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REGULATORY TOXICOLOGY

A comparison of the in vitro cyto- and neurotoxicity of brominated and halogen-free flame retardants: prioritization in search for safe(r) alternatives

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Abstract Brominated flame retardants (BFRs) are abundant persistent organic pollutants with well-studied toxicity. The toxicological and ecological concerns associated with BFRs argue for replacement by safe(r) alternatives. Though previous research identified the nervous system as a sensitive target organ for BFRs, the (neuro) toxic potential of alternative halogen-free flame retardants (HFFRs) is largely unknown. We therefore investigated the in vitro (neuro) toxicity of 13 HFFRs and three BFRs in dopaminergic pheochromocytoma (PC12) and neuroblastoma (B35) cells by assessing several cytotoxic and neurotoxic endpoints. Effects on cell viability and production of reactive oxygen species (ROS) were measured using a combined Alamar Blue and Neutral Red assay and a H_2-DCFDA assay, respectively, whereas effects on calcium homeostasis were measured using single-cell fluorescent Ca^{2+}-imaging. The majority of the tested flame retardants induced negligible cytotoxicity, except zinc hydroxystannate (ZHS) and zinc stannate (ZS). A considerable fraction of flame retardants affected ROS production (decabromodiphenyl ether (BDE-209), triphenylphosphate (TPP), aluminium trihydroxide (ATH), ammonium polyphosphate (APP), magnesium hydroxide (MHO), ZHS, ZS and melamine polyphosphate (MPP)). Interestingly, ATH, ZHS, ZS and montmorillonite (MMT) increased the basal intracellular calcium concentration ($[Ca^{2+}]_i$), whereas tetrabromobisphenol A (TBBPA), resorcinol bis (diphenylphosphate) (RDP), TPP, 9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide (DOPO), ATH, ZHS, ZS and MMT reduced depolarization-evoked increases in $[Ca^{2+}]_i$ as a result of inhibition of voltage-gated calcium channels. These combined data on the in vitro (neuro) toxicity of HFFRs in comparison with BFRs are essential for prioritization of safe(r) flame retardants. Though additional data are required for a complete (toxic) risk assessment, our data demonstrate that several HFFRs could be suitable substitutes for BFRs.

Keywords Brominated flame retardants (BFRs) · Halogen-free flame retardants (HFFRs) · In vitro neurotoxicology · Prioritization · Risk assessment · Substitution of BFRs

Introduction

Brominated flame retardants (BFRs) are widely used to reduce the likelihood of ignition of materials and/or decrease the rate of combustion, thereby increasing consumer safety. However, many BFRs are bioaccumulative, persistent organic pollutants (POPs; Covaci et al. 2011; Shaw et al. 2010) that have been found in increasing concentrations in the human food chain, human tissues and breast milk (Fängström et al. 2005; Hites 2004; Schantz et al. 2003). These findings argue for replacement of BFRs by less persistent alternatives.

Based on their application (mainly electrical appliances, furniture and textiles), halogen-free alternatives for the commonly used brominated polystyrene (BPS), decabromodiphenyl ether (BDE-209) and tetrabromobisphenol A (TBBPA) are already available. These halogen-free

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flame retardants (HFFRs) include the phosphorous flame retardants triphenylphosphate (TPP), resorcinol bis (diphenylphosphate) (RDP), bisphenol A bis(diphenylphosphate) (BDP), 9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide (DOPO) and aluminium diethyl-phos-phinate (Alpi); the inorganic halogen-free flame retardants and synergists aluminium trihydroxide (ATH), ammonium polyphosphate (APP), antimony trioxide (ATO), magnesium hydroxide (MHO), zinc hydroxystannate (ZHS) and zinc stannate (ZS); the nanoclay cloisite 30B (montmorillonite, MMT) and the nitrogen-based organic flame retardant melamine polyphosphate (MPP). Although some of these HFFRs are already in use and may even have considerable production volumes, information on their environmental behaviour is scarce and thus hampering proper risk assessment of these chemicals (for review see Waaijers et al. 2013b).

The nervous system is particularly vulnerable for the adverse effects of BFRs. For example, rodent studies report behavioural changes after developmental, neonatal or adult exposure to polybrominated diphenylethers (PBDEs), whereas other studies documented subtle structural and functional alterations in brains of PBDE-exposed animals (for reviews see Costa and Giordano 2007; Dingemans et al. 2011; Fonnum and Mariussen 2009). Both in vivo and in vitro studies indicate that in particular, the cholinergic system is affected by BFRs (Dingemans et al. 2011; Hendriks et al. 2012a; Viberg and Eriksson 2011), whereas also the HFFR ATH was shown to diminish cholinergic activity in rats (Bilkei-Gorzo 1993). Recently, we therefore studied modulation of human $\alpha_4\beta_2$ nicotinic acetylcholine receptors (nACh-R) as a measure for in vitro neurotoxicity to initially prioritize halogen-free alternatives for substitution of BFRs, demonstrating that nACh-R function is affected by several HFFRs (Hendriks et al. 2012b).

Additionally, previous in vitro studies indicated that BFRs can affect cell viability, oxidative stress, neuronal differentiation and migration, neurotransmitter release/uptake, neurotransmitter receptor function, and calcium (Ca^{2+}) homeostasis (Costa and Giordano 2007; Dingemans et al. 2011; Fonnum and Mariussen 2009; Hendriks et al. 2012a; Westerink 2013). The effects of BFRs on Ca^{2+} homeostasis appear to be due to store-mediated Ca^{2+} release and/or inhibition of voltage-gated calcium channels (VGCCs; Dingemans et al. 2009; Dingemans et al. 2010; Hendriks et al. 2012a; Westerink 2013).

Though some neurotoxic effects of HFFRs have been described, e.g. TPP-induced cytotoxicity in PC12 cells (Flaskos et al. 1994), ATH-induced neuritis in neuroblastoma cells (Zatta et al. 1992) and binding of ATH to *N*-methyl-D-aspartate (NMDA) receptors in human cerebral cortex (Hubbard et al. 1989), there is a general lack of data regarding the (neuro)toxic potency of HFFRs (for review see Waaijers et al. 2013b).

To better evaluate the suitability of HFFRs to replace BFRs from a neurotoxicological perspective, it is essential to collect data on a number of critical endpoints and to prioritize the HFFRs accordingly. We therefore investigated the effects of three BFRs (BPS, BDE-209 and TBBPA) and the above-mentioned HFFRs on three different but frequently used endpoints for in vitro neurotoxicity (cytotoxicity, production of reactive oxygen species (ROS) and changes in the intracellular calcium concentration ($[Ca^{2+}]_i$) using PC12 and B35 cells.

Materials and methods

Chemicals

RPMI 1640, DMEM, PenStrep, phosphate-buffered saline (PBS), Fura-2 AM and 2',7'-dichlorofluorescein diacetate (H_2-DCFDA) were obtained from Invitrogen (Breda, The Netherlands). All other chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), unless otherwise noted. Saline solutions for measurements of $[Ca^{2+}]_i$ and production of ROS were prepared with de-ionized water (Milli-Q; resistivity >10 MΩ*cm) and contained (in mM) 125 NaCl, 5.5 KCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES, 24 glucose and 36.5 sucrose (pH 7.3 with NaOH). The flame retardants (at the highest achievable purity) were purchased from different companies (see Supplemental Material, Fig. S1). The BFRs and phosphorous flame retardants were dissolved in purity-checked DMSO, and stock solutions of 100 mM (TBBPA, BDP, RDP, TPP and DOPO) or 10 mM (BPS and BDE-209) were further diluted to obtain final concentrations of 0.01 to 100 μM. The final concentration of DMSO in congener-containing saline was always kept below 0.1 % (v/v). The other HFFRs (Alpi, ATH, APP, ATO, Cloisite, MHO, MPP, ZHS and ZS) are poorly soluble in DMSO (or other solvents). Therefore, these compounds were directly dissolved in saline solution or culture medium at the maximal water solubility, as presented in Supplemental Material, Fig. S1 (S_{max}, measured by inductively coupled plasma mass spectrometry (ICP-MS), details not shown) and dilutions thereof. At least two concentrations per compound per endpoint were tested.

Cell culture

Rat PC12 pheochromocytoma cells and rat B35 neuroblastoma cells were cultured as described previously (Hendriks et al. 2012a) and outlined in the Supplemental Materials. Cells were subcultured one day prior to measurements of cell viability, ROS production or $[Ca^{2+}]_i$ on poly-L-lysine-coated cell culture materials.

Cell viability and ROS production

Effects of the flame retardants on cell viability were determined in PC12 and B35 cells by assessing mitochondrial activity and lysosomal integrity as independent measures of cytotoxicity using a combined Alamar Blue (AB) and Neutral Red (NR) assay as described previously (Hendriks et al. 2012a) and outlined in the Supplemental Materials. Effects of the flame retardants on ROS production were determined using a fluorescent H_2-DCFDA assay as described previously (Hendriks et al. 2012a) and outlined in the Supplemental Materials.

Single-cell fluorescent $[Ca^{2+}]_i$ imaging

$[Ca^{2+}]_i$ was measured using single-cell fluorescence microscopy in PC12 cells loaded with the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 AM as described previously (Hendriks et al. 2012a) and outlined in the Supplemental Materials. Briefly, cells were first superfused with saline and saline containing 100 mM K^+ to measure basal and depolarization-evoked $[Ca^{2+}]_i$, respectively. Next, cells were superfused with saline containing DMSO (0.1 %) or test compound and saline containing 100 mM K^+ in the presence of the test compound (see Fig. 3a for an example recording) to determine effects of flame retardants on basal and depolarization-evoked $[Ca^{2+}]_i$, respectively (see Fig. 3a for an example recording). Basal and depolarization-evoked $[Ca^{2+}]_i$ and effect of flame retardants thereon were quantified as outlined in the Supplemental Materials.

Data analysis and statistics

All data are presented as mean ± standard error of the mean (SEM) from the number of wells or cells (*n*) indicated, derived from 3–9 independent experiments (*N*). Cells exposed only to DMSO were used as control (set at 100 %), and effects of flame retardants on cell viability, ROS formation or $[Ca^{2+}]_i$ concentrations are expressed as % of control. Cells or wells that showed effects two times standard deviation (SD) above or below average were considered outliers and excluded from further analysis of cell viability, ROS production or calcium homeostasis. Since control cells show basal ROS production over time, these data are expressed as average percentage compared to the time-matched control values. For calcium imaging experiments, the individual cells (*n*) are used for statistical analysis as the individual cells rather than the different dishes (*N*) are the source of variation, indicating that statistically all cells are derived from the same population. Additionally, using the dish (*N*) as statistical unit rather than the cells (*n*) reduces the possibility to study single-cell calcium kinetics and oscillations (see also Heusinkveld and Westerink 2012).

Changes smaller than the standard deviation of control cells for the different assays, i.e. 15, 18, 10, 20 and 30 % for measurements of, respectively, AB, NR, ROS, basal $[Ca^{2+}]_i$ and the stimulation-evoked net TR, are not considered biologically relevant. These 'minimal relevant effect sizes' are indicated by the grey-shaded areas in the bar graphs. All relevant effects are statistically significant ($p < 0.05$; Student's *t* test, paired or unpaired where applicable). If applicable, the lowest observed effect concentration (LOEC) and concentration-dependence of the effects of the flame retardants were determined by one-way ANOVA and post hoc Bonferroni tests (calculated using Prism, GraphPad Software, La Jolla, CA, USA).

Results

Identification of effects of HFFRs on neuronal cell viability

The effects of HFFRs on neuronal viability, determined using a combined AB and NR assay in PC12 and B35 cells, are shown in Tables 1, 2, 3, 4. Exposure of PC12 and B35 cells to the BFRs BPS or BDE-209 up to 10 μM for 24 h did not affect cell viability (Table 1). As previously published (Hendriks et al. 2012a), TBBPA at 100 μM significantly decreased the cell viability in PC12 cells with both assays and in B35 cells with the NR assay. No overt cytotoxic effects were observed following exposure to the phosphorous flame retardants (see Fig. 1 for TPP and DOPO, and Table 2 for an overview), though 100 μM BDP induced a small increase in mitochondrial activity in PC12 and B35 cells, indicative for cell stress, and an increase in lysosomal activity in PC12 cells following exposure to 100 μM compared to control cells. The inorganic HFFRs ATO and MHO did not affect cell viability up to the maximal water solubility (S_{max}; also see Supplemental Material, Fig. S1; Table 3). ATH and APP affected cell viability only in B35 cells, whereas ZHS affected viability only in PC12 cells. ZS decreased cell viability in both cell lines and assays already at low concentrations (< 1 μM). The nanoclay MMT did not induce cytotoxic effects up to S_{max}, whereas exposure of PC12 cells to the nitrogen-based organic flame retardant MPP (70 μM) reduced lysosomal activity (Table 4). As summarized in Table 5, no overt cytotoxic effects were observed in the used neuronal cell lines, except for ZHS and ZS, which were able to reduce cell viability already at low concentrations.

Identification of HFFR-induced production of ROS

Oxidative stress occurs when ROS levels in the cell dramatically increase, which may result in significant damage to neuronal cells. An overview of HFFR-induced effects

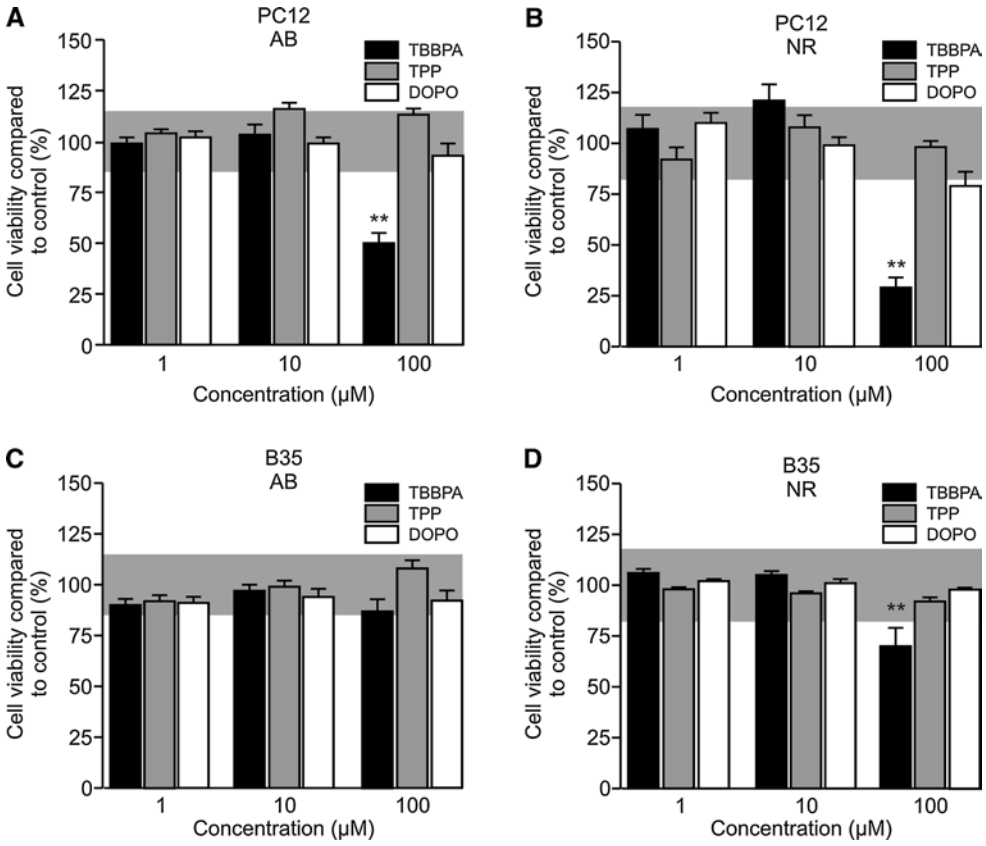

Fig. 1 Effects of 24-h exposure to TBBPA, TPP and DOPO on cell viability in PC12 and B35 cells. *Bar graphs*, representing cell viability determined using a combined *Alamar Blue* (AB; graph A and C) and *Neutral Red* (NR; graph B and D), demonstrate that TBBPA at 100 μM decreases cell viability in PC12 and B35 cells, while TPP and DOPO did not induce cytotoxicity up to 100 μM. *Bars* represent mean cell viability compared with controls (set at 100 %) ±SEM ($n = 27$–35 wells per concentration). *Grey-shaded areas* indicate minimal relevant effect sizes. $^{**}p < 0.001$ versus control

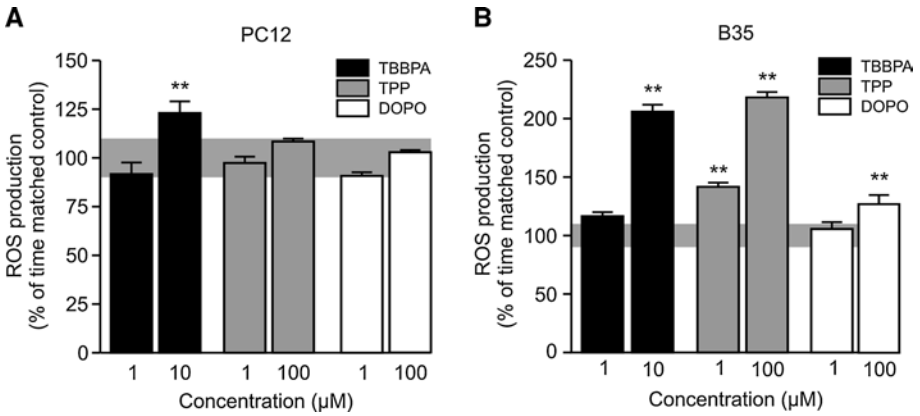

Fig. 2 ROS production induced by TBBPA, TPP and DOPO. Exposure to TBBPA and TPP at non-cytotoxic concentrations increases ROS production in PC12 (**a**) and B35 cells (**b**) over time (*graphs* show results after 24-h exposure, see Supplemental Material Fig. S2 for complete curves), while DOPO was able to affect ROS production only in B35 cells. *Bars* represent mean ROS production compared to time-matched controls (set at 100 %) ±SEM ($n = 32$–111 wells/concentration). *Grey-shaded areas* indicate minimal relevant effect sizes. $^{**}p < 0.001$ versus control

on ROS production following 24-h exposure is shown in Tables 1, 2, 3, 4. BPS up to 10 μM did not affect ROS levels compared to time-matched control PC12 or B35 cells (Table 1). BDE-209 at 1 μM increased ROS production in B35 cells, but not PC12 cells (Table 1). As shown previously (Hendriks et al. 2012a), ≥10 μM TBBPA increases ROS production in both cell lines (see also Fig. 2 and Fig. S2). BDP and RDP up to 100 μM did not affect ROS production, though ≥1 μM TPP and 100 μM DOPO both increased ROS production in B35 cells (Fig. 2 and Fig. S2; Table 2). Alpi (140 μM) induced an increase in ROS production in both cell lines (Table 2). Of the inorganic HFFRs, only APP was able to alter ROS production in PC12 cells, while all inorganic HFFRs (except

ATO) increased ROS production in B35 cells at non-cytotoxic concentrations (Table 3). B35 cells exposed to APP and ZS at cytotoxic concentrations resulted in a reduced, respectively, increased ROS production, which is probably the result of the cytotoxic effects at these concentrations (Table 2). In both cell lines, MMT up to S_{max} (0.2 μM) did not affect normal ROS production (Table 4). In B35 cells, but not PC12 cells, ≥0.35 μM MPP induced an increase in ROS production (Table 4).

Notably, Alpi (140 μM) and APP (700 μM) interact with H_2-DCFDA fluorescence under cell-free conditions (data not shown), possibly confounding the observed effects in the presence of cells.

Overall, B35 cells appeared more sensitive for disturbance of ROS production as 11 out of the 16 compounds were able to affect the normal ROS production to some extent, while in PC12 cells, only four compounds induced effects on ROS production.

Effects of HFFRs on basal $[Ca^{2+}]_i$ in PC12 cells

Since Ca^{2+} plays an essential role in multiple physiological and pathological processes, including cell viability (Orrenius et al. 2011), gene expression (Lyons and West 2011) and neurotransmission (Westerink 2006), we used single-cell fluorescent Ca^{2+}-imaging of Fura-2-loaded PC12 cells to investigate FR-induced effects on Ca^{2+}-homeostasis. PC12 cells have a high expression of voltage-gated Ca^{2+} channels (VGCCs) and are thus suitable to determine acute effects of exposure to BFR and HFFR on basal- and depolarization-evoked increases in $[Ca^{2+}]_i$.

PC12 cells have a low basal $[Ca^{2+}]_i$ of 0.12 ± 0.01 μM (n = 137), which rapidly and transiently increases to 1.9 ± 0.1 μM upon depolarization with 100 mM K^+ for 15 s (see Fig. 3). During a subsequent 5-min recovery period, $[Ca^{2+}]_i$ returned to near basal levels. Next, cells were exposed to 0.1 % DMSO, saline (controls) or saline containing different concentrations of flame retardants for 20 min to determine effects on basal $[Ca^{2+}]_i$ (see Fig. 3). Cells exposed to BPS and BDE-209 up to 10 μM have low basal $[Ca^{2+}]_i$ that is comparable to control cells, while TBBPA at ≥ 10 μM displayed a strong transient increase in basal $[Ca^{2+}]_i$ (Fig. 3 and Table 1; see also Hendriks et al. 2012a). RDP, BDP and DOPO did not affect basal $[Ca^{2+}]_i$, though 100 μM TPP and 279 μM Alpi increased basal $[Ca^{2+}]_i$ (see also Fig. 3 and Table 2). No effects on basal

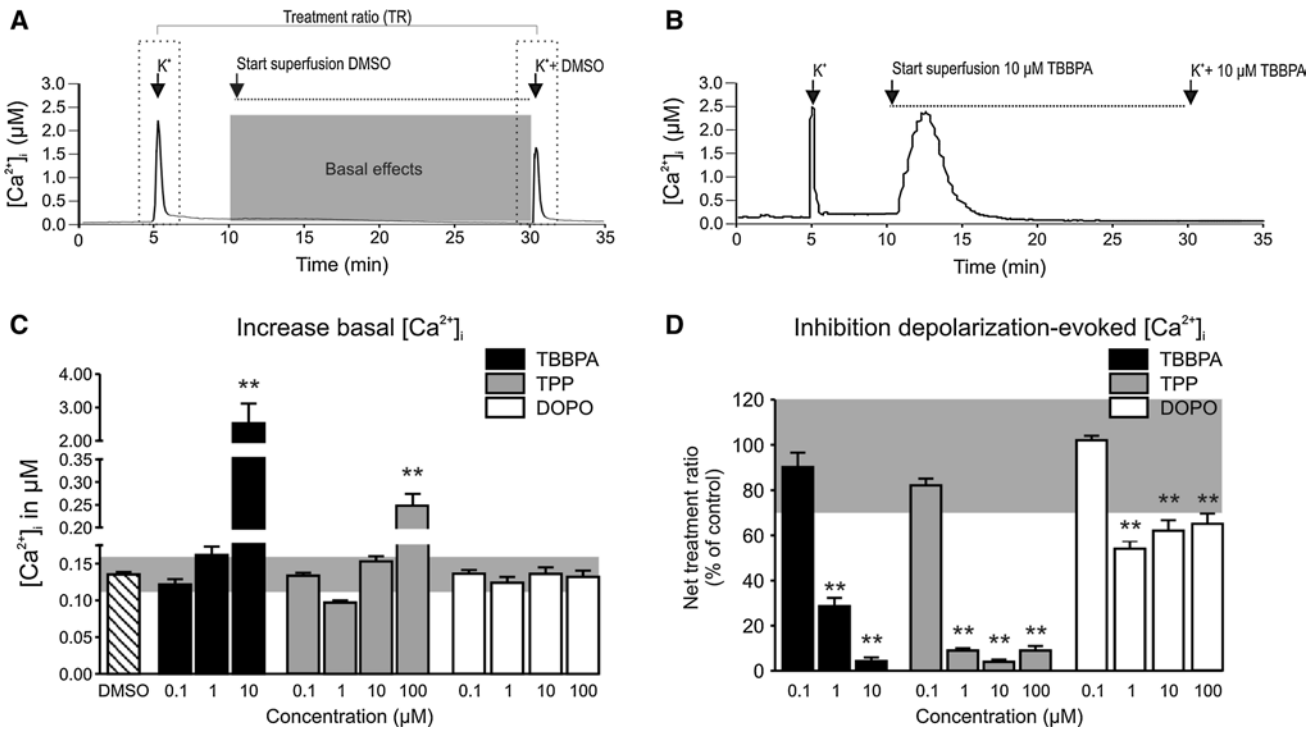

Fig. 3 Flame retardant-induced effect on $[Ca^{2+}]_i$ in PC12 cells. Example recording of single-cell $[Ca^{2+}]_i$ imaging from individual PC12 cells. In between two 15s depolarizations (100 mM K^+), cells were exposed for 20 min to 0.1 % DMSO (**a**) or external saline as control or different non-cytotoxic concentrations flame retardant (**b**, example recording during exposure to 10 μM TBBPA), resulting in a concentration-dependent increase of basal $[Ca^{2+}]_i$ and inhibition of the second depolarization-evoked increase in $[Ca^{2+}]_i$. *Bar graphs* illustrate the flame retardant-induced increase in basal $[Ca^{2+}]_i$ (**c**) and inhibition of the depolarization-evoked increase in $[Ca^{2+}]_i$ expressed as a net TR normalized to solvent-exposed control cells (**d**). *Grey-shaded areas* indicate minimal relevant effect sizes. n = 29–137 cells, $^{**}p < 0.001$ versus control

$[Ca^{2+}]_i$ were observed following exposure to APP, ATO, MHO, ZHS and ZS at non-cytotoxic concentrations, while an increase in basal $[Ca^{2+}]_i$ was observed in cells exposed to cytotoxic concentration ZHS and ZS (Table 3). ATH (1.9 μM) and MMT (0.4 μM) increased basal $[Ca^{2+}]_i$, whereas no effects were observed following MPP exposure (Tables 3 and 4). An overview of HFFR-induced effects on basal $[Ca^{2+}]_i$ is shown in Table 5.

HFFR-induced effects on depolarization-evoked $[Ca^{2+}]_i$ in PC12 cells

Following the 20-min exposure to saline or saline containing DMSO and/or flame retardant, cells were challenged for a second time with 100 mM K^+ to derive a net treatment ratio (net TR, see Supplemental Material, Materials and methods). In DMSO- or saline-exposed control cells, $[Ca^{2+}]_i$ increased to an average of 1.5 ± 0.1 μM during the second depolarization, i.e. 81 ± 2 % of the first depolarization (net TR, see Fig. 3a). Compared to control cells, BPS and BDE-209 did not affect the net TR, whereas the net TR was concentration-dependently reduced in cells exposed to ≥1 μM TBBPA (Fig. 3d and Table 1; see also Hendriks et al. 2012a), suggesting strong inhibition of VGCCs. All tested phosphorous flame retardants were able to reduce the second depolarization-evoked increase in $[Ca^{2+}]_i$, although TPP, DOPO (see also Fig. 3d; Table 2) and RDP were most potent.

Except ATO, all inorganic HFFRs were able to affect the net TR (Table 3), though MHO affected the depolarization-evoked increase in $[Ca^{2+}]_i$ only at the highest tested concentration. The strong reduction in net TR by ATH and ZS, and to a lesser extent by APP and ZS, suggests strong inhibition of the VGCCs by inorganic HFFRs. The nanoclay MMT was also able to strongly reduce the second depolarization-evoked increase in $[Ca^{2+}]_i$, already at low concentrations (Table 4). Contrary, for MPP, only a small inhibition of the depolarization-evoked increase was observed at a cytotoxic concentration (70 μM; Table 4), while no significant effect was observed at lower concentrations.

Overall, the depolarization-evoked increase in $[Ca^{2+}]_i$ in PC12 cells appears a sensitive endpoint within this in vitro

Table 1 Overview of the hazardous effects of the selected brominated flame retardants on several in vitro neurotoxic endpoints

			Brominated flame retardants					
			BPS		BDE-209		TBBPA	
			[BPS] (μM)	Effect size (%)	[BDE-209] (μM)	Effect size (%)	[TBBPA] (μM)	Effect size (%)
Cytotoxicity PC12 cells	AB	LOEC	>10	n/a	>10	n/a	100	50 ± 5
		MEC	n/a	n/a	n/a	n/a	100	50 ± 5
	NR	LOEC	>10	n/a	>10	n/a	100	29 ± 5
		MEC	n/a	n/a	n/a	n/a	100	29 ± 5
Cytotoxicity B35 cells	AB	LOEC	>10	n/a	>10	n/a	>100	n/a
		MEC	n/a	n/a	n/a	n/a	n/a	n/a
	NR	LOEC	>10	n/a	>10	n/a	100	70 ± 9
		MEC	n/a	n/a	n/a	n/a	100	70 ± 9
ROS	PC12	LOEC	>10	n/a	>10	n/a	10	123 ± 6
		MEC	n/a	n/a	n/a	n/a	100	167 ± 2
	B35	LOEC	>10	n/a	1	134 ± 7	10	206 ± 6
		MEC	n/a	n/a	1	134 ± 7	100	346 ± 5
Ca^{2+}-imaging PC12 cells	Basal	LOEC	>10	n/a	>10	n/a	10	2.2 ± 0.4
		MEC	n/a	n/a	n/a	n/a	100	11.7 ± 1.8
	Evoked	LOEC	>10	n/a	>10	n/a	1	28 ± 4
		MEC	n/a	n/a	n/a	n/a	100	0 ± 3
nACh-R	Antagonist	LOEC	>10	n/a	10	8 ± 1	3	19 ± 3
		MEC	n/a	n/a	10	8 ± 1	100	95 ± 1
Neurotoxic potency			Negligible		Low		Low	

Numbers indicate LOECs (lowest observed effect concentration) in μM; *MEC* maximal effect concentration in μM; *n/a* not applicable. The bottom row represents the neurotoxic potency according to the criteria presented in Table 5. Cytotoxicity effect size represents mean cell viability compared to control cells (%; see also Fig. 1); ROS effect size represents mean ROS production after 24 h (% of time-matched controls; see also Fig. 2). Basal Ca^{2+} effect size: mean $[Ca^{2+}]_i$ in μM (see also Fig. 3); depolarization-evoked Ca^{2+}: net treatment ratio (% of control; see also Fig. 3). TBBPA data were previously published by Hendriks et al. (2012a). nACh-R effect size: inhibition of the ACh-evoked response (%). nACh receptor data were previously published by Hendriks et al. (2012b). All data represent mean ± SEM

Table 2 Overview of the hazardous effects of the selected phosphorous flame retardants on several in vitro neurotoxic endpoints

			Phosphorous flame retardants									
			BDP		RDP		TPP		DOPO		Alpi	
			[BDP] (μM)	Effect size (%)	[RDP] (μM)	Effect size (%)	[TPP] (μM)	Effect size (%)	[DOPO] (μM)	Effect size (%)	[Alpi] (μM)	Effect size (%)
Cytotoxicity PC12 cells	AB	LOEC	100	123 ± 4	>100	n/a	>100	n/a	>100	n/a	>279	n/a
		MEC	100	123 ± 4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	NR	LOEC	10	123 ± 6	>100		>100		>100		>279	
		MEC	10	123 ± 6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Cytotoxicity B35 cells	AB	LOEC	100	124 ± 6	>100	n/a	>100	n/a	>100	n/a	>279	n/a
		MEC	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	NR	LOEC	>100	n/a	>100	n/a	>100	n/a	>100	n/a	>279	n/a
		MEC	n/a		n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
ROS	PC12	LOEC	>100	n/a	>100	n/a	100	122 ± 2	>100	n/a	140[a]	126 ± 3[a]
		MEC	n/a	n/a	n/a	n/a	100	122 ± 2	n/a	n/a	140[a]	126 ± 3[a]
	B35	LOEC	>100	n/a	>100	n/a	1	142 ± 4	100	128 ± 8	140[a]	154 ± 11[a]
		MEC	n/a	n/a	n/a	n/a	100	218 ± 5	100	128 ± 8	140[a]	154 ± 11[a]
Ca^{2+}-imaging PC12 cells	Basal	LOEC	>100	n/a	>100	n/a	100	0.25 ± 0.03	>100	n/a	279	1.54 ± 0.09
		MEC	n/a	n/a	n/a	n/a	100	0.25 ± 0.03	n/a	n/a	279	1.54 ± 0.09
	Evoked	LOEC	10	61 ± 4	1	15 ± 3	1	9 ± 1	1	57 ± 4	279	43 ± 3
		MEC	100	51 ± 9	100	3 ± 2	100	9 ± 2	100	69 ± 6	279	43 ± 3
nACh-R	Antagonist	LOEC	>100	n/a	100	10 ± 2	1	7 ± 1	>100	n/a	27.9	15 ± 3
		MEC	n/a	n/a	100	10 ± 2	100	78 ± 5	n/a	n/a	279	60 ± 6
Neurotoxic potency			Negligible		Low		Low		Low		Negligible	

Numbers indicate LOECs (lowest observed effect concentration) in μM; *MEC* maximal effect concentration in μM; *n/a* not applicable. The bottom row represents the neurotoxic potency according to the criteria presented in Table 5. Cytotoxicity effect size represents mean cell viability compared to control cells (%; see also Fig. 1); ROS effect size represents mean ROS production after 24 h (% of time-matched controls; see also Fig. 2). Basal Ca^{2+} effect size: mean $[Ca^{2+}]_i$ in μM (see also Fig. 3); depolarization-evoked Ca^{2+}: net treatment ratio (% of control; see also Fig. 3). nACh-R effect size: inhibition of the ACh-evoked response (%). nACh receptor data were previously published by Hendriks et al. (2012b). All data represent mean ± SEM

[a] Effects may be confounded due to dye–compound interaction

(neuro)toxicological screening, since eight of the 16 compounds were able to reduce the net TR (see Table 5 for an overview).

Rank ordering

An overview of the adverse effects of the selected flame retardants on in vitro cyto- and neurotoxic endpoints, including the results of effects on nACh-R function (Hendriks et al. 2012b), is shown in Table 5 (specific data per category of FRs are presented in Tables 1, 2, 3, 4). Full concentration–response curves could not be obtained for all endpoints, e.g. because of low solubility of the test compounds. Moreover, some endpoints, like ROS production and basal $[Ca^{2+}]_i$, do not have an absolute maximum, precluding calculation of true EC_{50} values. Classification of the flame retardant-induced effects on the different endpoints in this study is therefore based on the LOEC. Following the classification criteria as presented in Table 5, the flame retardants were ranked and subsequently combined to create an 'overall in vitro neurotoxic potency' per flame retardant ('neurotoxic potency', bottom rows of Tables 1, 2, 3 and 4). To prioritize the tested HFFRs for future testing and risk assessment, per endpoint scores were awarded and the total number of points per flame retardant was used to obtain a final in vitro (neuro)toxic rank order ('Final rank order', last column in Table 5). Based on this in vitro (neuro)toxicity study, three flame retardants were classified as having negligible neurotoxic potency (BPS, BDP, Alpi), eight as having low neurotoxic potency (BDE-209, TBBPA, RDP, TPP, DOPO, APP, ATO, MHO), one as having moderate neurotoxic potency (MPP) and four as having high neurotoxic potency in vitro (ATH, ZHS, ZS, MMT).

Discussion

Concerns about the adverse effects of brominated flame retardants (BFRs) on the environment and human health argue for replacement of these FRs. However, there is a

Table 3 Overview of the hazardous effects of the selected inorganic halogen-free flame retardants and synergists on several in vitro neurotoxic endpoints

			Inorganic halogen-free flame retardants and synergists											
			ATH		APP		ATO		MHO		ZHS		ZS	
			[ATH] (μM)	Effect size (%)	[APP] (μM)	Effect size (%)	[ATO] (μM)	Effect size (%)	[MHO] (μM)	Effect size (%)	[ZHS] (μM)	Effect size (%)	[ZS] (μM)	Effect size (%)
Cytotoxicity PC12 cells	AB	LOEC	>50	n/a	>1300	n/a	>1.6	n/a	>0.3	n/a	0.5	19 ± 2	0.6	32 ± 8
		MEC	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.5	19 ± 2	0.6	32 ± 8
	NR	LOEC	>50	n/a	>1300	n/a	>1.6	n/a	>0.3	n/a	0.005	70 ± 4	0.6	30 ± 4
		MEC	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.5	25 ± 2	0.6	30 ± 4
Cytotoxicity B35 cells	AB	LOEC	50	124 ± 4	13	69 ± 4	>1.6	n/a	>3100	n/a	>0.5	n/a	0.6	24 ± 4
		MEC	50	124 ± 4	1300	16 ± 2	n/a	n/a	n/a	n/a	n/a	n/a	0.6	24 ± 4
	NR	LOEC	>50	n/a	1300	14 ± 2	>1.6	n/a	>3100	n/a	>0.5	n/a	0.006	61 ± 7
		MEC	n/a	n/a	1300	14 ± 2	n/a	n/a	n/a	n/a	n/a	n/a	0.6	53 ± 10
ROS	PC12	LOEC	>1	n/a	700[a]	82 ± 4[a]	>15	n/a	>412	n/a	>0.4	n/a	>0.9	n/a
		MEC	n/a	n/a	700[a]	82 ± 4[a]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	B35	LOEC	0.01	121 ± 6	7[a]	120 ± 9[a]	>15	n/a	4.12	138 ± 9	0.4	127 ± 8	0.9	259 ± 13
		MEC	1	166 ± 25	700[a]	80 ± 4[a]	n/a	n/a	412	143 ± 9	0.4	127 ± 8	0.9	259 ± 13
Ca^{2+}-imaging PC12 cells	Basal	LOEC	1.9	0.27 ± 0.04	>1300	n/a	>29	n/a	>823	n/a	0.008	0.25 ± 0.01	1.8	0.26 ± 0.01
		MEC	1.9	0.27 ± 0.04	n/a	n/a	n/a	n/a	n/a	n/a	0.008	0.25 ± 0.01	1.8	0.26 ± 0.01
	Evoked	LOEC	0.019	30 ± 3	130	43 ± 3	>29	n/a	823	66 ± 3	0.008	41 ± 6	1.8	15 ± 4
		MEC	0.019	30 ± 3	1300	17 ± 2	n/a	n/a	823	66 ± 3	0.8	61 ± 3	1.8	15 ± 4
nACh-R	Antagonist	LOEC	1.9	13 ± 1	1300	55 ± 2	9.4	14 ± 2	204	20 ± 2	0.8	12 ± 4	>0.8	n/a
		MEC	1.9	13 ± 1	1300	55 ± 2	9.4	14 ± 2	204	20 ± 2	0.8	12 ± 4	n/a	n/a
Neurotoxic potency			High		Low		Low		Low		High		High	

Numbers indicate LOECs (lowest observed effect concentration) in μM; *MEC* maximal effect concentration in μM; *n/a* not applicable. The bottom row represents the neurotoxic potency according to the criteria presented in Table 5. Cytotoxicity effect size represents mean cell viability compared to control cells (%; see also Fig. 1); ROS effect size represents mean ROS production after 24 h (% of time-matched controls; see also Fig. 2). Basal Ca^{2+} effect size: mean $[Ca^{2+}]_i$ in μM (see also Fig. 3); depolarization-evoked Ca^{2+}: net treatment ratio (% of control; see also Fig. 3). nACh-R effect size: inhibition of the ACh-evoked response (%). nACh receptor data were previously published by Hendriks et al. (2012b). All data represent mean ± SEM

[a] Effects may be confounded due to dye–compound interaction

Table 4 Overview of the hazardous effects of the other selected halogen-free flame retardants and synergists on several in vitro neurotoxic endpoints

			Nanoclay		Nitrogen-based organic FR	
			MMT		MPP	
			[MMT] (μM)	Effect size (%)	[MPP] (μM)	Effect size (%)
Cytotoxicity PC12 cells	AB	LOEC	>0.4	n/a	>70	n/a
		MEC	n/a	n/a	n/a	n/a
	NR	LOEC	>0.4	n/a	70	49 ± 5
		MEC	n/a	n/a	70	49 ± 5
Cytotoxicity B35 cells	AB	LOEC	>0.4	n/a	>70	n/a
		MEC	n/a	n/a	n/a	n/a
	NR	LOEC	>0.4	n/a	>70	n/a
		MEC	n/a	n/a	n/a	n/a
ROS	PC12	LOEC	>0.2	n/a	>35	n/a
		MEC	n/a	n/a	n/a	n/a
	B35	LOEC	>0.2	n/a	0.35	124 ± 4
		MEC	n/a	n/a	35	149 ± 3
Ca^{2+}-imaging PC12 cells	Basal	LOEC	0.4	0.23 ± 0.01	>70	n/a
		MEC	0.4	0.23 ± 0.01	n/a	n/a
	Evoked	LOEC	0.004	2 ± 0	70	60 ± 6
		MEC	0.4	5 ± 1	70	60 ± 6
nACh-R	Antagonist	LOEC	0.004	12 ± 2	70	23 ± 3
		MEC	0.4	79 ± 4	70	23 ± 3
Neurotoxic potency			High		Moderate	

Numbers indicate LOECs (lowest observed effect concentration) in μM; *MEC* maximal effect concentration in μM; *n/a* not applicable. The bottom row represents the neurotoxic potency according to the criteria presented in Table 5. Cytotoxicity effect size represents mean cell viability compared to control cells (%; see also Fig. 1); ROS effect size represents mean ROS production after 24 h (% of time-matched controls; see also Fig. 2). Basal Ca^{2+} effect size: mean $[Ca^{2+}]_i$ in μM (see also Fig. 3); depolarization-evoked Ca^{2+}: net treatment ratio (% of control; see also Fig. 3). nACh-R effect size: inhibition of the ACh-evoked response (%). nACh receptor data were previously published by Hendriks et al. (2012b). All data represent mean ± SEM

general lack of data regarding the toxicity of suggested alternatives, including compounds that are already in use as alternative flame retardant (Waaijers et al. 2013b). In the present study, we therefore investigated the in vitro (neuro)toxic potential of several selected halogen-free flame retardants (HFFRs) in comparison with three widely used BFRs on several cytotoxic and neurotoxic endpoints. Except zinc hydroxystannate (ZHS) and zinc stannate (ZS), the tested flame retardants induced negligible cytotoxic effects on PC12 and/or B35 cells. A number of FRs induced an increase in ROS production. ROS is formed as a natural by-product of normal cell metabolism, but excess of ROS formation can result in oxidative stress that causes damage to DNA, proteins and membrane lipids, and may ultimately even induce apoptosis. Notably, ROS production was more frequently increased following exposure to FRs in B35 cells compared to PC12 cells, suggesting differences in, e.g. antioxidant capacities between these cell lines. Some compounds were able to disturb intracellular Ca^{2+} homeostasis; increases in basal $[Ca^{2+}]_i$ were observed in PC12 cells following exposure to TBBPA, ATH, ZHS, ZS, or MMT. A (prolonged) increase in $[Ca^{2+}]_i$ potentially affects essential cellular processes such as gene expression, protein phosphorylation, neurotransmission and caspase-mediated apoptosis. As summarized in Table 5, the depolarization-evoked increase in $[Ca^{2+}]_i$ appears a sensitive endpoint within this in vitro (neuro) toxicity screening since eight of the 16 tested compounds (TBBPA, RDP, TPP, DOPO, ATH, ZHS, ZS and MMT) were able to reduce the net TR. This indicates that these compounds inhibit VGCCs, comparable with PBDEs (Dingemans et al. 2011), PCBs (Langeveld et al. 2012) and TBBPA (Hendriks et al. 2012a). For the overall classification and rank order, the in vitro neurotoxic potential of the compounds on human $\alpha_4\beta_2$ nACh-R function as presented in our previous study (Hendriks et al. 2012b) was also taken into account.

The combined results from our in vitro neurotoxicity assessment indicate that the phosphorous flame retardants BDP (bisphenol A bis (diphenylphosphate)) and Alpi

Table 5 Overview of the hazardous effects of the selected flame retardants on several in vitro neurotoxic endpoints

Classification	Negligible potency	Low potency	Moderate potency	High potency
Criteria	LOEC ≥ 10 μM	1 μM ≤ LOEC < 10 μM	0.1 μM ≤ LOEC < 1 μM	LOEC < 0.1 μM
Points	0	1	2	3

		Brominated FRs			Phosphorous FRs					Inorganic HFFRs and synergist						Nano clay	Nitrogen-based organic FR
		BPS	BDE-209	TBBPA	BDP	RDP	TPP	DOPO	Alpi	ATH	APP	ATO	MHO	ZHS	ZS	MMT	MPP
Cytotox PC12 cells	AB																
	NR																
Cytotox B35 cells	AB																
	NR																
ROS	PC12																
	B35																
Ca^{2+}-imaging PC12 cells	Basal																
	Evoked																
nACh-R	Antagonist																
Final rank order		0	1	2	0	1	3	1	0	8	1	1	1	15	13	8	2

Classification criteria for rank ordering of the tested flame retardants, followed by an overview of the adverse effects of the selected flame retardants on several in vitro (neuro)toxic endpoints based on the LOEC (see Tables 1, 2, 3 and 4). Per endpoint, scores were awarded to the different flame retardants based on the LOEC. The total number of points per flame retardant was counted in order to obtain a final rank ordering. Note, the final rank order has at least the same potency as observed in one or more of the endpoints, despite the total number of points

(aluminium diethylphosphinate) (both negligible neurotoxic potency) as well as TPP (triphenylphosphate), RDP (resorcinol bis (diphenylphosphate)) and DOPO (9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide) (low neurotoxic potency, see Table 5) may be suitable for replacement of BFRs. However, previous studies indicate that TPP concentrations in house dust may be associated with altered hormone levels and decreased semen quality in men (based on sperm concentration, motility and morphology) (Meeker and Stapleton 2010). In addition, TPP was previously shown to exert in vitro neurotoxic effects, including modulation of neurotransmitter receptors (Flaskos et al. 1994; Gant et al. 1987; Hendriks et al. 2012b). Notably, TPP was reported to be present in human milk up to 11 ng/g lw (Sundkvist et al. 2010), which is at least twice as high as TBBPA milk concentrations (up to 4.1 ng/g lw; Abdallah and Harrad 2011; Cariou et al. 2008; Shi et al. 2009). Moreover, TTP has been measured in (dust of) houses, offices and cars at levels similar to or greater than those measured for PBDEs in the same samples (Brommer et al. 2012; Stapleton et al. 2009a). TPP is labelled by the European Chemicals Agancy (ECHA) as a compound with dangerous effects for the environment (ECHA Database Accessed 2012b). Consequently, despite the 'low neurotoxic potency' in the present study, TPP is from our point of view not considered as a suitable replacement for BFRs.

Toxicological and exposure data for the other phosphorous-based HFFRs are limited (for review see Waaijers et al. 2013b). Nonetheless, it is known that BDP and RDP may contain up to 5 % TPP as impurity (Clean Production Action 2007; Umwelt Bundes Amt 2001) and that TPP-like compounds may be formed as breakdown products of BDP and RDP. Additionally, one study identified the endocrine disruptor bisphenol A as a degradation product of BDP (Maine 2007). Degradation and metabolism are important factors to include in the risk assessment of flame retardants as it is well known that metabolites of, e.g. BDE-47, are more toxic than the parent compound (Dingemans et al. 2011).

A metal-based phosphorous flame retardant may dissociate into its ion constituents under some conditions. In case of Alpi, the ion constituent is aluminium, which has well-studied (neuro)toxic properties (Berthon 2002; Hu et al. 2007; Vijverberg et al. 1994; Wakui et al. 1990). However, it is unlikely that the levels of aluminium resulting from Alpi exposure reach levels high enough to cause serious health concerns.

Though no additional information is available in the public domain for Alpi, DOPO or the other tested phosphorous flame retardant, a recent study about the acute toxicity of HFFRs on the water flea *Daphnia magna* identified TPP also as highly toxic, whereas DOPO and Alpi were classified as having low toxicity (classification based on the

REACH criteria of the European Union). Due to solubility problems, it was hard to estimate the toxicity of BDP and RDP, though adverse effects were observed (Waaijers et al. 2013a). Thus, although BDP, RDP, DOPO and Alpi are rated as suitable alternatives with negligible or low neurotoxic potency based on our in vitro study, also for these compounds more research is needed, e.g. regarding human exposure, the environmental stability of the compound, its breakdown products and possible metabolites, before these HFFRs can be proposed as a safe(r) replacements of BFRs.

The inorganic metal-based flame retardants ATH (aluminium trihydroxide), ZS (zinc stannate) and ZHS (zinc hydroxystannate) together with the nanoclay MMT (montmorillonite) are the most toxic HFFRs tested in our in vitro study and are classified as having a high in vitro neurotoxic potency. Previous studies also report (neuro)toxic effects of ATH (Hendriks et al. 2012b; Hubbard et al. 1989; Waaijers et al. 2013a; Zatta et al. 1992). ATH is not expected to decompose under physiological conditions, and its toxic potency is therefore unlikely to be related to aluminium ions.

The observed neurotoxic effects of ZS and ZHS appear to be intrinsic to the compounds as these metals are, based on their stability and low solubility (Waaijers et al. 2013b), not expected to ionize in our test solutions to levels sufficiently high to induce toxicity. Moreover, metallic tin and its inorganic salts have a low toxicity (Cima 2011), and a neurotoxic excess of zinc (Wright and Baccarelli 2007) is unlikely at the concentrations used.

The nanoclay MMT, which consists of quartz (0.1–1 %) and alkyl quaternary ammonium bentonite (95–99 %), has a very poor solubility and low suspected bioavailability. Nevertheless, MMT has some reported neurotoxic effects (Banin and Meiri 1990; Murphy et al. 1993) and was also classified as having a moderate neurotoxic potency in our study. As such, ATH, ZS, ZHS and the nanoclay MMT seem less suitable as replacement of BFRs based on our in vitro study and the scarce available data on toxicity.

The inorganic metal-based FRs APP (ammonium polyphosphate), ATO (antimony trioxide) and MHO (magnesium hydroxide) were classified as having low neurotoxic potential based on our in vitro study. However, additional research for a complete risk assessment is needed as for instance ATO is rated as 'suspected of causing cancer but not sufficient for classification' (ECHA Database Accessed 2012a) and was recently classified as moderately toxic in *Daphnia magna* (Waaijers et al. 2013a).

APP was reported to break down rapidly in soil and sewage sludge into ammonia and phosphate (German Federal Environmental Agency 2001), while it undergoes slow hydrolysis with the release of ammonium phosphate when in contact with water (Clariant 2010). Considering the suspected low bioavailability of the polymer compared to monomeric ammonium phosphate, we assume that the observed moderate toxicity in our study is primarily due to monomers. In *Daphnia magna*, APP induced low toxicity (Waaijers et al. 2013a). Clearly, more toxicological and exposure studies are required to confirm the suitability of APP, ATO and MHO before these HFFRs can be proposed as a safe(r) replacements of BFRs.

Comparable with the polymer APP, MPP (melamine polyphosphate) has a suspected low bioavailability as polymer and MPP will dissociate in water into melamine and phosphoric acid. Melamine was shown to induce some neurotoxic effects, e.g. on voltage-gated sodium channels (Yang et al. 2011) and according to our in vitro study, MPP has a moderate neurotoxic potency.

Based on the in vitro endpoints in this prioritization study, the BFRs TBBPA (tetrabromobisphenol A) and BDE-209 (decabromodiphenyl ether) were classified as having low neurotoxic potency, whereas BPS (brominated polystyrene) was even classified as having negligible neurotoxic potency. Nevertheless, several in vitro and in vivo studies clearly demonstrate the adverse effects of PBDEs and TBBPA on the nervous system (for review see Dingemans et al. 2011). Notably, PBDE exposure is associated with changes in the motor function (Kicinski et al. 2012) and reduced psychomotor development index and full-scale IQ performance (Herbstman et al. 2008; Roze et al. 2009) in humans. Moreover, it is suggested that the fully brominated congener BDE-209 is metabolized into lower and more toxic brominated congeners, though the extent of these metabolic reactions in mammals, including humans, is still unclear (Costa and Giordano 2011; Stapleton et al. 2009b). In addition, several studies indicate that oxidative metabolism can increase the neurotoxic potency of a toxicant, including PBDEs (Dingemans et al. 2011). These findings emphasize the need for safe alternatives, but also indicate that our in vitro characterization should only be regarded as a tool for prioritization.

Future risk assessment of BFRs and HFFRs ideally should include the physical–chemical properties (e.g. molecular weight, log K_{ow} (a measure for lipophilicity), and water solubility) of the compound, production volumes, the presence in the environment, persistence, bioaccumulation, ecotoxicity, and in vitro as well as in vivo toxicity.

The combined data of our in vitro study indicate a high neurotoxic potency for ATH, ZHS, ZS and MMT, a moderate neurotoxic potency for MPP, a low neurotoxic potency for BDE-209, TBBPA, RDP, TPP, DOPO, APP, ATO and MHO, and negligible neurotoxic potency for BPS, BDP and Alpi. However, considering the current lack of toxicological information and exposure data regarding the suggested HFFRs, it is necessary to further study the proposed alternative flame retardants in vitro as well as in vivo to confirm the low risk of some of these HFFRs for the

environment and human health. Following such additional research, and taking into account the above-mentioned concerns, the HFFRs that are classified here as having negligible or low neurotoxic potency may thus be selected as viable alternatives for replacement of BFRs.

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