, Saikumar P. Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane. *J. Biol. Chem.* 2001; 276:18361–74. [PubMed: 11279112]
- Nagata S. Apoptotic DNA fragmentation. *Exp. Cell Res.* 2000; 256:12–8. [PubMed: 10739646]
- Nakari T, Pessala P. In vitro estrogenicity of polybrominated flame retardants. *Aquat. Toxicol.* 2005; 74:272–9. [PubMed: 16024102]
- Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods.* 1991; 139:271–9. [PubMed: 1710634]

- Nomura K, Imai H, Koumura T, Kobayashi T, Nakagawa Y. Mitochondrial phospholipid hydroperoxide glutathione peroxidase inhibits the release of cytochrome c from mitochondria by suppressing the peroxidation of cardiolipin in hypoglycaemia-induced apoptosis. *Biochem. J.* 2000; 351:183–93. [PubMed: 10998361]
- O'Dowd C, Mothersill CE, Cairns MT, Austin B, McClean B, Lyng FM, Murphy JE. The release of bystander factor(s) from tissue explant cultures of rainbow trout (*Onchorhynchus mykiss*) after exposure to gamma radiation. *Radiat Res.* 2006; 166:611–7. [PubMed: 17007552]
- Ott M, Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. Cytochrome c release from mitochondria proceeds by a two-step process. *Proc. Natl. Acad. Sci. U. S. A.* 2002; 99:1259–63. [PubMed: 11818574]
- Petit JM, Maftah A, Ratinaud MH, Julien R. 10N-nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria. *Eur J Biochem.* 1992; 209:267–73. [PubMed: 1396703]
- Petrosillo G, Ruggiero FM, Pistolesi M, Paradies G. Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-heart submitochondrial particles via cardiolipin peroxidation. Possible role in the apoptosis. *FEBS Lett.* 2001; 509:435–8. [PubMed: 11749969]
- Poot M, Pierce RH. Detection of changes in mitochondrial function during apoptosis by simultaneous staining with multiple fluorescent dyes and correlated multiparameter flow cytometry. *Cytometry.* 1999; 35:311–7. [PubMed: 10213196]
- Pozarowski P, Grabarek J, Darzynkiewicz Z. Flow cytometry of apoptosis. *Curr. Protoc. Cell Biol.* 2004;8. Chapter 18, Unit 18. [PubMed: 18228448]
- Schecter A, Johnson-Welch S, Tung KC, Harris TR, Papke O, Rosen R. Polybrominated diphenyl ether (PBDE) levels in livers of U.S. human fetuses and newborns. *J. Toxicol. Environ. Health A.* 2007; 70:1–6. [PubMed: 17162494]
- Schecter A, Papke O, Joseph JE, Tung KC. Polybrominated diphenyl ethers (PBDEs) in U.S. computers and domestic carpet vacuuming: possible sources of human exposure. *J. Toxicol. Environ. Health A.* 2005; 68:501–13. [PubMed: 15805045]
- Schecter A, Papke O, Tung KC, Staskal D, Birnbaum L. Polybrominated diphenyl ethers contamination of United States food. *Environ. Sci. Technol.* 2004; 38:5306–11. [PubMed: 15543730]
- Shao J, Eckert ML, Lee LE, Gallagher EP. Comparative oxygen radical formation and toxicity of BDE 47 in rainbow trout cell lines. *Mar. Environ. Res.* 2008a; 66:7–8. [PubMed: 18400291]
- Shao J, Stapleton PL, Lin YS, Gallagher EP. Cytochrome p450 and glutathione s-transferase mRNA expression in human fetal liver hematopoietic stem cells. *Drug Metab. Dispos.* 2007; 35:168–75. [PubMed: 17050647]
- Shao J, White CC, Dabrowski MJ, Kavanagh TJ, Eckert ML, Gallagher EP. The role of mitochondrial and oxidative injury in BDE 47 toxicity to human fetal liver hematopoietic stem cells. *Toxicol. Sci.* 2008b; 101:81–90. [PubMed: 17916640]
- Sjodin A, Patterson DG Jr, Bergman A. A review on human exposure to brominated flame retardants--particularly polybrominated diphenyl ethers. *Environ. Int.* 2003; 29:829–39. [PubMed: 12850099]
- Stapleton HM, Baker JE. Comparing polybrominated diphenyl ether and polychlorinated biphenyl bioaccumulation in a food web in Grand Traverse Bay, Lake Michigan. *Arch. Environ. Contam. Toxicol.* 2003; 45:227–34. [PubMed: 14565581]
- Stapleton HM, Dodder NG, Offenberg JH, Schantz MM, Wise SA. Polybrominated diphenyl ethers in house dust and clothes dryer lint. *Environ. Sci. Technol.* 2005; 39:925–31. [PubMed: 15773463]
- Tjarnlund U, Ericson G, Orn U, de Wit C, Balk L. Effects of two polybrominated diphenyl ethers on rainbow trout (*Oncorhynchus mykiss*) exposed via food. *Mar. Environ. Res.* 1998; 46:107–112.
- Tomer A, Harker LA, Burstein SA. Flow cytometric analysis of normal human megakaryocytes. *Blood.* 1988; 71:1244–52. [PubMed: 3359043]
- van Boxtel AL, Kamstra JH, Cnijn PH, Pieterse B, Wagner JM, Antink M, Krab K, van der Burg B, Marsh G, Brouwer A, Legler J. Microarray analysis reveals a mechanism of phenolic polybrominated diphenylether toxicity in zebrafish. *Environ. Sci. Technol.* 2008; 42:1773–9. [PubMed: 18441834]

- Wood CM, Kelly SP, Zhou B, Fletcher M, O'Donnell M, Eletti B, Part P. Cultured gill epithelia as models for the freshwater fish gill. *Biochim. Biophys. Acta.* 2002; 1566:72–83. [PubMed: 12421539]
- Wright MM, Howe AG, Zaremborg V. Cell membranes and apoptosis: role of cardiolipin, phosphatidylcholine, and anticancer lipid analogues. *Biochem Cell Biol.* 2004; 82:18–26. [PubMed: 15052325]
- Yu K, He Y, Yeung LW, Lam PK, Wu RS, Zhou B. DE-71-induced apoptosis involving intracellular calcium and the Bax-mitochondria-caspase protease pathway in human neuroblastoma cells in vitro. *Toxicol. Sci.* 2008; 104:341–51. [PubMed: 18453545]
- Zha H, Aime-Sempe C, Sato T, Reed JC. Proapoptotic protein Bax heterodimerizes with Bcl-2 and homodimerizes with Bax via a novel domain (BH3) distinct from BH1 and BH2. *J. Biol. Chem.* 1996; 271:7440–4. [PubMed: 8631771]
- Zhou T, Ross DG, DeVito MJ, Crofton KM. Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol. Sci.* 2001; 61:76–82. [PubMed: 11294977]
- Zhou T, Taylor MM, DeVito MJ, Crofton KM. Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol. Sci.* 2002; 66:105–16. [PubMed: 11861977]
- Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial ROS-induced ROS release: an update and review. *Biochim. Biophys. Acta.* 2006; 1757:509–17. [PubMed: 16829228]

Figure 1. Effect of BDE 47 on cell viability as measured by propidium iodide (PI) fluorescence Gill cells were incubated at 19 °C for 24 hours with 0.0 (0.2% DMSO control), 0.2, 0.8, 3.2, 12.5 and 50 μM BDE 47. Cells showing an increase in nuclear PI staining following treatment with BDE 47 indicate an increase in nuclear membrane permeability associated with cell death. Data represent mean $\pm$ SEM of 3 or more experiments. Asterisks denote significant treatment-related effects ($p \leq 0.05$).

Figure 2.

Change in redox status associated with increasing concentration of BDE 47. Gill cells were exposed to increasing concentrations of BDE 47 in 0.2% DMSO for 24 hours and the effect on cellular redox status was determined by measuring NAD(P)H autofluorescence via flow cytometry. Data represent mean $\pm$ SEM of 3 or more experiments with asterisks denoting significant differences between controls and BDE 47-exposed cells ($p \leq 0.05$).

Figure 3. Alteration of forward angle light scatter (FS) and side light scatter (SS) properties following treatment with BDE 47

A reduction in the FS signal and an increase in the SS signal were observed at 12.5 μ M and 50 μ M following a 24 hour exposure to BDE 47 reflecting the early stages of apoptosis.

Figure 4. BDE 47-induced apoptosis in RTgill-W1 by sub-G1 analysis

Panel A. Exposure to BDE 47 caused significant increases in sub-G1 DNA content of gill cells at 3.2, 12.5 and 50 μM concentrations (* $p < 0.01$) at 24 hours post exposure. Data represent mean $\pm$ SEM of 3 experiments. In panel B are representative cytograms for cells from treatments involving DMSO (controls), 3.2 μM, 12.5 μM, and 50 μM BDE 47 exposure groups from a single experiment 24 hr following exposure.

Figure 5. Analysis of mitochondrial membrane potential following exposure to BDE 47
 Gill cells were incubated at 19 °C for 24 hours with 0.0 (0.2% DMSO control), 0.2, 0.8, 3.2, 12.5 and 50 μM BDE 47. Panel A: Measurement of mitochondrial membrane potential ($\Delta\psi_m$) using JC-1. Cells show an increase in membrane depolarization with treatment as seen by an increase in green fluorescence (JC-1 monomers). Panel B: MitoTracker red, which accumulates in polarized mitochondria, is reduced at the highest treatment level indicating a reduction in mitochondrial membrane potential. Data represent mean $\pm$ SEM of 3 or more experiments. Asterisks denote significant treatment-related effects ($p \leq 0.05$).

Figure 6. Loss of mitochondrial membrane integrity following exposure to BDE 47

Gill cells were incubated at 19 °C for 24 hours with 0.0 (0.2% DMSO control), 0.2, 0.8, 3.2, 12.5 and 50 μM BDE 47. The marked decrease of NAO fluorescence in cells at 50 μM BDE 47 indicates a decrease in mitochondrial membrane integrity. Data represent mean $\pm$ SEM of 3 or more experiments. Asterisks denote significant treatment-related effects ($p \leq 0.05$).