Determination of environmentally relevant exposure concentrations of polybrominated diphenyl ethers for *in vitro* toxicological studies

Rong-Guo Wei^a, Ya-Xian Zhao^{a,b}, Peng-Yan Liu^b, Zhan-Fen Qin^{a,*}, Shi-Shuai Yan^a, Yan Li^{a,b}, Xiao-Fei Qin^a, Xi-Juan Xia^a, Xiao-Bai Xu^a, Min-Chan Yan^c

^a State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-environmental Sciences, Chinese Academy of Sciences, Beijing, China

^b College of Chemistry and Environmental Science, HeBei University, Baoding, Hebei, China

^c Taizhou Hospital, Taizhou City, Zhejiang Province 317000, China

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ABSTRACT

Toxicological studies at environmentally relevant concentrations are essential for understanding ecotoxic and health risks of pollutants such as polybrominated diphenyl ethers (PBDEs). However, no information is available on what exposure levels of PBDEs *in vitro* studies are environmentally relevant. We exposed MCF-7, HepG2, H295R and PC12 cells to BDE-47, and measured BDE-47 concentrations in the cells after exposure. We also used the percentile method to summarize literature data on environmental exposure levels of biotic tissues to PBDEs. The exposure concentration that resulted in a BDE-47 burden in cells close to the 90th percentile of PBDEs levels in tissues was assigned as the upper limit for the environmentally relevant concentration. Exposure to 1 nM BDE-47 resulted in PBDEs burdens in MCF-7, HepG2 and H295R cells close to the 90th percentile but PBDEs burdens in PC12 cells were higher than the 90th percentile. In consideration of the high exposure levels in PBDE-polluted areas, we concluded that the highest environmentally relevant exposure concentration of PBDEs in culture media should be approximately 10 nM for MCF-7, HepG2 and H295R cells, and <10 nM for PC12 cells. These results provide an approximate reference for setting environmentally relevant exposure concentrations of PBDEs for studies *in vitro*.

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1. Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of flame retardants used extensively in a variety of commercial and household products, such as electronic equipment, plastics, construction materials, coatings and textiles (Costa and Giordano, 2007; Stapleton et al., 2009). PBDEs have been found in various environmental and biotic samples, including human tissues (Alcock et al., 2003; Costa and Giordano, 2007; Darnerud et al., 2001; Stapleton et al., 2009; Watanabe and Sakai, 2003). In recent years, the potential adverse effects of PBDEs in animals and humans have caused increasing concern. Numerous studies have shown that PBDEs and polychlorinated biphenyls (PCBs) have a wide range of toxicities, such as thyroid disrupting activity and developmental neurotoxicity (Costa and Giordano, 2007; Hallgren and Darnerud, 2002; Harju et al., 2007; Song et al., 2009).

Abbreviations: BDE-47, 2,2',4,4'-tetrabrominated diphenyl ether; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; PBDEs, polybrominated diphenyl ethers.

* Corresponding author. Address: P.O. Box 2871, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. Tel.: +86 10 62919177; fax: +86 10 62923563.

E-mail address: qinzhansen@rcees.ac.cn (Z.-F. Qin).

To date, available information on PBDEs toxicity has mainly been derived from studies at high doses, which are generally several orders of magnitude higher than actual environmental exposure levels for animals and humans (Suvorov and Takser, 2008). There is limited information on adverse effects of PBDEs in animals and humans at low doses and especially at environmentally relevant doses. High dose studies have predominated in toxicology because scientists assume these studies can indicate the target of low dose exposure. However, some environmental toxicants display non-linear dose–response relationships, which means they cause different effects at different doses, and the impacts at low doses may not occur at high doses (Andrade et al., 2006; Narita et al., 2007; Wetherill et al., 2002; Wozniak et al., 2005). Recently, PBDEs have been reported to display apparent non-monotonic effects in experimental rats. Suvorov et al. (2008) found that exposure to a low dose (2 µg/kg) of 2,2',4,4'-tetrabrominated diphenyl ether (BDE-47) seemed to more effectively induce hyperactivity in rat offspring compared to high doses (20 and 200 µg/kg). Talsness et al. (2008) reported that exposure to low doses of BDE-47 (140 µg/kg body weight) caused an increase in ovarian weight in experimental rats but high doses (700 µg/kg) had no significant effect. Therefore, toxicological studies at doses higher than environmental exposure levels might contribute little to the

understanding of ecotoxic and health risks associated with PBDEs. Therefore, toxicological studies at environmentally relevant doses are essential.

In vitro studies are important in toxicology and are frequently used to study PBDEs toxicity. In general, previous studies of PBDEs *in vitro* have incorporated micromolar (μM) levels in the culture media, although several reports have used nanomolar (nM) levels (Barber et al., 2006; Cantón et al., 2006; Dingemans et al., 2007, 2008; Fery et al., 2009; Giordano et al., 2008, 2009; He et al., 2008a,b, 2009; Hu et al., 2007; Madia et al., 2004; Mercado-Feliciano and Bigsby, 2008; Peters et al., 2004, 2006; Stavenes Andersen et al., 2009; Song et al., 2009; Stapleton et al., 2009; Tagliaferri et al., 2010; Zhang et al., 2007). None of these studies measured the cellular PBDE concentrations but it is assumed that the concentrations used may not be environmentally relevant because they are much higher than the picomolar (pM) or lower levels typically found in environmental water or body fluids (Frederiksen et al., 2009; Gill et al., 2004; Streets et al., 2006; Wu et al., 2008). It is important to establish exposure concentrations of PBDEs for *in vitro* toxicological studies that will result in environmentally relevant concentrations in culture cells. Human breast carcinoma MCF-7 cells, human adrenocortical carcinoma H295R cells, human hepatoma HepG2 cells and rat pheochromocytoma PC12 cells are the most commonly used cells in PBDEs toxicological studies. In this study, we exposed culture cells to BDE-47, which is a main PBDEs component in various environmental and biotic samples, and investigated its accumulation in the cells. As the available literature on environmental exposure levels of PBDEs in biotic tissues had not been summarized previously, we used the percentile method to do this. Based on the 90th percentile level of PBDEs, we determined the PBDEs exposure concentration that would result in environmentally relevant burdens in cells for studies *in vitro*.

2. Materials and methods

2.1. Chemicals

BDE-47 was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Dimethyl sulfoxide (DMSO) and dextran were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Trypsinase was purchased from Invitrogen (Steinheim, Germany). Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Carlsbad, CA). All other consumables for cells were obtained from Invitrogen Life Technologies, unless otherwise stated.

Solutions of BDE-71 for use as a surrogate standard and EO-5278 to quantify the BDE-71 were purchased from Cambridge Isotope Laboratories (Andover, MA). Pesticide grade *n*-hexane, methylene dichloride, and nonane were purchased from Tedia (Fairfield, OH). Anhydrous sodium sulfate, sulfuric acid, sodium hydroxide, and anhydrous ethanol (Beijing Chemical Factory, Beijing, China) were of analytical grade.

2.2. Cell culture

HepG2 cells (Cell Bank of Chinese Academy of Sciences) were cultured in DMEM containing 10% fetal bovine serum (FBS, Hyclone). Rat pheochromocytoma PC12 cells (Cell Bank of Chinese Academy of Sciences) were cultured in DMEM supplemented with 7.5% FBS and 7.5% horse serum (Hyclone). MCF-7 cells (ER⁺ human mammary carcinoma), kindly provided by Professor Ana M. Soto (Tufts University School of Medicine), were cultured in DMEM containing 5% FBS. H295R adrenocortical carcinoma cells (ATCC, Manassas, VA) were cultured in a 1:1 mixture of DMEM and Ham's F-12 Nutrient mixture (Sigma–Aldrich Chemical Co., St. Louis, MO),

supplemented with 1% Insulin Transferrin Selenium (ITS) (BD Biosciences, San Jose, CA) Premix, 2.5% Nu-Serum (BD Biosciences), and 1.2 g/L Na_2CO_3 . HepG2, MCF-7 and H295R were grown in 25 cm² cell culture flasks (Corning, United States), while PC12 were grown in 25 cm² cell culture flasks coated with 50 mg/mL of poly-L-lysine (Sigma–Aldrich Chemical Co.). All cells were cultured under a humidified atmosphere of 5% CO_2 /95% air at 37 °C, and subcultured upon reaching 80–90% confluence.

2.3. Exposure of cells to BDE-47

A stock solution of BDE-47 (1 mg/mL) was prepared in DMSO, and diluted with culture medium to a range of concentrations (0.1–100 nM) for treatments. In all cases, the DMSO volume was no more than 0.001% of the total volume. The vehicle control cells were exposed to 0.001% DMSO.

All cells were trypsinized and plated in 25 cm² cell culture flasks (without poly-L-lysine coating) at a seeding density of 1.5×10^4 cells/mL (except for PC12) with the culture media containing a range of concentrations of BDE-47. The seeding density of PC12 was 3.3×10^4 cells/mL and 2.6×10^4 cells/mL for 3 days and 6 days, respectively. Exposure times were generally within 6 days in the most of previous *in vitro* studies (Barber et al., 2006; Fery et al., 2009; He et al., 2008a,b; Hu et al., 2007; Peters et al., 2004, 2006; Song et al., 2009). So, we chose 3 days and 6 days as exposure time in this study. The culture medium was changed every 3 days. Each treatment was carried out twice with two replicate flasks each time.

2.4. Collection and extraction of cell samples

After 3 days or 6 days, the cells were collected according to the following method. Firstly, the culture medium was removed from the flask. Culture flasks and attached cells were then washed with phosphate buffered saline (PBS) three times. The cells were trypsinized and transferred to clean glass centrifuge tubes, followed by centrifugation at 1000 rpm for 5 min. Harvested cells were allowed to air dry and then weighed. The cell samples were spiked with BDE-71 as a surrogate standard. The mixture sample was extracted by sonicating twice in 15 mL of hexane for a period of 4 min, followed by centrifugation. Lipids were removed from the extracts with 8 g of acidified silica gel (44% concentrated sulfuric acid, w/w), and the PBDE mixture was then eluted with 100 mL of hexane. The extract was concentrated to 2 mL using a rotary evaporator, and then concentrated further to approximately 0.1 mL under a nitrogen stream. Finally, the extract was transferred to GC vials. Throughout the extraction, cleanup and analysis procedure, amber glassware or aluminum foil wrapping was used to protect the analytes from light.

2.5. PBDEs analysis

All samples were analysed for PBDEs by an Agilent model 6890N gas chromatograph coupled with a model 5973i mass spectrometer (GC/MS) (Agilent Technologies, Palo Alto, CA) using electron-capture negative-ionization (ECNI) in the selected-ion monitoring mode. The GC/MS operating conditions were as described by Liang et al. (2008). The ions monitored for BDE-47 were *m/z* 79 and 81 (bromide ions) in the GC/ECNI-MS mode. BDE-47 was quantified using an external standard (EO-5278) with a multi-level calibration curve spanning the range of anticipated analyte concentrations in the samples.

2.6. Quality assurance and quality control

Recovery of the BDE-71 surrogate standard ranged from 90% to 100%, therefore analyte values were not corrected for recovery. A procedural blank was run in parallel with every batch of six samples. The limit of detection (LOD) was based on the peak-to-peak noise of the baseline near the analyte peak of a spiked sample. LOD for BDE-47, defined as a signal of three times the noise level, was 0.06 ng/g wet-weight in the present study.

2.7. Literature data on environmental exposure levels of biotic tissues to PBDEs

We conducted a literature search (keyword, PBDEs; limits, animals and humans; date, September 28, 2009) of the National Library of Medicine (NLM) PubMed database for all articles addressing PBDEs levels in biotic samples. There are the inter-species and inter-tissues differences in PBDE bioaccumulation. Based a great deal of data, however, PBDEs concentrations in various biotic tissues are mainly related to background exposure levels of PBDEs rather than biological difference among species and tissues. Therefore, inter-species and inter-tissues difference was neglected to give an overview of PBDEs concentrations in biotic tissues in this study as well as other reports (Gauthier et al., 2008; Helgason et al., 2009; Hites, 2004; Shaw and Kannan, 2009; Tanabe et al., 2008). From the results of this search, we selected papers that reported PBDEs concentrations on a wet-weight basis in biotic tissues, including liver, brain, blood, and kidney. Data on fat and egg were excluded because these two tissues are not generally considered as targets of toxicants. We also excluded any data from the whole body of some animals, such as fish. This was due to the potential overestimation of PBDEs burdens in these biotic samples because the PBDEs could be absorbed by the body surface but not enter the body tissues. In addition, data from a laboratory experiment were also excluded from the subsequent analysis as we only included data from natural environments. Forty papers met our selection criteria and were retained for analysis (Table 1). Most of the data were related to the general environment, while some data were from environments polluted with PBDEs from production, application or disposal. PBDEs levels in biotic tissues from polluted regions are obviously higher than those from the general environment, and consequently we summarized the data from these environments separately. The percentile method is a useful method for estimating background concentrations of chemical pollutants in biotic and environmental samples (Chen et al., 2010; Isosaari et al., 2006). The 90th percentile level as well as the 95th percentile level is used to estimate the upper extremes of pollutant concentrations. In this study, we used this method to summarize the environmental exposure levels of PBDEs because PBDEs concentrations among various biotic tissues varied by orders of magnitude. The 25th, 50th, 75th and 90th percentiles of PBDEs levels were calculated in each group using Microsoft Excel. We used minimum and maximum PBDEs values to calculate the percentiles if primary data of PBDEs was unavailable in some cases. The 90th percentile of PBDEs levels was assigned as the upper limit of the environmental exposure levels of PBDEs in biotic tissues. The exposure concentration in culture media that resulted in a BDE-47 burden in cells close to the 90th percentile of PBDEs levels was assigned as the approximate upper limit of the environmentally relevant concentration.

2.8. Data analysis

All statistical analyses were performed using Microsoft Excel XP and SPSS software version 13.0 (SPSS Inc., Chicago, IL). One-way ANOVA was used to test significant differences between PBDEs

accumulation in the cells. A *p*-value of <0.05 was considered significant.

3. Results

Only minimal amounts of lower brominated congeners arising from BDE-47 were found in all cell samples. We did not analyse samples for BDE-47 metabolites because the *in vitro* metabolism of BDE-47 has been reported to be minimal, even in hepatocytes (Stapleton et al., 2009). Therefore, the PBDEs burdens in cells that we report are BDE-47 concentrations. Exposure of the cells to 0.1–100 nM BDE-47 in the culture medium resulted in a concentration-dependent accumulation in the cells (Table 2, Fig. 1). BDE-47 burdens in all cells were at ppb levels (0.96–142.70 ng/g) following both 3 days and 6 days of exposure to 0.1–1 nM BDE-47. BDE-47 concentrations in PC12 cells were higher than those in other cells. Following 10 nM exposure, BDE-47 concentrations in PC12 cells reached ppm levels (1557.67–1757.43 ng/g), while remaining at ppb levels (204.56–726.68 ng/g) in the other cell lines. After exposure to 100 nM BDE-47 for 3 days, BDE-47 concentrations in all cells reached ppm levels (2897.24–6263.29 ng/g). BDE-47 concentrations in all cells following exposure for 6 days were too high to measure BDE-47 concentrations. We did not measure this dose by further diluting because all we consider was which dose was environmentally relevant. Time-dependent accumulation was observed in all cells except for PC12 cells, with exposure for 6 days resulting in higher burdens than exposure for 3 days (Fig. 2). In comparison, BDE-47 burdens in PC12 cells were nearly equivalent after both 3 and 6 days of exposure to each concentration.

PBDEs levels in biotic tissues vary widely from 0 to 2687 ng/g wet-weight (Table 1). The 25th, 50th, 75th and 90th percentiles of PBDEs levels were 0.16, 1.81, 10.68 and 55.35 ng/g, respectively, in the general environment, and 6.90, 48.20, 436.00 and 675.30 ng/g, respectively, in polluted regions (Table 3). After exposure to 1 nM BDE-47 for 3 days, BDE-47 concentrations in MCF-7, HepG2 and H295R cells were 18.21, 27.5 and 18.14 ng/g, respectively. While after exposure for 6 days, BDE-47 concentrations in MCF-7, HepG2 and H295R cells were 43.26, 52.15 and 43.46 ng/g, respectively, which were comparable to the 90th percentile in the general environment (55.35 ng/g, Table 3). Therefore, approximately 1 nM and lower concentrations in culture media were determined to be relevant to general environmental exposure of biotic tissues to PBDEs. However, exposure of PC12 cells to 1 nM BDE-47 for 3 days and 6 days resulted in BDE-47 burdens of 128.47 and 142.70 ng/g, respectively (Table 3). These results are two to three times higher than the 90th percentile in the general environment (55.35 ng/g). Therefore, for PC12 cells the environmentally relevant exposure level should be lower than 1 nM in the culture medium. When considering exposure levels of PBDEs in polluted regions, the environmentally relevant concentrations were determined to be approximately 10 nM and lower for MCF-7, HepG2 and H295R cells and lower than 10 nM for PC12 cells.

4. Discussion

There have been numerous toxicological studies on PBDEs using *in vitro* culture cells, such as MCF-7, HepG2, H295R and PC12 cells. However, none of these studies have determined the PBDEs concentration in the cells following exposure. The toxicological effects observed in these studies have been directly correlated to the exposure concentration of PBDEs in culture media rather than the final PBDEs burden in the cells. In this study, we measured for the first time PBDEs burdens in cells following exposure and associated PBDEs burdens in cells with exposure concentrations in culture media. It should be noted that the detection of PBDEs

Table 1

Literature data on concentrations of PBDEs (ng/g wet-weight) in biotic tissues in the general environment and PBDEs-polluted areas.

	Matrix	Country/area	Sampling years	Total PBDEs	Mean/median	Reference
G ^a	Fish, beef, pork, chick meat	Japan		0.018–1.72		Ohta et al. (2002)
G	Maternal and cord blood plasma	Stockholm, Sweden	2000–2001	0.001–0.058	0.024	Guvenius et al. (2003)
G	Glaucous gull	Svalbard and Bjørnøya	1995–1999	2.0–25.0		Herzke et al. (2003)
G	Fish liver, muscle	Belgian north sea		0.06–128.0		Voorspoels et al. (2003)
G	Salmon muscle	Canadian	2002	0.14–5.5	1.5	Tittlemier et al. (2004)
G	Trout liver, muscle	Greenland and European	2000–2001	0.069–11.0		Vives et al. (2004)
G	Black guillemot liver	Greenland	2000	0.66–9.5	1.71	Vorkamp et al. (2004)
G	Fish muscle	Kahramanmaras, Turkey	2003	nd ^c –6.7		Erdogrul et al. (2005)
G	Gibel carp liver			nd–12.0		
	Carp liver	Belgium	2002	0.3–7.3	1.0	Hoff et al. (2005)
	Eel liver			0.8–39.8	5.2	
G	Polar bear liver	Beaufort Sea, Alaska	1993–2002	<0.2–2.0	1.0/0.82	Kannan et al. (2005)
		Chukchi Sea, Alaska		<0.2–2.7	0.88/0.60	
G	Glaucous gull plasma	Norwegian Arctic	2004	8.23–67.5		Verreault et al. (2005)
	Polar bear plasma		2002	2.65–9.72	5.38	
G	Rock cod muscle	Ross Sea, Antarctica	2000–2002	0.02–0.06	0.05	Corsolini et al. (2006)
G	Buzzard muscle	Flanders, Belgium	2003–2004	0.4–104	5.7	Jaspers et al. (2006)
	Buzzard liver			<0.1–310	2.8	
G	Bald eagle plasma	British Columbia	2001–2003	0.62–18.87	4.73	McKinney et al. (2006)
G	Salmon tissue	Southern Chile	2004	0.9–2.0	1.46	Montroy and Barra (2006)
G	Poultry, beef, pork	Texas, America	2003–2004	0.041–1.28		Schecter et al. (2006)
G	Glaucous gull plasma	Bear Island, Norwegian Arctic	2002	3.1–71.8	17.36	Verreault et al. (2006)
G	Penguin blood	Antarctica	2004	0.002–1.726		Corsolini et al. (2007)
G	Fish	America	2003–2004	0.04–38.0		Hayward et al. (2007)
G	Bream muscle	Czech Rivers	2005	3.2–20.8	9.8/9.5	Hajslóvá et al. (2007)
	Chub muscle			0.9–36.0	7.5/4.9	
	Perch muscle			0.9–6.8	3.8/3.9	
G	Polar cod liver	Barents Sea east of Svalbard	2004	0.88–2.24	1.50/1.50	Haukas et al. (2007)
	Black guillemot liver			1.55–6.33	3.45/2.91	
G	Glaucous gull liver			19.9–186	59.1/41.4	
	Buzzard brain	Switzerland	2003–2005	nd–31		Naert et al. (2007)
	Sparrow hawk brain			4.6–168		
	Cormorant brain			nd–1.3		
	Blackbird brain			nd–27		
G	Glaucous gull plasma	Bear Island, Norwegian Arctic	2004	8.23–67.5	20.23	Verreault et al. (2007a)
G	Glaucous gull plasma	Norwegian Arctic	2006	2.49–54.5	12.52	Verreault et al. (2007b)
G	Fish muscle	Western US Parks	2003–2005	0.01–9.5	1.1	Ackerman et al. (2008)
G	Antarctic fish muscle, liver	Ross Sea, Antarctica	2005	0.001–1.1		Borghesi et al. (2008)
G	Swordfish muscle	Southern Thyrrenian Sea, Italy	2005	<0.004–1.91	0.612/0.387	Corsolini et al. (2008)
	Swordfish liver			0.225–11.206	2.218/0.916	
G	Polar bear blood	East, Greenland	1999–2001	0.5–1.9	1.2	Gebbink et al. (2008)
	Polar bears brain			<0.5–7.6	2.9	
	Polar bears liver			14–103	40	
G	Sea otters liver	America	1995–1998	0.5–63	31.7	Kannan et al. (2008)
G	Lynx liver	Norway	1993–2002	0.14–12.24		Mariussen et al. (2008)
	Grouse liver		1990–2000	0.021–0.326		
	Moose liver		1995	nd–0.047		
	Deer liver		1995–1996	0.039–0.106		
G	Catfish fillet	Southern Mississippi	2006	0.3–11.4		Staskal et al. (2008)
G	Flat fish muscle	Scotland	2004	nd–1.67		Webster et al. (2008)
	Plaice liver		1999–2005	2.08–65.64		
	Trout liver		2005	1.11–9.59		
	Trout muscle		2002–2005	nd–1.22		
G	Ringed seal liver	Svalbard	2007	0.33–4.5	1.1	Routti et al. (2009)
		Baltic	2002–2007	2.8–35.0	7.1	
P ^b	Freshwater fish fillet	Washington State	1997	nd–174		Johnson and Olson (2001)
P	Fish muscle	Scheldt Estuary		0.08–6.9		Voorspoels et al. (2003)
	Fish liver			15–984		
P	Fish muscle	Cinca River, Spain	2002	0.2–436		Eljarrat et al. (2004)
	Fish liver			0.1–446		
P	Pelagic fish plasma	Detroit River	2001–2002	0.932–9.124		Valters et al. (2005)
	Benthic fish plasma			0.155–21.069		
P	Bald eagle plasma	Southern California	2001–2003		30.91	McKinney et al. (2006)
P	Sea otter liver	California Coast	1992–2002	0.2–862	82.8 /27.2	Kannan et al. (2007)
P	Tilapia, bighead carp	Guiyu and Hong Kong	2004	3.84–2687		Luo et al. (2007a)
P	Grass carp muscle	Nanyang River, Guiyu, China	2007	45.2–53.1		Luo et al. (2007b)
	Mud carp muscle			48.0–96.0		
	Crucian carp muscle			70.9–570.0		
	Silver carp muscle			89.8–614.9		
	Carp muscle			458.1–765.9		
P	Herring gull brain	Great Lakes, Canada	1991, 2003	15.44–142.65		Crump et al. (2008)
P	Hen kidney	Taizhou, China	2006	10.0–67.2	48	Liang et al. (2008)
	Hen spleen			48.2–226.3	155	
P	Plaice liver	Scotland	1996–2005	4.1–536.1		Webster et al. (2008)

^a G: general environment.^b P: PBDEs-polluted area.^c nd: levels below limit of detection.

Table 2

BDE-47 accumulation (ng/g wet-weight) in cells following exposure to BDE-47.

Treatment	MCF-7		HepG2		H295R		PC12	
	3 days	6 days	3 days	6 days	3 days	6 days	3 days	6 days
0	nd ^a	nd	nd	nd	nd	nd	nd	nd
0.1 nM	1.84 ± 0.87	3.59 ± 0.92	2.89 ± 0.30	7.16 ± 2.76	0.96 ± 0.21	7.81 ± 1.74	14.02 ± 2.04	15.35 ± 2.24
1.0 nM	18.21 ± 4.69	43.26 ± 11.14	27.5 ± 11.19	52.15 ± 18.04	18.14 ± 4.95	43.46 ± 16.74	128.47 ± 33.09	142.70 ± 36.75
10.0 nM	204.56 ± 45.97	698.96 ± 157.08	319.01 ± 81.35	701.62 ± 178.21	225.38 ± 58.58	726.68 ± 232.03	1557.67 ± 350.06	1757.43 ± 394.93
100.0 nM	3021.87 ± 543.94		4080.41 ± 770.95		2897.24 ± 501.27		6263.29 ± 1034.22	

^a nd: levels below limit of detection. **Fig. 1.** Graph illustrating concentration-dependent accumulation of BDE-47 in MCF-7, HepG2, H295R and PC12 cells following exposure to BDE-47 for 3 days (A) and 6 days (B). **Fig. 2.** Graph illustrating time-dependent accumulation of BDE-47 in MCF-7, H295R and HepG2 cells, and non-time-dependent accumulation in PC12 cells, after exposure for 3 days and 6 days at the same exposure concentrations.

within a cell does not provide an indication of the tissue PBDEs concentrations. This is because not all the PBDEs will have entered the cells, as it is possible that many have only adsorbed onto the cell surface. Similarly, with the literature data on PBDEs in biotic tissues, all the PBDEs detected in the tissues do not necessarily enter the cells. In this manner the data on PBDEs burdens in cells and

the data on PBDEs burdens in biotic tissues are similarly incomplete. So, PBDEs burdens in cells in this study had comparability with PBDEs burdens in biotic tissues in the literature.

In this study, as expected, we found a concentration-dependent accumulation of BDE-47 in all cells. The accumulation of BDE-47 with increased exposure concentration suggested that BDE-47

Table 3

Summary of literature data as percentiles of PBDEs levels (ng/g wet-weight) in biotic tissues in the general environment and polluted areas.

	Minimum	25th	50th	75th	90th	Maximum
General environment	0	0.16	1.81	10.68	55.35	310
Polluted area	0	6.90	48.20	436.00	675.30	2687

accumulation did not reach saturation in the exposure concentration range 0.1–100 nM. This indicates that a highest exposure concentration should be limited so that PBDEs burdens in cells do not largely exceed typical PBDEs environmental exposure levels. We found that BDE-47 concentrations in MCF-7, HepG2 and H295R cells exposed to 1 nM BDE-47 for 3 days reached environmental exposure levels typical of biotic tissues from the general environment. This was even more pronounced for cells exposed to 1 nM BDE-47 for 6 days. In PC12 neuronal cells, exposure to 1 nM BDE-47 for 3–6 days resulted in PBDEs burdens that were higher than the environmentally relevant level. Results from previous studies have shown that exposure to 0.01–10 μ M PBDEs for 2–6 days will result in much higher PBDEs burdens in cells than typical environmental exposure levels in biotic tissues. In summary, to ascertain the ecotoxic and health risks of PBDEs we suggest that the highest concentrations of PBDEs in culture media should be <10 nM for PC12 cells and approximately 10 nM for other cells. These guidelines apply even when considering PBDE-polluted regions with high PBDEs burdens.

Although it is not a new concept, the study of environmentally relevant doses (concentrations) has been the focus of recent attention (Bardullas et al., 2009; Kozul et al., 2009). Myers et al. (2009) concluded that high dose experiments could not be used to predict low dose results. They also suggested that previous acceptable exposure levels for some toxicants that were based on high dose results should be reevaluated in environmentally relevant dose studies, especially for those involving the endocrine system. The National Institute of Environmental Health Sciences (NIEHS) is also focusing on the environmentally relevant dose (Birnbaum, 2009). In this study, our results provide an approximate reference to choose an environmentally relevant exposure concentration of PBDEs for *in vitro* studies. PBDEs concentrations in cells following exposure will vary among different studies due to diversity in exposure conditions and times. Thus, we suggest that during *in vitro* toxicological studies on PBDEs the final concentration of PBDEs in the cells should be determined after exposure, which will facilitate explanation of the dose–response relationship and environmental significance.

It is known that there are small differences in PBDEs accumulation among different cell lines. However, it was an unexpected result that PC12 neuronal cells had a BDE-47 accumulation approximately one order of magnitude higher than other cells after 3 days exposure at the same exposure concentration. This suggests that PC12 cells can accumulate BDE-47 more rapidly than other cells. PBDEs accumulation in MCF-7, H295R and HepG2 cells increased with exposure time. However, BDE-47 accumulation in PC12 cells was not time-dependent with similar concentrations observed after exposure for 3 days and 6 days at the same exposure concentration. BDE-47 accumulation in PC12 cells was rapid and reached a plateau within 3 days of exposure, whereas accumulation in MCF-7, HepG2 and H295R cells did not reach a plateau even after 6 days. Increasing the BDE-47 exposure concentration by one order of magnitude (0.1–100 nM) caused a comparable increase in the BDE-47 burdens in MCF-7, HepG2 and H295R cells but not to saturation. Between 0.1 and 10 nM, BDE-47 accumulation in PC12 cells increased comparably to the order of magnitude by which the exposure concentration was increased. However, when the exposure concentration was increased to 100 nM, the

BDE-47 burden in PC12 cells was four times higher than the value for exposure at 10 nM (Fig. 1). This may suggest that BDE-47 saturation could occur with a further increase in exposure concentration. These results suggest that neuronal cells might have different accumulation kinetics from epithelia cells. Mundy et al. (2004) reported that the accumulation of BDE-47 in primary cultures of rat neocortical cells *in vitro* increased linearly with time for the first 60 min. Between 90 and 120 min the rate of accumulation decreased and saturation began at 120 min. Further studies could investigate why PBDE accumulation in neural cells *in vitro* can reach a plateau within a short time, and whether this phenomenon occurs *in vivo*. This could aid understanding of PBDEs accumulation in the brain and its neurotoxicity. Furthermore, the results could be helpful for choosing exposure concentration and exposure time for *in vitro* and *in vivo* toxicological studies on PBDEs.

5. Conclusion

In vitro exposure to 1 nM BDE-47 for up to 6 days resulted in environmentally relevant burdens in MCF-7, HepG2 and H295R cells and much higher burdens in neuronal PC12 cells. To investigate toxicity of PBDEs at an environmentally relevant concentration, we suggest that the highest PBDEs exposure concentrations should be <10 nM for PC12 cells and approximately 10 nM for other cells. These guidelines are applicable even when considering exposure in PBDE-polluted areas. *In vitro* toxicological studies measuring the PBDEs burdens in cells after exposure are necessary to investigate the dose–response relationship and environmental significance. We found that PBDEs accumulation reached a plateau within a short time in PC12 cells but not in other cells, which suggests that neuronal cells might have different accumulation kinetics to other cells.

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